Interactions between Fat and Dachsous and the regulation of planar cell polarity in the *Drosophila* wing

Hitoshi Matakatsu and Seth S. Blair*

Department of Zoology, University of Wisconsin, 250 North Mills Street, Madison, WI 53706, USA
*Author for correspondence (e-mail: ssblair@wisc.edu)

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Summary

It was recently suggested that a proximal to distal gradient of the protocadherin Dachsous (Ds) acts as a cue for planar cell polarity (PCP) in the *Drosophila* wing, orienting cell-cell interactions by inhibiting the activity of the protocadherin Fat (Ft). This Ft-Ds signaling model is based on mutant loss-of-function phenotypes, leaving open the question of whether Ds is instructive or permissive for PCP. We developed tools for misexpressing *ds* and *ft* in vitro and in vivo, and have used these to test aspects of the model. First, this model predicts that Ds and Ft can bind. We show that Ft and Ds mediate preferentially heterophilic cell adhesion in vitro, and that each stabilizes the other on the cell surface. Second, the model predicts that artificial gradients of Ds are sufficient to reorient PCP in the wing; our data confirms this prediction. Finally, loss-of-function phenotypes suggest that the gradient of *ds* expression is necessary for correct PCP throughout the wing. Surprisingly, this is not the case. Uniform levels of *ds* drive normally oriented PCP and, in all but the most proximal regions of the wing, uniform *ds* rescues the *ds* mutant PCP phenotype. Nor are distal PCP defects increased by the loss of spatial information from the distally expressed *four-jointed* (*fj*) gene, which encodes putative modulator of Ft-Ds signaling. Thus, while our results support the existence of Ft-Ds binding and show that it is sufficient to alter PCP, *ds* expression is permissive or redundant with other PCP cues in much of the wing.

Key words: Adhesion, Cadherin, Frizzled, *Drosophila*

Introduction

The *fat* (*ft*) and *dachsous* (*ds*) genes in *Drosophila* encode large, cadherin-related proteins (Clark et al., 1995; Mahoney et al., 1991). These proteins differ from classic cadherins in several respects (reviewed by Tepass et al., 2000). Ft and Ds have unusually large numbers of extracellular cadherin repeats (34 and 27, respectively). Ft also contains five EGF and two laminin-A globular-like domains. Their cytoplasmic domains also differ markedly from those of classic cadherins, although there are some limited similarities to the β-catenin binding sites in classic cadherins (Clark et al., 1995). Unlike classic cadherins, Ft and Ds are concentrated apcial to but not within the adherens junctions (Ma et al., 2003). Ft and Ds are required for several processes during *Drosophila* development, including the regulation of growth and the proximodistal patterning of appendages (Bryant et al., 1988; Buratovich and Bryant, 1997; Clark et al., 1995; Garoia et al., 2000). They also are required for the correct orientation of planar cell polarity (PCP) in the eye, wing and abdomen (Adler et al., 1998; Casal et al., 2002; Ma et al., 2003; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002).

In PCP, cells of an epithelial sheet are polarized perpendicular to the apical-basal axis (Adler, 2002; Eaton, 2003; Fanto and McNeill, 2004; Strutt, 2003; Uemura and Shimada, 2003). In the *Drosophila* wing, the hairs produced by each epithelial cell normally point distally (see Fig. 3A). In one view, PCP in the wing relies in part upon signals passed from cell to cell via the Frizzled (Fz) PCP pathway, using the 'core' planar polarity proteins (Fig. 1E) (Tree et al., 2002). From 18-30 hours after pupariation (AP), the core planar polarity proteins are redistributed to the proximal [Prickle (Pk), Van Gogh (Vang, also known as Strabismus)], distal [Fz, Dishevelled (Dsh)], or proximal and distal [Flamingo (Fmi), also known as Starry night] faces of individual cells (Axelrod, 2001; Bastock et al., 2003; Shimada et al., 2001; Strutt, 2001; Tree et al., 2002; Usui et al., 1999). This redistribution requires mutual interactions between these proteins, both within and between cells. The interaction between cells is thought to ensure that neighboring cells have a similar polarization.

However, these cell-cell interactions must be oriented in some fashion. It was recently proposed that this orienting cue or bias is provided by Ds and Ft (Ma et al., 2003; Yang et al., 2002). Ft is uniformly expressed in the wing blade (Garoia et al., 2000; Ma et al., 2003), and thus is unlikely to provide any polarity information on its own. Ds, by contrast, is expressed at higher levels in the proximal wing, and thus it has been proposed that a proximal to distal gradient of Ds provides the polarity cue for much or all of the wing (Ma et al., 2003) (reviewed in Fanto and McNeill, 2004). Although it is difficult to detect more than low levels of uniform of Ds in the distal wing at 5 hours AP (Strutt and Strutt, 2002) (Fig. 1A-C), by 17 hours AP strong Ds expression extends into the distal wing (see Fig. 6H), forming a proximal to distal gradient down the center of the wing by 26 hours AP (Fig. 1D) (see also Ma et

al., 2003). Loss of *ft* or *ds* results in PCP defects throughout most of the wing, the redistribution of Fmi and Dsh to the wrong faces of cells, and the propagation of errors in Fz signaling for longer distances (Adler et al., 1998; Ma et al., 2003; Strutt and Strutt, 2002).

According to one view, Ft and Ds act, not as adhesion molecules, but as receptor (Ft) and ligand (Ds). Because clones lacking Ft or Ds function have opposite effects on the orientation of hairs in neighboring wild-type cells, it was hypothesized that Ds inhibits Ft, creating an opposing distal to proximal gradient of Ft activity (Fig. 1E) (Ma et al., 2003), similar to what is proposed in the eye (Yang et al., 2002). Examination of protein distribution in loss-of-function clones suggests that Ft and Ds interact either directly or indirectly in the wing, which is suggestive of a receptor-ligand relationship (Ma et al., 2003; Strutt and Strutt, 2002) (see below). Although Ft activity is as yet hypothetical, the intracellular domain of Ft can bind to the *Drosophila* homolog of mammalian Atrophin (encoded by *grunge*); the similarity between ft^{-} and $grunge^{-}$ PCP phenotypes suggests that Grunge may have a role in mediating some or all of the activity of Ft (Fanto et al., 2003).

Another mechanism for regulating Ft activity may be provided by the distally expressed Four-jointed (Fj) protein (Fig. 1B,C,E) (Brodsky and Steller, 1996; Villano and Katz, 1995; Zeidler et al., 2000). Like *ds* and *ft* mutants, null alleles of *fj* cause proximodistal defects in wing and leg. Although *fj* null homozygotes have little effect on PCP, creating artificial boundaries or gradients of *fj* expression can alter PCP, suggesting that it acts as a redundant cue (Zeidler et al., 1999; Zeidler et al., 2000). Clones lacking *fj* affect anti-Ft and anti-Ds staining in a manner consistent with a reduced interaction between Ds and Ft (Ma et al., 2003; Strutt and Strutt, 2002). Recent evidence indicates that Fj acts in the Golgi, suggesting that Fj affects the interaction between Ft and Ds by modifying the forms generated or the cellular localization of these proteins (Strutt et al., 2004).

