Cleavage and secretion is not required for Four-jointed function in *Drosophila* patterning

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Summary

***four-jointed* (*fj*) is required for proximodistal growth and planar polarity in *Drosophila* tissues. It encodes a predicted type II transmembrane protein with putative signal peptidase sites in its transmembrane domain, and its C terminus is secreted. Fj has therefore been proposed to act as a secreted signalling molecule. We show that Fj protein has a graded distribution in eye and wing imaginal discs, and is largely localised to the Golgi in vivo and in transfected cells. Forms of Fj that are constitutively secreted or anchored in the Golgi were assayed for function in vivo. We find that cleavage and secretion of Fj is not necessary for activity, and that Golgi-anchored Fj has increased activity over wild type. *fj* has similar phenotypes to those caused by mutations in the cadherin-encoding genes *fat* (*ft*) and *dachsous* (*ds*). We show that *fj* interacts genetically with *ft* and *ds* in planar polarity and proximodistal patterning. We propose that Fj may act in the Golgi to regulate the activity of Ft and Ds.**

Key words: Four-jointed, *Drosophila*, Planar polarity, Cadherins

Introduction

The *four-jointed* (*fj*) gene of *Drosophila* is involved in proximodistal (PD) patterning and regulation of planar polarity in many adult tissues. In homozygous *fj* flies, the wings and legs are shorter than normal (Tokunaga and Gerhart, 1976; Villano and Katz, 1995; Brodsky and Steller, 1996). Wings show a specific reduction in the distance between the anterior and posterior crossveins (Fig. 3B), whereas in legs, the femur, tibia and first three tarsal segments are truncated, and the T2 and T3 tarsal segments are fused (Fig. 5D). Mosaic analysis has shown a lack of joint structures in wild-type tissue adjacent to *fj* clones, indicating that *fj* acts non-autonomously in PD patterning (Tokunaga and Gerhart, 1976).

Although PD patterning defects can be observed in homozygous *fj* animals, strong defects in planar polarity are only evident around clones of mutant tissue. In wild-type wings, each cell produces a single hair which points distally. In and around *fj* mutant clones, hair polarity is disrupted (Zeidler et al., 2000). Polarity in the eye is manifested in the orientation of groups of cells, the ommatidia. The orientation of the ommatidia is dependent on the position in the eye, with those in the dorsal half of the eye pointing in the opposite direction and having opposite chirality to those in the ventral half (Wolff and Ready, 1993). In *fj* homozygous mutants, a very small proportion of ommatidia (0.3%) have wrong orientation and chirality (Fig. 7H). A more striking phenotype is seen on the polar boundary of *fj* clones, where several rows of ommatidia are inverted on the dorsoventral (DV) axis (Zeidler et al., 1999). *fj* clones also show planar polarity defects in the abdomen (Zeidler et al., 2000; Casal et al., 2002). In all three cases, non-autonomous phenotypes are seen, with the polarity of wild-type cells being affected by adjacent mutant tissue.

The *fj* transcript has been shown to be expressed in gradients in the eye and wing (Villano and Katz, 1995; Brodsky and Steller, 1996; Zeidler et al., 2000). Together with the polarity phenotypes in clones, this has led to models whereby *fj* is required for production of a gradient of a signalling molecule, on the PD axis of the wing, the DV axis of the eye and the AP axis of the abdomen, which regulates the orientation of hairs, bristles and ommatidia (Zeidler et al., 1999; Zeidler et al., 2000; Casal et al., 2002).

Cloning of *fj* revealed that it encodes a predicted type II transmembrane protein, with two putative cleavage sites for signal peptidase in its transmembrane domain (Villano and Katz, 1995; Brodsky and Steller, 1996). Cleavage at these sites is predicted to result in release and secretion of the C terminus of Fj. Cleavage of Fj has been demonstrated in in vitro translation experiments and in larval tissue (Villano and Katz, 1995; Buckles et al., 2001), and the C-terminus of Fj is secreted into the supernatant in transfected tissue culture cells (Buckles et al., 2001). As Fj functions non-autonomously, it has been proposed to act as a secreted signalling molecule, analogous to the cleaved type II transmembrane protein Hedgehog (Lee et al., 1992; Tabata and Kornberg, 1994).

The *ds* and *ft* genes encode atypical cadherins (Clark et al., 1995; Mahoney et al., 1991), and have mutant phenotypes similar to *fj* in both PD patterning and planar polarity (Adler et al., 1998; Casal et al., 2002; Yang et al., 2002; Rawls et al., 2002; Strutt and Strutt, 2002; Ma et al., 2003). Recent experiments have indicated complex crossregulatory interactions between *fj*, *ds* and *ft* (Yang et al., 2002; Ma et al.,

2003). Furthermore, Ds and Ft protein localisation is altered on the boundaries of *fj* mutant clones (Strutt and Strutt, 2002; Ma et al., 2003). These observations suggest that these molecules may act in a common pathway.

Using modified forms of Fj, we now demonstrate that, contrary to previous models, secretion of Fj is not necessary for its functions in planar polarity and PD patterning. Instead Fj shows highest activity when localised to the Golgi, consistent with a role in modulating the activity of other signalling molecules most likely via post-translational modification.

Materials and methods

Fly strains

Alleles used are described in FlyBase. *Omb-GAL4* (Lecuit et al., 1996), *Act-GAL4* (Ito et al., 1997) and *UAS-Fj* (Zeidler et al., 1999) have been described. Clones were generated using the FLP/FRT system (Xu and Rubin, 1993) and marked with *white* in the adult eye and *arm-lacZ* (Vincent et al., 1994) in discs. Overexpression clones in the eye were generated using *P[w+, Act>y+>GAL4]* (Ito et al., 1997) with the *white* minigene removed by imprecise excision (gift of Eric Spana) and marked using *P[ry+, UAS-white]* (D.S., unpublished).

Molecular biology

Constructs were generated by PCR-based methods and amplified regions verified by sequencing. Fj^{Un} is the full-length Fj ORF (Brodsky and Steller, 1996; Villano and Katz, 1995), with amino acid substitutions V87F and A91M. CD2-Fj is amino acids 1-34 of the CD2 ORF (Williams et al., 1987) and amino acids 102-583 of Fj. GNT-Fj is amino acids 1-121 of human GalNAcT3 (Bennett et al., 1996), a glycine residue, and amino acids 109-583 of Fj. Fj^{Un}-FLAG and GNT-Fj-FLAG have the FLAG octapeptide at the C terminus. GNT-Fjx1 is amino acids 1-121 of human GalNAcT3, linker residues GPDL and amino acids 36-437 of mouse Fjx1 (Ashery-Padan et al., 1999). Constructs were inserted into the vectors pUAST (Brand and Perrimon, 1993) for expression in flies and pMK33b for expression in *Drosophila* S2 cells under control of the metallothionein promoter.

Genomic rescue constructs were made using P1 clone DS08374, which spans the *fj* gene. *fj-24kb* is a 24.4 kb *Sal*I-*Spe*I fragment, containing 18.3 kb genomic sequences upstream of the *fj* ORF, and 4.3 kb downstream sequences. *fj-14kb* is a 14.8 kb *Kpn*I-*Spe*I fragment, with 8.7 kb sequences upstream of the *fj* ORF, and 4.3 kb downstream. Both constructs were made in pCasper4. Modified *fj* isoforms were made by inserting 6 bp (GGCCGC) in the Cavener consensus of the *fj* gene (GC-GGCCGC-AAAATG), to create a *Not*I site, and by replacing the N terminus of *fj* up to the internal *Bgl*II site at amino acid 110 with modified Fj sequences. Insertion of this *Not*I site did not affect the rescue in vivo by an otherwise wild-type *fj* gene (data not shown).

Antibodies, immunostaining and western blotting

Antibodies were raised in rabbits against the C terminus of Fj, using a His-tagged bacterially expressed peptide containing amino acids 485-583 and affinity purified against a GST-tagged peptide for the same region. Rat antibodies against Fj were raised against a His-tagged peptide containing amino acids 111-433 and used for immunofluorescence without purification. Other primary antibodies used were rabbit anti-βGAL (Cappel), rabbit anti-Ci (Alexandre et al., 1996) and mouse monoclonal anti-Golgi (Calbiochem) (Stanley et al., 1997). Immunofluorescence was carried out using secondary antibodies conjugated to Cy2, RRX (Jackson), Alexa488 or Alexa568 (Molecular Probes). For western blotting, HRP-conjugated secondary antibodies (Dako) were used with Dura (Pierce) chemiluminescent detection.

Tissue culture

Drosophila S2 cells were grown in Schneiders medium (Gibco) with FCS and transfected with hygromycin-resistant pMK33 vectors by the calcium phosphate method. Transfected cells were selected by growth in the presence of 200 μg/μl hygromycin for several weeks. Expression was induced by addition of $CuSO_4$ 24 hours prior to harvesting.

Tissue and cell extracts and immunoprecipitations

Third instar larval extracts were prepared by dissecting larval head complexes (consisting of the imaginal discs and brain plus associated tissue) into SDS-PAGE sample buffer on ice. The equivalent of 1-2 head complexes were loaded per lane for western blotting. For immunoprecipitation from S2 cells, cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% Triton X-100 and precipitations carried out in the presence of 1% Triton X-100 using FLAG-M2-Affinity Resin (Sigma).