The Ft-Ds signaling model is based on loss-of-function phenotypes, as the very large size of these molecules has made the construction of misexpression constructs difficult. We have, however, developed fully functional constructs for the misexpression of full-length Ft and Ds, and we use these to test various predictions of the model. First, Ft and Ds must bind preferentially. Although the changes in protein distribution caused by loss-of-function clones are consistent with a heterophilic interaction (Ma et al., 2003; Strutt and Strutt, 2002), this has never been directly demonstrated. We show that Ft and Ds mediate preferentially heterophilic cell adhesion in vitro and in vivo. Second, artificial gradients of *ft* and *ds* expression in the wing should be sufficient to reorient PCP throughout the wing; again, we show that this prediction is largely met.

The extensive PCP defects induced by loss of *ds* suggest that the gradient of *ds* expression acts as a global cue required for orienting PCP throughout the wing. However, it remains possible that *ds* acts, not as an instructive cue, but as a permissive factor, and that the crucial polarity information is being provided other cues. We have therefore tested the role of the *ds* gradient by driving uniform *ds* misexpression. Surprisingly, we find that a gradient of *ds* is not necessary for correct PCP in all but the most proximal region of the wing. In most of the wing, uniform *ds* expression can rescue the *ds* mutant PCP phenotype. This is true even in the absence of any putative redundant information from Fj. Thus, other unknown cues are sufficient to orient PCP in absence of information from the pattern of *ds* transcription.

We have also used misexpression to test the timing of Ds activity in PCP, by analyzing whether Ds acts during the stages when the core polarity proteins are being redistributed, or whether it acts during an earlier, recently identified period of Fz activity (Strutt and Strutt, 2002). If the loss of *fz* is limited to this early period, distinct PCP defects are produced that are similar to those observed in *ds* mutants, but differ from the typical PCP defects observed in *fz* mutants. Because of this similarity in phenotypes, it was suggested that Ds acts during this early period (Eaton, 2003; Strutt and Strutt, 2002). Our data supports this hypothesis.

Finally, we show that Ft misexpression is sufficient to drive PCP in an orientation opposite that that caused by Ds, and that the activity of ectopic Ft depends partly, but not wholly, on the presence of Ds and Fj.

Materials and methods

Fly strains

ds^{05142} (Bloomington Stock Center); $fj^{9\text{-}II}$ (Villano et al., 1995); fj^{d1} (Brodsky et al., 1996); $ft^{G\text{-}rv}$ and ft^{fd} (Bryant et al., 1988); UAS-*fj* (Zeidler et al., 1999); *UAS-fz-GFP* and *actin-fz-GFP* (Strutt, 2001); *AyGal4 UAS-GFP* (Ito et al., 1997); $tub\text{-}gal80^{ts}$ (McGuire et al., 2003); and dsh^{1} *tub-gal4*, *en-gal4*, *sal-gal4*, *ptc-gal4*, *dll-gal4*, *da-gal4* and *ap-gal4*.

Molecular biology

UAS-ds contains the full-length *ds* coding sequence, reconstructed from fragments amplified by RT-PCR (Takara). *UAS-ft* contains full-length *ft* genomic sequence, reconstructed from DNA fragments obtained from P1 clones DS06482 and DS06843, or amplified from genomic DNA by PCR. All constructs were confirmed by sequencing and cloned into pUAST (Brand and Perrimon, 1993). Detailed information is available by request. To induce expression in S2 cells, we co-transfected *UAS-ft* or *UAS-ds* with pAWGal4 (gift from Y. Hiromi), which drives Gal4 under the control of an actin promoter in S2 cells. To examine the effects of *ft* or *ds* misexpression in vivo, UAS constructs were injected into w^{1118} embryos and transfectants were isolated using standard methods. In western blots of transfected S2 cells, we detected bands corresponding to full-length and processed Ft and Ds proteins (data not shown); each construct can substantially rescue the PCP defects of the corresponding mutant.

In vitro studies

S2 cells were co-transfected with UAS constructs and pAWGal4. To identify transfectants UAS-GFP was co-transfected into S2 cells. Cell aggregation assays were performed as described (Usui et al., 1999; Oda et al., 1994). Cells were normally agitated on a rotator at 50 or 100 rotations per minute (rpm); 150 rpm was used for high rates of agitation.

Immunostaining

S2 cells were fixed in 4% formaldehyde in PBS for 30 minutes at room temperature, with rhodamine-labeled phalloidin (Molecular Probes, 1:2000) added as a counterstain. Wing discs and pupal wings were fixed in 4% formaldehyde in Brower fix buffer lacking EGTA at room temperature for 40 minutes. The following primary antibodies were used: rat anti-Ds (1:20,000) (Yang et al., 2002), rabbit anti-Ds (1:100) (Strutt and Strutt, 2002), rat anti-Ft (1:2000) (Yang et al., 2002), mouse anti-DSRF (Geneka, 1:1000), rabbit anti-pMad (1:2000) (Tanimoto et al., 2000) and mouse anti-βgal (Developmental

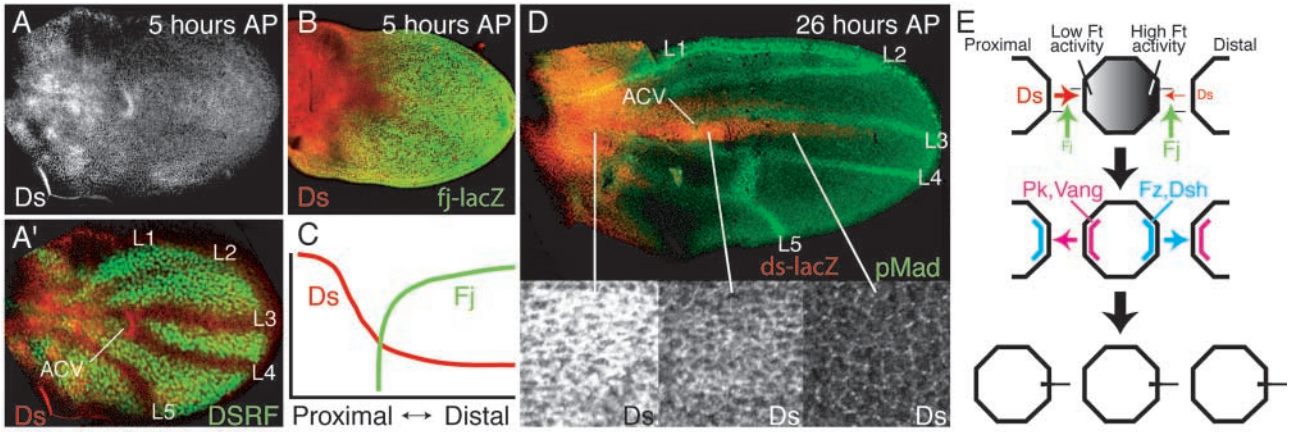

Fig. 1. Ds and Fj expression, and the signaling model. In this and subsequent figures anterior is up and proximal is to the left. (A,A′) Anti-Ds staining (A, white; A′, red) in wing at 5 hours AP. The primordia of longitudinal veins one through five (L1-L5) and the anterior cross vein (ACV) are shown in A′ by the absence of anti-DSRF staining (green). (B) *fj-lacZ* (green) is expressed in the distal wing at 5 hours AP, in a region complementary to the region of high anti-Ds staining (red). (C) Expression levels of Ds and *fj-lacZ* at 5 hours AP. (D) *ds-lacZ* expression (red) and anti-Ds staining (white) in the ds^{05142}/+ wing at 26 hours AP. Top panel is stained with anti-pMad (green) to identify the veins; lower panels show relative levels of Ds at indicated positions. Broad stripes of Ds expression extend out along the center of the wing, forming a proximal to distal gradient. (E) Signaling model. Ds on one cell binds to Ft on the adjacent cells and inhibits its activity; Fj inhibits this inhibition. Ft activity in the central cell is polarized by the higher levels of Ds and the lower levels of Fj on the proximal side. Polarized Ft activity biases the subsequent Fz signaling between cells, leading to the polarized distribution of proteins to the proximal (Pk, Vang) or distal (Dsh, Fz) faces of the cell, and to the formation of hairs on the distal faces.