Results

Fj protein localisation

The expression pattern of *fj* transcripts has previously been characterised, both by in situ hybridisation and by using *fj* enhancer traps driving *lacZ* expression (Villano and Katz, 1995; Brodsky and Steller, 1996; Zeidler et al., 2000). To examine the protein expression pattern and subcellular localisation of Fj, we raised antibodies against the C-terminal region of Fj. The distribution of Fj protein largely mirrors that of the mRNA. In the eye disc, Fj protein is expressed in a gradient ahead of the morphogenetic furrow, high at the equator and low at the poles of the eye (Fig. 1A,B). In the leg disc, weak Fj staining can be detected in concentric rings, possibly corresponding to segmental boundaries (Fig. 1C), while very little Fj can be detected in early pupal legs (Fig. 1D) consistent with the failure to detect mRNA at this stage (Villano and Katz, 1995).

In the wing disc, Fj expression is localised in the wing pouch (Fig. 1E-G). Protein levels are graded, being low in peripheral regions and highest towards the centre of the pouch, which will give rise to the distal adult wing. Expression is downregulated in a stripe in the approximate position of the dorsoventral (DV) compartment and also in a stripe along the anteroposterior (AP) compartment boundary. Co-staining with Ci antibody (Alexandre et al., 1996) that marks the anterior compartment shows that this downregulation occurs in a few rows of cells just inside the anterior compartment. Overall, expression levels are lower in the posterior compartment. Fj protein persists in the early pupal wing, where expression is largely limited to the distal tip (Fig. 1H). Interestingly, *fj* mutants show the strongest PD patterning defects and non-autonomous polarity phenotypes in more proximal regions of the wing (Villano and Katz, 1995; Zeidler et al., 2000) and not towards the tip of the wing where Fj is localised in early pupal stages. Taken together with the lower levels of Fj expression seen in the early pupal wing when compared with the third instar wing disc, this suggests to us that the crucial stage for Fj gradient expression in wing patterning may be in the third instar.

To study the subcellular localisation of endogenous Fj protein, higher magnification images were taken of expression

in the wing pouch (Fig. 1I,J). Fj is principally localised in discrete spots inside the cell, the majority of which colocalise with a Golgi marker. In more basal regions of the cell some large spots are seen that do not colocalise with the Golgi marker (Fig. 1J), and additionally faint staining is also seen in the cytoplasm.

Analysis of modified Fj proteins

Although Fj protein can be cleaved and secreted in vitro and in vivo (Villano and Katz, 1995; Buckles et al., 2001), the significance of this is unclear. As we observe localisation of Fj protein in the Golgi (Fig. 1I), this raised the question of whether cleavage and secretion of Fj is required for its function. To investigate this, we designed a series of Fj transgenes, in which Fj localisation and processing is altered (Fig. 2A).

The method of von Heijne (von Heijne, 1986) predicts two possible sites for signal peptidase cleavage following amino acids 89 and 93. These predicted sites were eliminated by altering the '-3' amino acid, to generate the construct $Fj^{Uncleavable}$ (Fj^{Un}). However, an alternative method for predicting signal sequence cleavage [SignalP (Nielsen et al., 1997)] suggests that this 'uncleavable' form may nevertheless be cleaved following residue 97. In addition, a constitutively-secreted Fj variant $CD2^{Signal}$-Fj (CD2-Fj) and a Golgi-retained form GalNAcT3-Fj (GNT-Fj) were created by fusing either the rat CD2 signal sequence (Williams et al., 1987) or the transmembrane domain and Golgi retention signal of the GalNAc-T3 glycosyltransferase enzyme (Bennett et al., 1996) respectively to the C terminus of Fj, downstream of its predicted cleavage sites.

We used two systems to determine if the Fj^{Un}, CD2-Fj and GNT-Fj proteins displayed the expected intracellular localisation: either transfection into *Drosophila* S2 cells, or expression in the large salivary gland cells of transgenic animals using the GAL4-UAS system.

A single major Fj protein band of the expected size, was detected on western blots of extracts from larvae expressing the Fj constructs expressed under control of the actin promoter (Fig. 2B). In addition, expressing wild-type Fj produces a minor, lower molecular weight band, which probably represents the cleaved C terminus. Prominent higher molecular weight bands are seen in GNT-Fj extracts, which most likely represent glycosylated forms. In S2 cells, Fj protein is partially cleaved, and its secreted C terminus can be detected in the supernatant (Buckles et al., 2001). Similarly, we were able to detect the C terminus of Fj in the medium of cells expressing CD2-Fj (data not shown). In addition, expression of Fj^{Un} in S2 cells resulted in a low level of cleavage and secretion, which could be detected by immunoprecipitation of a tagged form from a large volume of medium (Fig. 2C), confirming the SignalP cleavage prediction. Nevertheless, cleavage is impaired relative to wild-type Fj, as the cleaved C terminus is not detected in Fj^{Un} larval extracts. By contrast, GNT-Fj did not show secretion of the Fj C-terminus into the medium.

In agreement with the colocalisation of endogenous Fj with

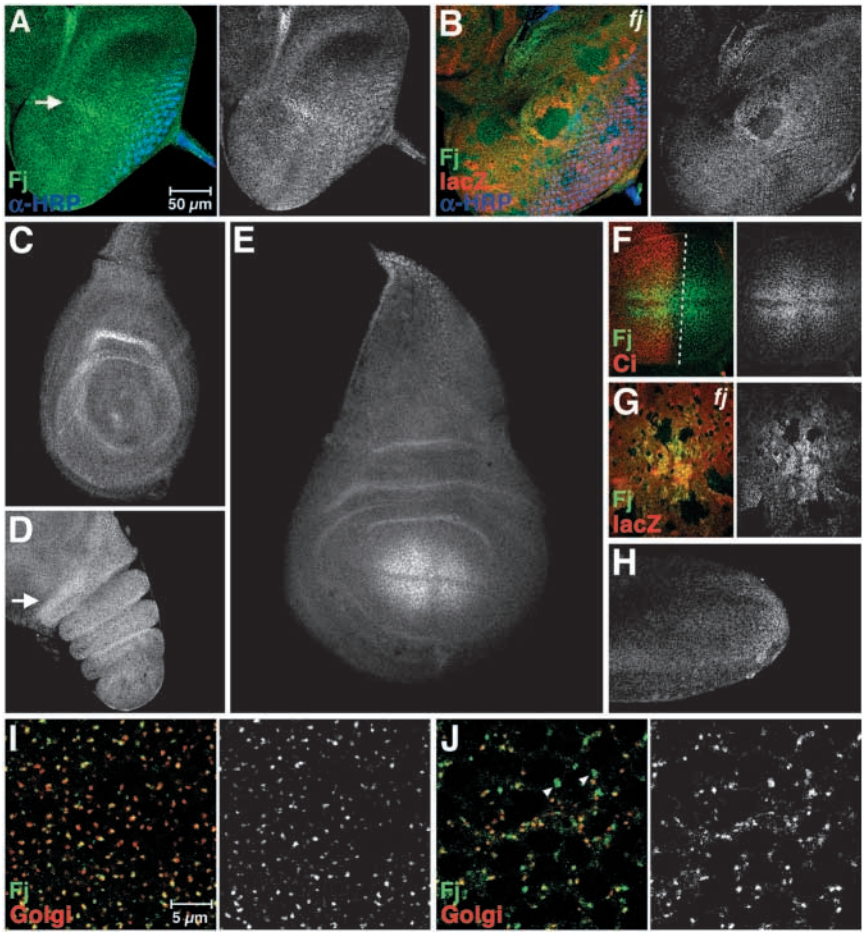

Fig. 1. Localisation and regulation of Fj protein. Confocal images showing protein distribution revealed by immunofluorescence in third instar imaginal discs (A-C,E-G,I-J) or 4.5 hour prepupal leg (D) or wing (H). Fj expression (green or white) was detected using a rat polyclonal sera (A-H) or affinity-purified rabbit antibodies (I,J) against the C-terminal domain of Fj. Anti-HRP to reveal photoreceptor neurones in blue (A,B). *lacZ* clonal marker in red (B,G). Ci in red (F) and Golgi marker in red (I,J). A-H are all at the same magnification (scale bar in A), I and J are 10× higher magnification. Posterior is towards the right and dorsal is upwards, except where noted. (A) Wild-type eye disc. Arrow indicates peak expression in position approximating to DV midline. (B) Eye disc containing fj^{d1} clones (lack of red *lacZ* staining), showing that graded signal on DV axis is specific for Fj. (C) Wild-type leg disc. (D) Wild-type prepupal leg, distal down. Fj expression levels are low, but a stripe can be seen in the first tarsal segment (arrow). (E) Wild-type wing disc. (F) Wild-type wing pouch, costained for Ci in the anterior compartment, position of AP compartment boundary indicated by the broken line. (G) Wing pouch containing fj^{d1} clones (lack of red staining), showing that staining is specific for Fj. (H) Prepupal wing, distal towards the right. (I,J) Cells in the centre of wing pouch, at apical levels above nuclei (I) and more basally (J), co-stained for Golgi marker (red). In basal sections, some spots of Fj expression fail to colocalise with the Golgi marker (arrowheads).

a Golgi marker in wing imaginal disc cells (Fig. 1I), immunostaining of ectopically expressed wild-type Fj protein in salivary glands and S2 cells revealed that it overlaps, but does not entirely co-localise with a Golgi marker (Fig. 2D,H). Therefore, as in the wing, some Fj is either in a Golgi compartment not marked by the anti-Golgi antibody used, or is in an adjacent compartment of the secretory pathway. Fj^{Un} localises similarly to wild-type in S2 cells (Fig. 2J). GNT-Fj, as expected, is tightly localised to the Golgi (Fig. 2G,K), completely overlapping with the marker used. By contrast, the constitutively-secreted form of Fj (CD2-Fj) shows less colocalisation with the Golgi in both systems (Fig. 2E,I), being seen additionally in many spots within the cell (which may represent secretory vesicles), and in salivary glands being efficiently secreted into the duct (Fig. 2F).