Studies Hybridoma Bank, 1:1000). Fluorescent secondary antibodies were visualized using a Biorad MRC 1024 confocal microscope.

Timed induction of patterned *ds* misexpression

sal-gal4/tub-gal80^{ts}; *UAS-ds*/+ embryos or white prepupae were collected and reared at either 20°C or 30°C. Larvae or pupae were upshifted to 30°C or downshifted to 20°C to induce or repress *ds* expression, respectively. For each time span we scored more than fifty adults for temperature shifts before pupariation and more than sixteen adults for temperature shifts at or after pupariation.

Results

Binding between Ft and Ds

Almost all of the classic cadherins mediate homophilic cell adhesion (Takeichi, 1995). To test whether the same was true of Ft or Ds, we transfected *ft* and *ds* constructs into *Drosophila* S2 cells, which normally show little or no cell aggregation (Fig. 2A). Although transfection of *Drosophila E-cadherin* (*DE-cadherin*; also known as *shotgun*) (Oda et al., 1994) or the protocadherin encoded by *flamingo* (Usui et al., 1999) elicited cell aggregation (cell clusters of >100 or 50 cells, respectively), we were unable to elicit any detectable homophilic aggregation amongst *ft*-transfected cells or *ds*-transfected cells (Fig. 2B, and data not shown). By contrast, cells co-transfected with *ft* and *ds* aggregated, forming clusters of approximately 50 cells (Fig. 2C). The aggregation was weaker that the homophilic adhesion between cells expressing *DE-cadherin*, but similar to the aggregation between cells expressing *flamingo*, as measured by their sensitivity to the rate of agitation. At higher rates of agitation (see Materials and methods) cells expressing *DE-cadherin* still aggregated, whereas aggregation between cells expressing *flamingo*, or cells co-transfected with *ft* and *ds*, was lost. The aggregation between cells co-transfected with *ft* and *ds* was inhibited by the addition of EGTA, suggesting that, as with classic Cadherins, aggregation is dependent on Ca^{2+} (Fig. 2D). Similarly, cells separately transfected with *ft* and *ds* aggregated when placed together (Fig. 2G-J).

Ft and Ds also stabilized each other in vitro and in vivo. In unaggregated S2 cells transfected with *ft* or *ds*, most of the anti-Ft and anti-Ds staining was localized in internal vesicle-like structures, and we could detect only low levels at the cell surface (Fig. 2E,F). Similarly, in clusters containing both *ft*- and *ds*-transfected cells, we detected only low levels of Ds or Ft at the interface between the *ds*-expressing or the *ft*-expressing cells (Fig. 2G,H, and data not shown). However, anti-Ft and anti-Ds staining was substantially stronger at the interface between *ft*-transfected and *ds*-transfected cells (Fig. 2G-J). In vivo, anti-Ft staining was heightened in *ds*-overexpressing regions in late third instar wing discs (Fig. 2K,L), and anti-Ds staining was heightened in *ft*-overexpressing regions (Fig. 2M,N). These effects were post-transcriptional, as we could not detect any equivalent changes in mRNA levels with in situ hybridization (data not shown). Clones overexpressing one protein also appeared to alter the distribution of the other in adjacent wild-type cells, ‘capping’ the protein to the surface facing the clone while reducing levels on the other surfaces of the cell (Fig. 2L,N).

These changes in protein stability and distribution are consistent with our own and previously reported results from ft^{-}, ds^{-} and ds^{-} ft^{-} double mutant clones (Ma et al., 2003; Strutt and Strutt, 2002) (data not shown). In the wing pouch Ds is reduced in ft^{-} clones, whereas Ft is more diffuse (although the overall staining is elevated) in ds^{-} clones. Moreover, Ft and Ds are redistributed at mutant clone boundaries in a manner consistent with the capping of proteins on the cell surface. For example, wild-type cells at the boundaries of ds^{-} ft^{-} double mutant clones lose both anti-Ft and anti-Ds staining on the surface facing the clone (Ma et al., 2003). Thus, both the in

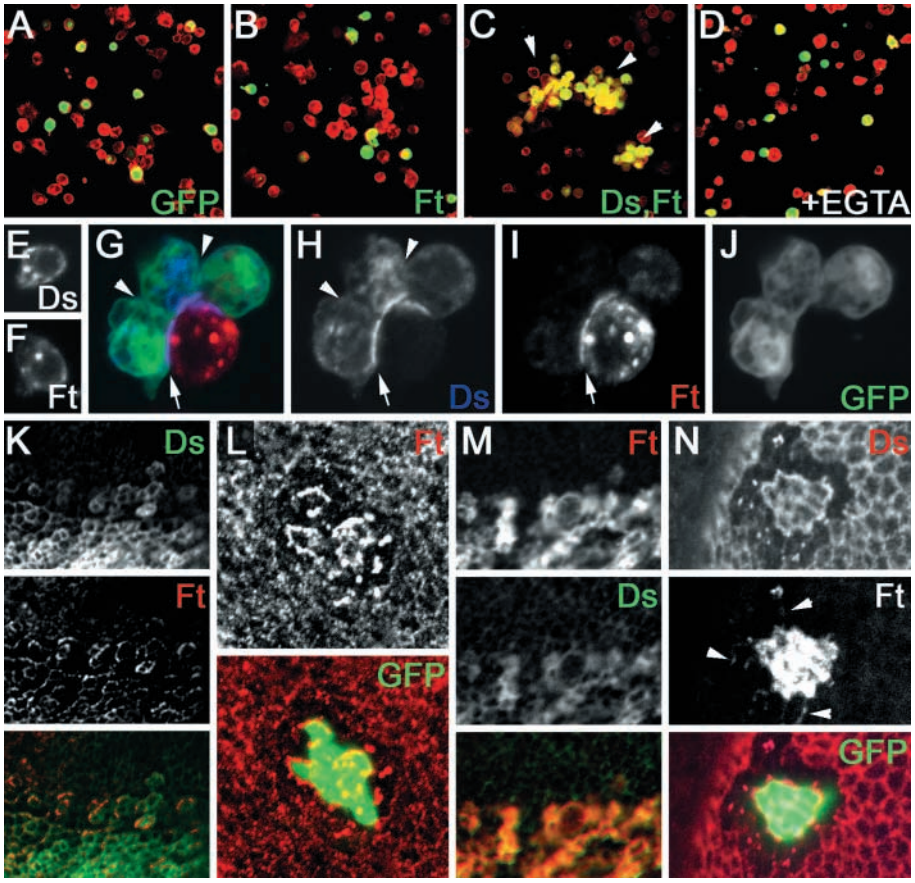

Fig. 2. Interactions between Ds and Ft. (A) S2 cells transformed with *gfp* (green) alone do not adhere. (B) Cells co-transfected with *ft* and *gfp* do not adhere. (C) Cells co-transfected with *ds*, *ft* and *gfp* form large clusters (arrowheads). (D) The addition of 1 mM EGTA to cells co-transfected with *ds*, *ft* and *gfp* blocks adhesion. (E,F) Cells transformed with only *ds* (E) or *ft* (F) have only low levels of Ds or Ft on the cell surface. (G-J) Mixtures of cells separately transformed with *ft* (I, anti-Ft, red) and with *ds* and *gfp* (H, anti-Ds, blue; J, GFP, green) adhere. Adhering cells have higher levels of anti-Ds and anti-Ft staining at the interface between *ds*-expressing and *ft*-expressing cells (G-I, arrows), but low levels of anti-Ds staining at the interface between *ds*-expressing cells (G,H; arrowheads). (K-N) Changes in Ft or Ds stability and distribution induced by misexpression of *ds* or *ft* in late third instar wing discs. (K,L) Misexpression of *ds* in the posterior using *en-gal4* (K) or in clones using *FLPout-gal4* (L; identified by *UAS-GFP*, green) causes heightened anti-Ft staining at the cell surface. Wild-type cells at the interface with the *ds*-expressing clone (L) had a lower than normal anti-Ft staining, but staining was higher at the interface with the clone. (M,N) Misexpression of *ft* in the posterior using *en-gal4* (M), or in clones using *FLPout-gal4* (N; identified by *UAS-GFP*, green), causes heightened anti-Ds staining at the cell surface. Wild-type cells at the interface with the *ft*-expressing clone (N) had a lower than normal anti-Ds staining, but staining was higher at the interface with the clone. This was noted especially in clones in the prospective notum and wing hinge. Some *ft*-misexpressing filopodia or cell remnants extend from the clone (arrowheads) and have high levels of Ds.

vitro and in vivo results indicate a preferentially heterophilic interaction between Ft and Ds. This type of binding is unusual for cadherin family members, and is consistent with the ligand-receptor relationship proposed by the signaling model.

Patterned Ds expression is sufficient to reorient PCP and Fz redistribution

To test whether Ds is sufficient to reorient PCP in the wing, we used the UAS-GAL4 system to drive patterned *UAS-ds* expression. We observed PCP defects if we used drivers that drove expression in the *spalt* (*sal*) pattern, which forms a gradient orthogonal to the proximodistal axis of the wing (Fig. 3B,E). Similarly, misexpressing *ds* in the *Distal-less* (*Dll*) pattern, which forms a distal to proximal gradient that is opposite to the endogenous *ds* expression pattern, partially reversed hair polarity (Fig. 3C). The defects were similar to those caused by misexpressing *fz* using *dll-gal4* (Adler et al., 1997) or *sal-gal4* (Fig. 3F). PCP defects could also be induced by creating sharp boundaries of *ds* misexpression, using either the posterior-specific driver *engrailed* (*en*) (Fig. 4B) or *patched* (*ptc*), which is expressed at high levels in cells just anterior to the fourth longitudinal vein (data not shown). *en-gal4* and *ptc-gal4* induced PCP defects in wild-type tissue adjacent to the region of misexpression. Similar non-autonomy was found surrounding *ds* mutant clones, indicating that the PCP defects are propagated to adjacent cells (Adler et al., 1998; Ma et al., 2003; Strutt and Strutt, 2002). Thus, an artificial gradient or boundary of Ds misexpression can alter wing hair polarity.

From 18 to 30 hours AP, the Fz protein is redistributed to the distal face of each cell in the wing (Fig. 1E) (Strutt, 2001). Some polarity mutations prevent this polarization, but ds^{-} and ft^{-} simply reorient the polarization without disrupting it (Ma et al., 2003; Strutt and Strutt, 2002). Similarly, patterned *ds* misexpression reoriented the polarization of Fz-GFP within cells without disrupting it (Fig. 3G,H). The adult PCP defects induced by localized *ds* misexpression depended on the presence of intact Fz/Dsh PCP signaling, as misexpression of *ds* in the dsh^{1} mutant background generated PCP defects resembling those of dsh^{1} rather than those of *ds* misexpression (data not shown). Misexpression of *ds* also did not generate the multiple wing hair phenotypes that normally result from misexpression of *fz* (Fig. 3E; compare with Fig. 3F). Thus, Ds affects the direction, rather than the presence, of polarized Fz redistribution.

Correct PCP in the absence of a Ds gradient

The data above indicate that Ds is sufficient to reorient PCP. However, that does not test whether the endogenous gradient of Ds is required for PCP. We therefore used strong misexpression of *ds* to override the endogenous *ds* pattern and

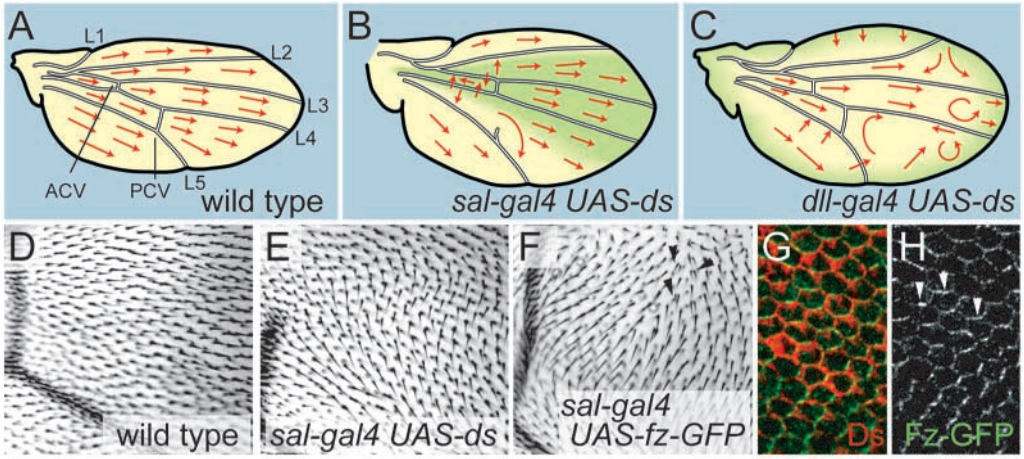

Fig. 3. Wing hair polarity after patterned *ds* misexpression. (A-C) Diagrams of hair polarity (red arrows) in adult wings. Approximate regions of Gal4-driven gene misexpression are shown in green. (A) Wild type. Veins are labeled as in Fig. 1, with the addition of the posterior cross vein (PCV). (B,C) Gradients of *ds* misexpression commonly reorient hairs from regions of high to low expression. (B) *UAS-ds/+*; *sal-gal4/+*. (C) *UAS-ds/+*; *dll-gal4/+*. (D-F) Hair polarity in a region just distal to the PCV. (D) Wild type. (E) *UAS-ds/+*; *sal-gal4/+*. (F) *sal-gal4/+*; *UAS-fz-GFP/+* induces multiple wing hair phenotype (arrowheads). (G,H) Redistribution of Fz-GFP (green, white) by Ds (red) in *sal-gal4/+*; *UAS-ds/+* wing at 26 hours AP; region shown is posterior to the PCV. The normally distal (right) Fz localization within each cell has frequently changed to the posterior (H, arrowheads).

create wings with uniform *ds* expression. We used either Gal4 driven from a *tubulin* promoter (*tub-gal4*), looked at the posterior of wings with posteriorly-expressed *engrailed* (*en*)-*gal4*, or looked at dorsal wings with dorsally-expressed *apterous* (*ap*)-*gal4*.