In vivo activity of modified Fj proteins

We have demonstrated that wild-type Fj protein localises in vivo to the Golgi apparatus, although a subset also appears to be cleaved and secreted. In addition, we have made modified forms of Fj which are either efficiently secreted or tightly retained in the Golgi. These constructs were tested for in vivo function in transgenic flies, by overexpressing using the GAL4-UAS system.

Expression of wild-type Fj using *omb-GAL4* results in a wing phenotype very similar to loss-of-function phenotypes, as has been shown for other drivers (Zeidler et al., 2000; Buckles et al., 2001). In wings of both *fj* mutants and flies overexpressing Fj, there are proximodistal patterning defects which result in the anterior and posterior crossveins being closer together (blue line in Fig. 3B and 3D, compare with 3A). In addition, the distal region of the wing is shorter in *omb-GAL4, UAS-fj* flies. Overexpression of Fj^{Un}, CD2-Fj and GNT-Fj all give a similar phenotype (Fig. 3E,F), despite their different subcellular localisations. Quantification of the distance between the two crossveins (the 'intervein distance') shows a trend for GNT-Fj to give the most severe phenotypes, and CD2-Fj the mildest phenotypes (Fig. 3F). These results suggest that Fj protein is most active when it is retained in the Golgi, rather than when it is secreted.

A similar overexpression assay was used to determine whether the modified forms of Fj were active in planar polarity. If wild-type Fj is expressed at the poles of the eye using *omb-GAL4*, then several rows of ommatidia on the dorsal and