Wing PCP was largely unaffected by the presence of uniformly misexpressed *ds* (*tub-gal4*, Fig. 4C; posterior of *en-gal4*, Fig. 4B; dorsal of *ap-gal4*, data not shown). This was true even though the levels of anti-Ds staining being driven were at (*en-gal4*, Fig. 2K) or well above (*tub-gal4*, Fig. 4F; compare with Fig. 4E) the high levels observed in the proximal regions of the wild-type wing. To rule out the possibility of signaling from the endogenous Ds, we repeated these experiments in a ds^{05142} mutant background, which has a strong PCP phenotype (Fig. 4A) and lacks detectable cell surface anti-Ds staining. Uniform misexpression of Ds (*tub-gal4* or posterior of *en-gal4*) almost completely rescued the ds^{05142} PCP phenotype distal to the anterior cross vein, without inducing any additional PCP defects (Fig. 4D,H, and data not shown). Thus, distal to the anterior cross vein, PCP must depend on cues other than the pattern of *ds* transcription.

We were unable, however, to rescue ds^{05142} PCP defects in the most proximal regions of the wing (Fig. 4D,J). This is the region of the wing that normally has high Ds expression at 5 hours AP (Fig. 1A-C), and thus may require higher levels of Ds activity than could be supplied by misexpression. However, as noted above, the levels of anti-Ds driven using *tub-gal4* were well above normal proximal levels (Fig. 4E,F). Moreover, raising the temperature at which the larvae were reared to 30°C, which results in higher activity of the cold-sensitive Gal4, did not reduce the proximal PCP phenotype (data not shown). As there is also a sharp gradient of Ds expression at the distal boundary of this expression domain (Fig. 1A-C), we favor an alternative explanation, that the Ds gradient observed here is locally required for normal PCP (see Discussion).

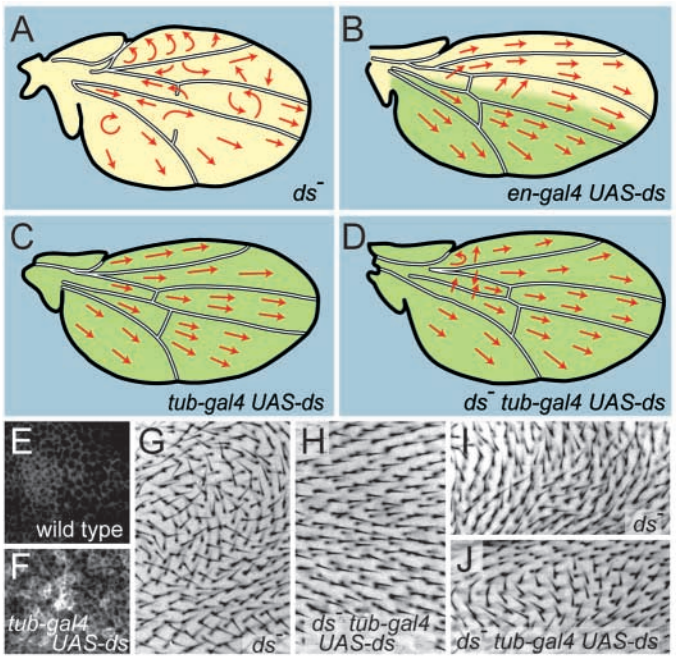

Fig. 4. Wing hair polarity in regions of uniform *ds* misexpression. (A-D) Diagrams of hair polarity in adult wings. (A) ds^{05142}. (B) *en-gal4/+*; *UAS-ds/+*. Hair polarity is normal except near the boundary of misexpression. (C) *tub-gal4/UAS-ds*. Hair polarity is normal. (D) ds^{05142}; *tub-gal/UAS-ds*. Hair polarity is rescued (compare with A), except in the proximal wing. (E,F) Comparison of anti-Ds staining in the proximal region of 5 hour AP wings in wild type (E) and *tub-gal4*/*UAS-ds* (F). Wings were stained in the same well and photos were taken using identical settings. (G-J) Comparison of hair polarity between ds^{05142} (G,I) and ds^{05142}; *tub-gal/UAS-ds* (H,J) wings. Uniform *ds* expression rescues the PCP defect in the distal region between L3 and L4 (G,H), but not in the proximal region just posterior to L1 (I,J).

The Fj gradient plays only a minor role in PCP

What orients PCP in the more distal portions of the wing? One redundant cue suggested by previous studies is the distally expressed Fj (Fig. 1B,C). As discussed above, Fj may be required in vivo for strong interactions between Ft and Ds. Co-transfection of *fj* into cells co-transfected with *ft* and *ds* did not detectably enhance their aggregation or reduce their sensitivity to high rates of agitation. This did not appear to

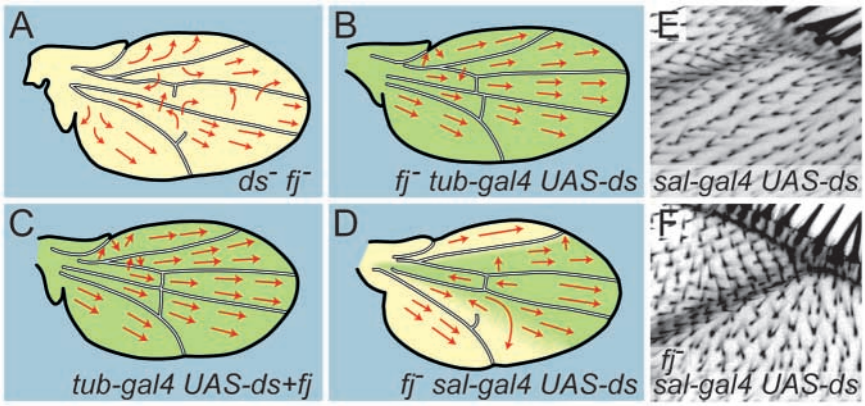

Fig. 5. The Fj gradient plays only a minor role in PCP. (A-D) Hair polarity in adult wings. (A) ds^{05142} fj^{d1}. Polarity is not appreciably worse than in ds^{05142} (see Fig. 4A). (B) *UAS-ds/+*; fj^{d1}; *tub-ga4/+* and (C) *tub-gal4/UAS-ds UAS-fj*. Polarity is normal, except in the proximal wing. (D,F) *UAS-ds/+*; fj^{d1} *sal-gal4*/fj^{d1}. (E) *UAS-ds*; *sal-gal4/+*. (E,F) The hair polarity near the tip of L2 was abnormal in a fj^{d1} background (also compare D with Fig. 3B).

be due to endogenous Fj, as we could not detect Fj in untransfected S2 cells by western blot. However, subtle quantitative effects would likely be difficult to detect in this assay. It is possible that S2 cells do not respond to *fj* in a normal manner; for instance, if Fj regulates the membrane localization of Ft or Ds in epithelial cells, this may differ in the non-epithelial S2 cells, as they lack any obvious apical-basal polarity.

If Fj does modulate Ft-Ds interactions in vivo, a gradient of Fj could spatially regulate the activity of even uniformly misexpressed Ds, accounting for the failure of uniform Ds to disrupt PCP. However, our tests using a null allele of *fj* indicate that the contribution of *fj* to wing PCP is minor. ds^{05142} fj^{d1} double mutant wings did not show an appreciably stronger PCP defect than that observed in ds^{05142} wings (Fig. 5A; compare with Fig. 4A). Moreover, uniform misexpression of *ds* in a *fj* null background caused only minor PCP defects in the proximal wing (*tub-gal4*, Fig. 5B; posterior of *en-gal4*, not shown), and PCP in most of the wing was normal. Overriding the endogenous gradients with simultaneous uniform misexpression of *fj* and *ds* had no effect on PCP (*tub-gal4*, Fig. 5C; posterior of *en-gal4*, data not shown). Similarly, the PCP defects caused by patterned *ds* misexpression using *sal-gal4* were only slightly more extensive in a *fj* null background (Fig. 5D,F; compare with Fig. 3B and Fig. 5E). Thus, the wing must have some sources of polarizing information that can act in the absence of a gradient of *ds* or *fj* transcription.

The timing of Ds activity in PCP

Does Ds act during the polarized redistribution of the core polarity proteins, or at an earlier stage? To answer this question, we made use of the PCP defects induced by patterned *ds* misexpression using *sal-gal4*, and regulated the timing of misexpression with a temperature-sensitive version of the Gal4 inhibitor Gal80 (McGuire et al., 2003).

When we initiated misexpression during larval stages we observed PCP defects. Defects were rare if induction occurred after 0 hours AP (Fig. 6A). Induction at 0 hours AP resulted in scattered ectopic anti-Ds staining at 5 hours AP at 30°C (the equivalent of 6 hours AP at 25°C; Fig. 6D), and strong misexpression at 22 hours AP at 30°C (the equivalent of 24 hours AP at 25°C; Fig. 6E). Thus, PCP was usually normal despite the misexpression of *ds* during the stage when the polarized redistribution of core polarity proteins occurs (18-30 hours AP at 25°C).

Conversely, if we induced misexpression early but suppressed misexpression beginning in mid-third instar, few PCP defects were induced; however, if we did not begin suppression until later larval or early pupal stages, strong PCP defects were observed (Fig. 6B). If the suppression of misexpression began at 0 hours AP, ectopic anti-Ds staining was still visible at 5 hours AP at 20°C (the equivalent of 4 hours AP at 25°C; Fig. 6F), but was undetectable at 24 hours AP at 20°C (the equivalent of 17-19 hours AP at 25°C; Fig. 6G). Thus, PCP defects were induced despite the apparently normal *ds* expression during the stage of Fz redistribution.

The effects of Ft misexpression on PCP

As Ft is uniformly expressed in the wing, it is not thought to play an instructive role in wing PCP. Nonetheless, we used misexpression to answer two questions raised by the Ft-Ds signaling model. First, is Ft misexpression sufficient to drive PCP in an orientation opposite that caused by Ds? Second, how does the activity of ectopic Ft depend on the presence of Ds and Fj?

UAS-ft showed substantial activity in vivo: when driven with a low-level driver (*da-gal4*) it rescued the pupal lethality of $ft^{G\text{-}rv}$/ft^{fd} heterozygotes and showed substantial rescue of the mutant wing PCP and tarsal segment defects (data not shown). Unfortunately, driving stronger expression with *tub-gal4* in wild-type wings resulted in larval lethality prior to the stage when PCP defects could be assessed. However, driving *ft* misexpression with *en-gal4* (posterior) or *ap-gal4* (dorsal) was not lethal, and resulted in strong PCP defects. These defects were observed not only near the boundaries of misexpression, but also within regions of apparently uniform misexpression in the posterior or dorsal wing blade (Fig. 7A,I,J for *en-gal4*, Fig. 7B for *ap-gal4*). Driving *ft* expression centrally using *ptc-gal4* also resulted in strong PCP defects (Fig. 7C,K), as did driving *ft* expression in a gradient orthogonal to the proximodistal axis using *sal-gal4* (Fig. 7D,L). Interestingly, all of these PCP defects were observed in a central region near the two cross-veins. This is also the region that shows the most common PCP defects in *ft* mutant clones (Strutt and Strutt, 2002). As expected from the Ft-Ds signaling model, which posits high Ft activity in the distal wing, driving high distal expression with *dll-gal4* did not induce any PCP defects (data not shown).

The Ft-Ds signaling model hypothesizes that Ds inhibits Ft activity, and that Fj modulates that inhibition. Thus, hairs

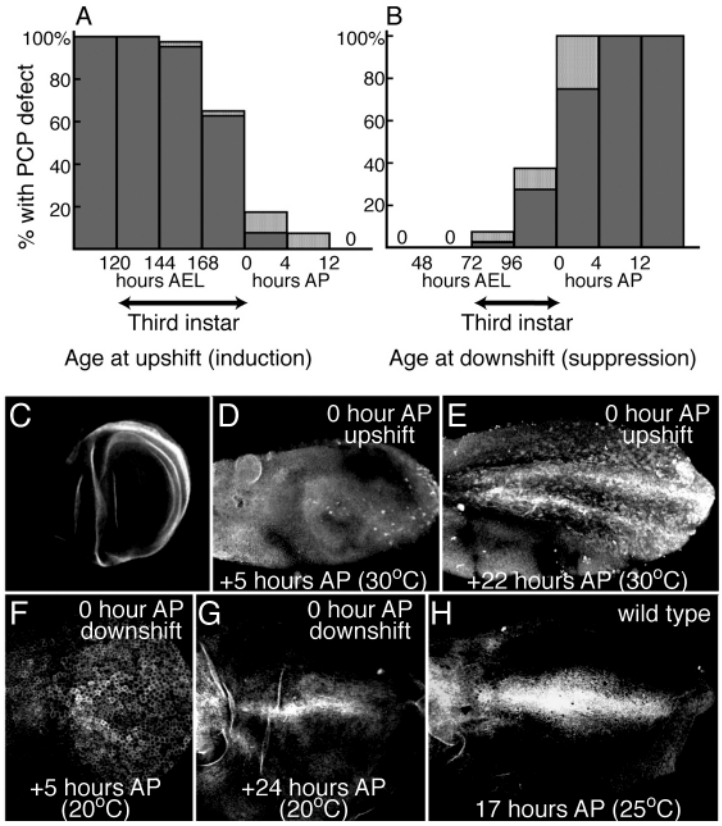

Fig. 6. Timed misexpression of Ds, induced in *sal-gal4*/*tub-gal80^{ts}*; *UAS-ds*/+ wings using temperature upshifts (induction) or downshifts (suppression). (A,B) Percentage of flies showing PCP defects. Dark gray indicates strong defects; light gray, weak defects. The relationship between the absolute age of the larvae or pupae and the developmental stage was altered by the different rearing temperatures, thus the approximate third instar stages are indicated. Flies reared continuously at 20°C did not show any adult PCP defects or ectopic anti-Ds staining in wing discs (C), whereas those reared continuously at 30°C showed strong hair reorientation (similar to that seen in Fig. 3B,E). (D-H) Anti-Ds staining in pupal wings. A temperature upshift at 0 hours AP induced scattered ectopic anti-Ds staining by 5 hours AP at 30°C (D, approximately equivalent to 6 hours AP at 25°C) and strong misexpression in the broad *sal-gal4* pattern by 22 hours AP at 30°C (E, approximately equivalent to 24 hours AP at 25°C). A temperature downshift at 0 hours AP did not suppress ectopic anti-Ds staining by 5 hours AP at 20°C (F, approximately equivalent to 4 hours AP at 25°C) but did by 24 hours AP at 20°C (G, approximately equivalent to 17-19 hours AP at 25°C). The narrow stripe of Ds expression in G is also observed in wild-type wings at this stage (H), and differs in level and extent from that driven by *sal-gal4* (E).