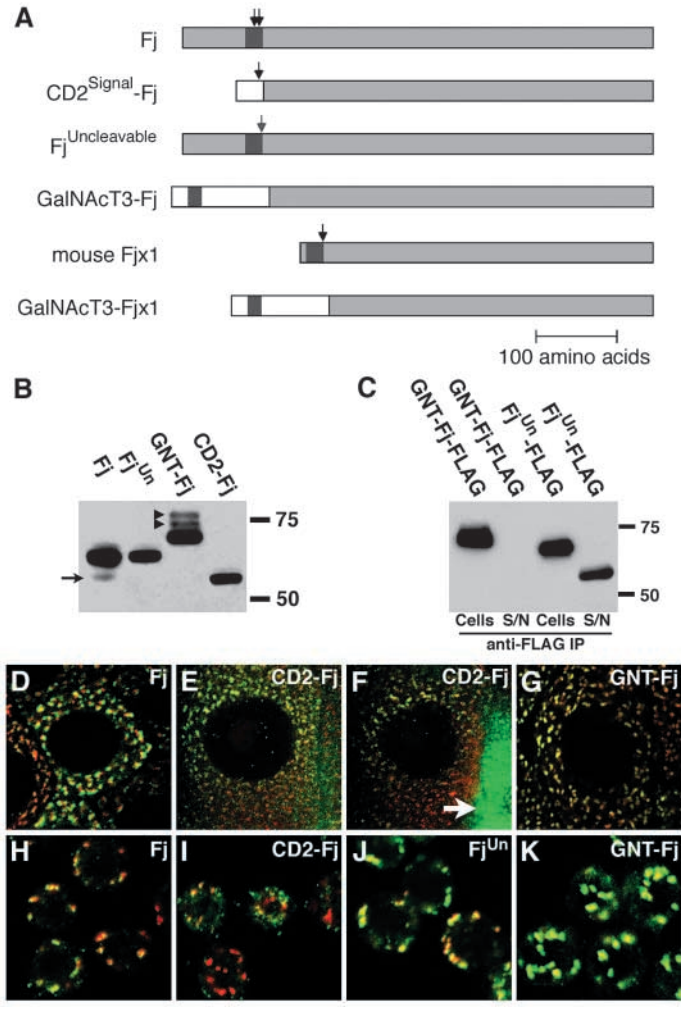

Fig. 2. Modified forms of Fj that are efficiently secreted or retained in the Golgi. (A) Diagrams of modified forms of *Drosophila* Fj and mouse Fjx1 used in this study. Fj and Fjx1 sequences shown in light grey, sequences of heterologous genes in white. Putative transmembrane domains predicted by the Kyte-Doolittle and TMHMM methods (Kyte and Doolittle, 1982; Sonnhammer et al., 1998) are shown in dark grey. Black arrows indicate predicted signal peptidase cleavage sites by the method of von Heijne (von Heijne, 1986), the grey arrow shows the cleavage site in Fj^{Un} predicted by SignalP (Nielsen et al., 1997). Forms of Fj^{Un} and GNT-Fj with a C-terminal FLAG-tag were also made for immunoprecipitation experiments. (B) Western blot of third instar larval extracts expressing different forms of Fj under the *Actin5C* promoter using the GAL4-UAS system. The blot was probed with an affinity-purified rabbit antibody raised against the C terminus of Fj. The major bands seen for Fj, Fj^{Un} and GNT-Fj are the correct sizes predicted for full-length uncleaved protein, whereas that for CD2-Fj is consistent with the predicted size of the cleaved form. The arrow indicates a minor band seen in Fj-expressing extracts, that probably corresponds to the cleaved form previously reported (Villano and Katz, 1995; Buckles et al., 2001). The arrowheads indicate higher molecular weight bands seen in GNT-Fj extracts, that are probably glycosylated forms. We assume the putative glycosylated forms are most prominent in the GNT-Fj extract, because this molecule is most tightly Golgi-localised. (C) Western blot of immunoprecipitated proteins from cells and tissue culture supernatant of S2 cells overexpressing GNT-Fj-FLAG or Fj^{Un}-FLAG. FLAG-tagged protein was immunoprecipitated from ~50× excess of cell lysate (relative to that needed to produce an equivalent signal by direct western analysis) and an equivalent amount of tissue culture supernatant. Under these conditions, the volume of affinity matrix used is oversaturated by the amount of FLAG-tagged protein in the cell extracts, but not by the amount in the supernatants. These results suggest that less than 1% of Fj^{Un}-FLAG is secreted. Protein was detected using an affinity-purified rabbit antibody raised against the C terminus of Fj. (D-K) Confocal images of third instar salivary gland cells expressing different forms of Fj under the *Actin5C* promoter using the GAL4-UAS system (D-G) or S2 cells expressing proteins under the metallathionein promoter (H-K). Fj (green) is detected by immunofluorescence using an affinity-purified rabbit antibody raised against the C terminus of Fj. Golgi (red) is marked using an antibody against an integral Golgi protein (Stanley et al., 1997). (F) A more basal view of the same cell shown in E in which secreted Fj is seen at high levels in the gland duct (arrow). Such secreted Fj is not detected for the other constructs.

ventral edges of the eye are inverted (Zeidler et al., 1999) (Fig. 4B). Overexpression of Fj^{Un}, CD2-Fj and GNT-Fj give similar inversions of polarity (Fig. 4C-E). Thus, all three modified forms of Fj are active in polarity as well as PD patterning, and, furthermore, the direction of the polarity phenotypes demonstrate that none of the three forms are acting as dominant negatives.

The eye polarity phenotype was further investigated by inducing clones overexpressing Fj using *Act>>GAL4*. Overexpressing wild-type Fj results in non-autonomous inversions of one or two rows of ommatidia on the equatorial boundary of the clone (Zeidler et al., 1999) (Fig. 4F). Inversions were also seen when CD2-Fj was overexpressed, although the phenotype was weak in all the clones analysed (Fig. 4G). Significantly, although GNT-Fj is not secreted, overexpressing it in clones gave a very strong non-autonomous phenotype, whereby in some clones several rows of ommatidia beyond the equatorial boundary are inverted (Fig. 4H). This demonstrates that Fj functions non-autonomously, even when not capable of being secreted.

In addition, we tested if the mouse homologue of Fj, Fjx1 (Ashery-Padan et al., 1999), could provide Fj activity when overexpressed. However, no overexpression phenotypes were observed with a variety of drivers, even if the protein was retained in the Golgi using the GNT retention signal (Fig. 3F, and not shown).

Rescue of *fj* mutants by modified forms of Fj

Although Golgi-retained Fj is most active in overexpression assays, the secreted CD2-Fj still gives significant phenotypes. This could be explained by the fact that the GAL4-UAS system results in high levels of expression, and that a large amount of CD2-Fj is always passing through the Golgi/secretory pathways. Therefore, we developed a more sensitive assay for Fj activity, by making rescue constructs using genomic DNA from around the *fj* locus.