should point towards regions of high Ft or Fj activity, but away from regions of high Ds activity. In most regions of the wing this prediction was met. For example, hairs near the boundary of *en-gal4*-driven or *ptc-gal4*-driven *ft* misexpression tended to point posteriorly (Fig. 7A,C,I,K), whereas anterior cells near the boundary of *en-gal4*-driven *ds* misexpression tended to point anteriorly (Fig. 4B). However, some exceptions were also observed, where hairs appeared to point towards regions of low *ft* expression. Some of this may be due to propagation of PCP errors from within the domains of misexpression to boundary regions. However, it should also be remembered that Ft and Ds stabilize each other (Fig. 2), and thus regions misexpressing high levels of one protein may also have high levels of the other. The effects of *fj* misexpression on PCP are weak, but hairs do reorient towards region of high *fj* (Zeidler et al., 2000) (Fig. 7G). Because endogenous Fj may mask the effects of *fj* misexpression, we tried the same experiments in a *fj* mutant background; this increased the strength of the PCP phenotype (compare Fig. 7H,P with Fig. 7G,O).

If Ds spatially regulates Ft activity, then the PCP defects induced by uniform *ft* mixexpression might be reduced by a reduction in Ds levels. Indeed, in a ds^{05142} background, the posterior PCP defects induced by the posterior expression of *ft* were weakened (Fig. 7E,M). However, they were not eliminated, despite the absence of detectable cell surface Ds in the ds^{05142} background, indicating that the misexpressed Ft has substantial activity even in the absence of its putative ligand. Similarly, if Fj normally plays a role strengthening the Ds-Ft interaction, the effects of misexpressing *ft* should be reduced in a fj^{-} background. As expected, posterior PCP defects were weakened in fj^{-} wings (Fig. 7F,N).

Discussion

The *ds* gradient: sufficient but not necessary

Several of our gain-of-function findings are consistent with previous loss-of-function findings, and support the model that Ft-Ds signaling is sufficient to influence wing PCP. Ft and Ds preferentially bind in vitro. Patterned misexpression of *ds* is sufficient to alter wing PCP, consistent with its proposed role as a ligand. The effects of *ft* or *ds* misexpression on the direction of hair polarization are usually the opposite of those previously reported from *ft* or *ds* loss of function. The direction of hair polarity induced by ectopic *ft* or *ds* are usually opposite from each other, consistent with the proposal that Ds binding inhibits Ft activity. Finally, the effects of Ft misexpression are reduced in a *ds* mutant background, consistent with the proposed role of Ft as a receptor.

Nonetheless, our data also show that the proximal to distal gradient of *ds* expression is not necessary for PCP throughout the wing, despite the distal defects observed in loss-of-function *ds* mutants. Instead, our experiments show that uniform *ds* misexpression can rescue the PCP defects caused by a *ds* mutation in all but the most proximal portions of the wing. Thus, *ds* is permissive for PCP in most of the wing, and there must be another polarity cue in the distal wing that is sufficient to orient PCP in the presence of uniformly transcribed *ds*. Our experiments indicate that this distal cue is not provided by the distally expressed Fj protein: distal PCP is not disrupted either by uniform misexpression of both *ds* and *fj*, or by uniform misexpression of *ds* in a *fj* null mutant.

It remains possible that the distal cue functions by regulating Ft-Ds signaling. Our studies tested the PCP inputs from the