We initially tested a 24 kb rescue construct, which consists of 17 kb of genomic sequence upstream and 4 kb downstream of the *fj* transcribed region. This transgene appeared to contain most of the regulatory elements required to rescue a *fj* mutant. One copy of the *fj-24kb* transgene gave significant rescue of the wing PD patterning defect (Fig. 5A, compare with female wings in Fig. 3A and 3B), whereas there was complete rescue with two copies (Fig. 5B). Rescue of the leg PD patterning defect was also seen with this construct (Fig. 5C-F). Rescue of *fj* polarity phenotypes are harder to assay, as *fj* only shows such phenotypes in clones. Nevertheless, we think it likely that *fj-24kb* contains the enhancers necessary for polarity patterning at least in the eye for two reasons. First, loss-of-function clones of *fj-24kb* in a *fj* null background phenocopy *fj* loss of function clones (Fig. 5G); and second, the *mini-white* gene present in the rescue construct is expressed in a gradient in the eye, similar to that of *fj* itself (Fig. 5H).

A *fj-14kb* transgene (7 kb genomic sequences upstream and 4 kb downstream of the *fj* gene) also gave partial rescue of the *fj* wing PD patterning defect (Fig. 6C, compare with males in Fig. 6A,B). The degree of rescue was similar for several independent insertions, and was not significantly improved by increasing the copy number (Fig. 6D). No rescue of the leg PD patterning defect was seen (data not shown). The pattern of gene expression driven by this 14 kb region of genomic DNA was tested by inserting a *lacZ* gene in place of the 5′ end of *fj* (*lacZ(fj)-14kb*). As expected from the rescue phenotypes, expression was observed in the wing pouch of the third instar imaginal disc (data not shown).

As the *fj-14kb* construct gave an intermediate rescue phenotype, we selected this to test the relative activities of the modified forms of Fj. The efficiency of rescue varied significantly depending on the protein assayed: in particular, three independent insertions of the *CD2-fj-14kb* transgene only poorly rescued the PD growth defect of *fj* (intervein length 64-73% of wild type, compared with 85-87% with wild-type *fj-14kb*), while fj^{Un}*-14kb* rescued similarly to *fj-14kb* (Fig. 6E). In addition, one insertion of *GNT-fj-14kb* gave full rescue of the fj^{d1} wing phenotype, suggesting that the activity of GNT-Fj is higher than that of wild-type Fj.

Interestingly, most of the transgenic lines carrying a single copy of the *GNT-fj-14kb* transgene had a dominant PD patterning defect in the wing and leg (Fig. 6F, and not shown). In flies with two copies of the transgene, the wing phenotype was as strong as that of *fj* null mutants (Fig. 6G). No dominant phenotypes were observed with multiple insertions of *fj-14kb*, again suggesting that GNT-Fj protein has greater activity. Therefore, these results confirm those seen with the overexpression assay, supporting the conclusion that constitutive secretion of Fj reduces its activity, while Golgi-retained Fj is hyperactive.

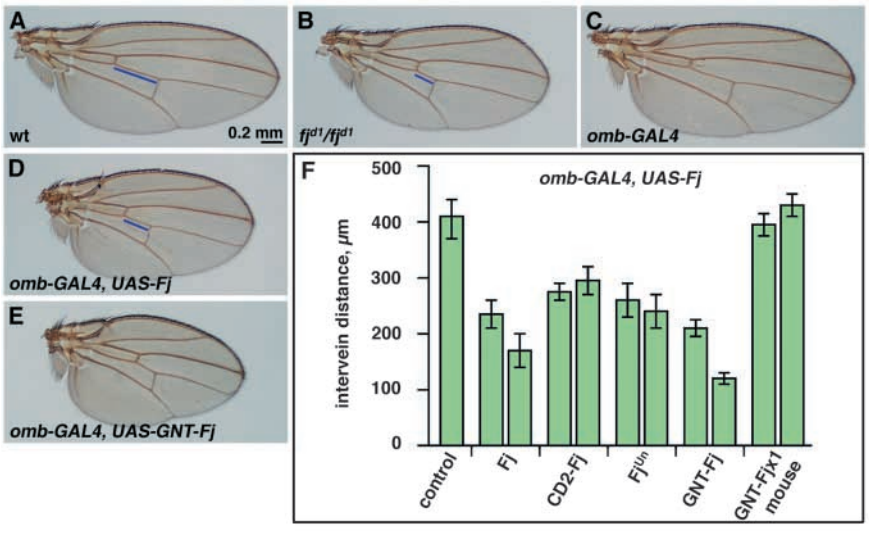

Fig. 3. Overexpression of Fj variants in the wing. (A-E) Wings from wild-type (A), fj^{d1}/fj^{d1} (B), *omb-GAL4* (C), *omb-GAL4,UAS-fj* (D) and *omb-GAL4, UAS-GNT-fj* (E) females, raised at 25°C. The blue lines in A, B and D indicate the region between the anterior and posterior crossveins, which is reduced in fj^{d1}/fj^{d1} flies or flies overexpressing Fj. (F) The intervein distance in females overexpressing Fj variants using the *omb-GAL4* driver. Results are shown for an *omb-GAL4* control, and two independent insertions of each variant form of Fj, and are an average of the intervein distance in wings from at least six flies; the vertical bars are the standard deviation between wings.