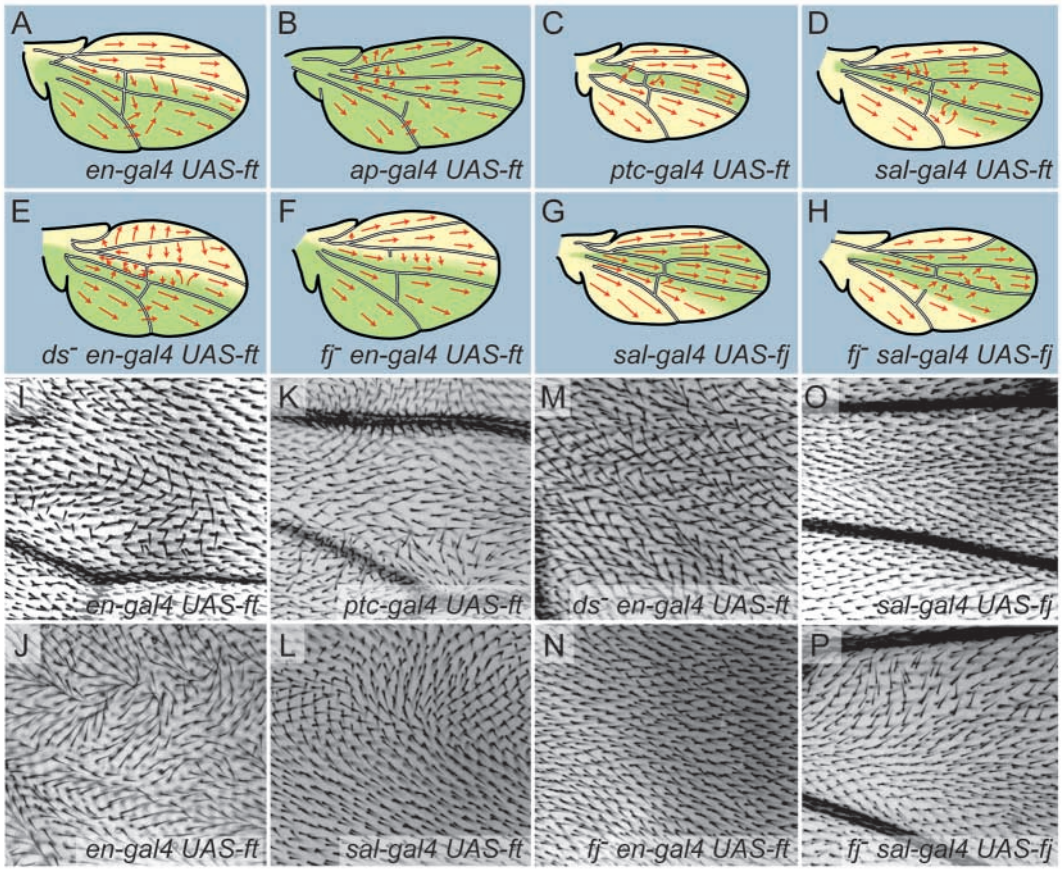

Fig. 7. Wing hair polarity after *ft* and *fj* misexpression. (A-H) Hair polarity in adult wings. (A) *UAS-ft/+*; *en-gal4/+*. (B) Dorsal hairs in *UAS-ft/+*; *ap-gal4/+*. (C) *UAS-ft/+*; *ptc-gal4/+*. (D) *sal-gal4/+*; *UAS-ft/+*. (E) *UAS-ft/+*; ds^{05142} *en-gal4*/ds^{05142}. (F) *UAS-ft/+*; fj^{d1} *en-gal4*/fj^{d1}. (G) *sal-gal4/+*; *UAS-fj/+*. (H) fj^{d1} *sal-gal4*/fj^{d1}; *UAS-fj/+*. (I-P) Examples of wing hair polarity. The regions shown are between distal L3 and L4 for I and K, posterior to the PCV for J, L, M and N, and between distal L4 and L5 for O and P. (I,J) *en-gal4/+*; *UAS-ft/+*. (K) *UAS-ft/+*; *ptc-gal4/+*, (L) *sal-gal4/+*; *UAS-ft/+*. Hairs are often repolarized towards regions with higher *ft* expression (A,C,I,K, near the anteroposterior boundary), although exceptions are observed. Hairs near the PCV are repolarized within regions of uniform misexpression (A,B,J). (M) *UAS-ft/+*; ds^{05142} *en-gal4*/ds^{05142}. Posterior polarity defects are decreased in the *ds* mutant background (compare with J), but are not eliminated; the polarity differs from that in the same region of the *ds* mutant (Fig. 4A). (N) *UAS-ft/+*; fj^{d1} *en-gal4*/fj^{d1}. Posterior polarity defects are decreased by the loss of *fj* (compare with J). (O) *sal-gal4/+; UAS-fj/+*. (P) fj^{d1} *sal-gal4*/fj^{d1}; *UAS-fj/+*. The hair polarity induced by *fj* misexpression was normal between L4 and L5 (O), but was altered in the fj^{d1} background (P). Hairs point towards regions with higher *fj* expression (H,P).

patterns of *ds* and *fj* transcription, but unknown factors might post-transcriptionally regulate the forms of Ds or Ft protein produced, or their availability at the cell surface. It also is possible that Ft activity is spatially regulated by binding partners other than Ds. *ft* mutants have stronger PCP and disc overgrowth defects than do *ds* mutants, and misexpression of *ft* still causes PCP defects in a *ds* mutant lacking detectable cell surface protein.

Alternatively, the cue may be provided by a mechanism that is completely independent of Ft or Ds. One often-proposed candidate is signaling via the *Drosophila* Wnts, especially given their patterned (distal or marginal) expression (Baker, 1988; Gieseler et al., 2001; Janson et al., 2001; Kozopas and Nusse, 2002) and the involvement of the Wnt receptor Fz in PCP. Vertebrate Wnt7a also helps regulate PCP in the inner ear (Dabdoub et al., 2003). However, although the misexpression of *Drosophila wnt4* can disrupt wing PCP (Lawrence et al., 2002), PCP defects have not been reported in *Drosophila* Wnt mutants.

Is a Ds gradient required in the proximal wing?

Although a *ds* gradient is not required for PCP in most of the wing, it is possible that such a gradient is required locally in the portion of the wing near and proximal to the anterior cross vein. We were unable to rescue proximal *ds* mutant PCP defects with uniform Ds expression, and our data suggests that this was not simply a failure caused by insufficient Ds levels. This is the location of a strong proximal to distal boundary or gradient of endogenous Ds expression at 5 hours AP (see Fig. 1A-C). Thus, we favor the view that this sharp Ds gradient acts as a PCP cue in the proximal wing. If so, this indicates that the cues that orient PCP in the wing are not generally distributed; rather, the wing may be a patchwork of different regions that rely on different cues. This would provide a mechanism for locally altering PCP during evolution without globally affecting polarity in the wing.

Mechanisms

The hypothesis that Ft acts as a receptor and Ds acts as a ligand

for PCP is based, not only on the uniform expression pattern Ft, but also on epistasis experiments in the eye, where the PCP activity of *ds* clones appears to depend on the presence of *ft* (Yang et al., 2002). Wing PCP can also be disrupted by the expression of a truncated form of Ds lacking its intracellular domain, which is consistent with Ds acting as a ligand (H.M. and S.S.B., unpublished).

However, we have shown here that misexpressed Ft retains PCP activity in a *ds* mutant that eliminates detectable cell surface Ds. Thus, Ft activity is apparently not strictly dependent on patterned Ds expression. Again, this is consistent with the greater severity of *ft* mutant phenotypes compared with *ds*, and with our finding that uniform misexpression of *ft* but not *ds* can cause PCP defects. As we do not have any evidence for homophilic Ft binding, the unbound Ft molecule may have basal PCP activity. Alternatively, low-level homophilic binding or heterophilic binding to some unknown ligand may activate Ft in the absence of Ds.

It is not yet known how Ft-Ds interactions regulate the polarized redistribution of the core polarity proteins in the older pupal wing. The cytoplasmic domains of Ft and Ds contain potential regions for β-catenin binding (Clark et al., 1995), and *ft* and *ds* mutants can enhance the effects of β-catenin (Armadillo) misexpression (Greaves et al., 1999). However, although expression of *DE-cadherin* in vitro results in a detectable concentration of Armadillo at the cell membrane (Oda et al., 1994), we have not detected similar effects after expression of *ft* or *ds* (data not shown). Moreover, clones homozygous for a strong *armadillo* mutation do not affect PCP (Axelrod et al., 1998). It has also been suggested that the cytoplasmic domain of Ft binds to and changes the activity of Grunge, the *Drosophila* homolog of the Atrophin transcriptional co-repressor (Fanto et al., 2003), but it is not known whether this interaction is altered by Ft-Ds binding.

Our studies examining the timing of Ds activity suggest that its effects on the polarization of the core polarity proteins are likely to be indirect, as Ds acts before the polarized redistribution of the core polarity proteins within cells can be detected. Patterned misexpression of Ds at later stages, during the time of core protein polarization, had no effect on PCP. The period sensitive to *ds* misexpression is roughly congruent with the period of early Fz activity identified by Strutt and Strutt (Strutt and Strutt, 2002); if loss of Fz is limited to a period from 6 to 24 hours AP it leads to distinct, *ds*-like PCP defects. Thus, early Fz and Ds activity may be linked, or they may share a common target.

The only known sign of cell polarization during the stages sensitive to Ds and early Fz activity is the redistribution of the Widerborst PP2A regulatory subunit from the anterior-proximal side to the distal side of wing cells at some time between 8 and 18 hours AP (Hannus et al., 2002). Reductions in Widerborst activity can disrupt the polarized redistribution of Fmi and Dsh, suggesting an instructive role. However, Widerborst polarization is not affected by ectopic Fz expression, making it less likely that Widerborst polarization mediates early Fz activity.

Heterophilic protocadherins

A final interesting feature of our results is the preferentially heterophilic binding we observed between Ft and Ds in vitro. This result is consistent with our own and previous in vivo analyses of protein distribution within and adjacent to *ft* and *ds* mutant and overexpression clones (Ma et al., 2003; Strutt and Strutt, 2002) (this study). With the exception of the desmosomal cadherins (Chitaev et al., 1997; Syed et al., 2002), this kind of binding is unusual for cadherin-like proteins.

A number of mammalian Fat-like (Fat1, Fat2, Fat3, XP_227060) and Ds-like (Protocadherin 16, Cdh23) proteins have been identified (Bolz et al., 2001; Bork et al., 2001; Cox et al., 2000; Dunne et al., 1995; Mitsui et al., 2002; Nakajima et al., 2001; Nakayama et al., 1998; Ponassi et al., 1999). Mutations and knockouts have been examined for a few of these; however, conjectures about the bases of the mutant phenotypes have largely assumed that these proteins mediate homophilic cell adhesion (Bolz et al., 2001; Bork et al., 2001; Ciani et al., 2003; Wilson et al., 2001). It will be interesting to see whether the preferentially heterophilic interactions observed in *Drosophila* are preserved in similar mammalian proteins.

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