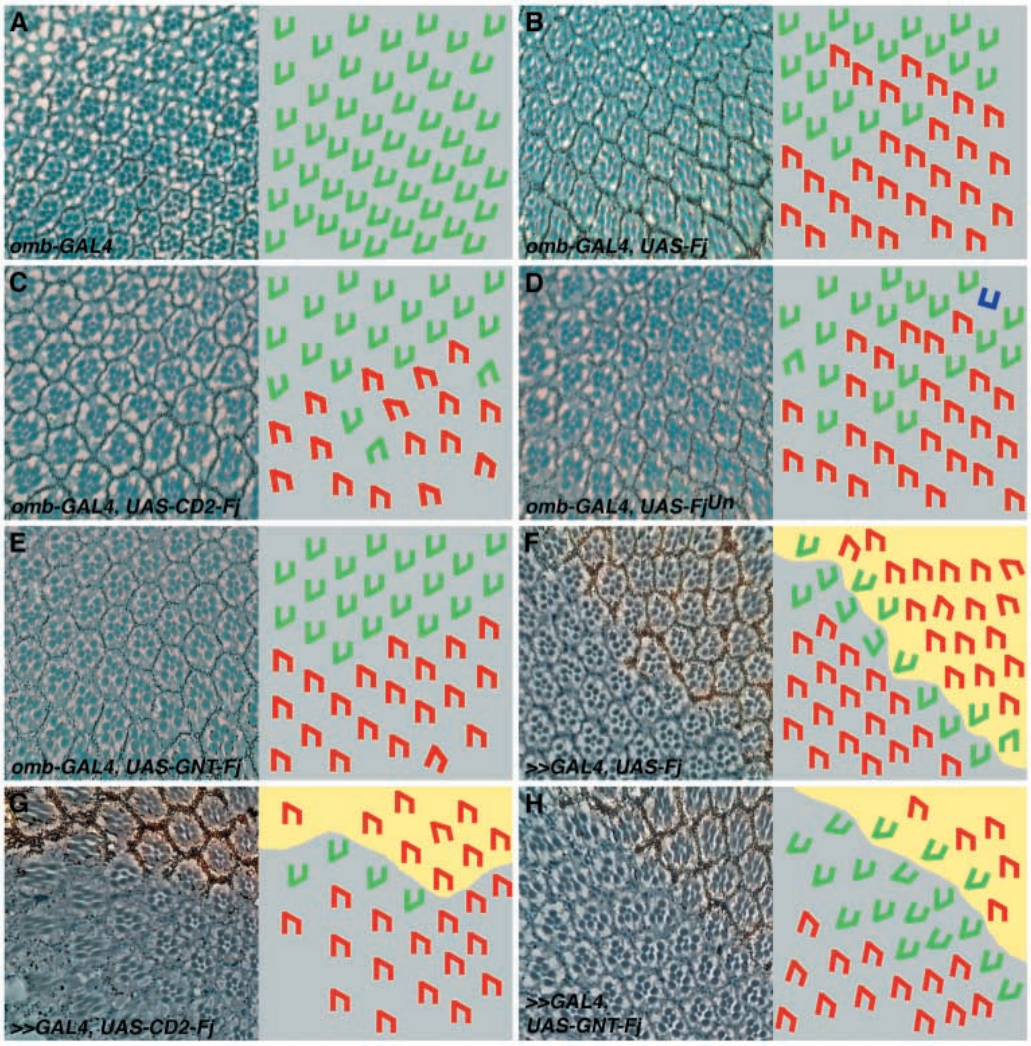

Fig. 4. Overexpression of Fj variants in the eye. Adult eye sections are shown on the left, and diagrams on the right. Dorsal is upwards and anterior is left. Dorsal-type ommatida are shown in red, ventral-type ommatidia in green and achiral ommatidia in blue. (A-E) Ventral eye sections from females: *omb-GAL4* control (A); or *omb-GAL* driving expression of wild-type *fj* (B), *CD2-fj* (C), fj^{Un} (D) or *GNT-fj* (E). In a wild-type eye, all ommatidia in this region of the eye have ventral chirality (A), but overexpression of all *fj* variants leads to inversions of polarity on the ventral margin of the eye (red ommatidia). (F-H) Act>>GAL4 overexpression clones, of wild-type *fj* (F), *CD2-fj* (G) and *GNT-fj* (H). Regions where Fj is overexpressed are marked by the presence of pigment in the eye sections and by yellow shading in the diagrams. All Fj variants show non-autonomous inversions of polarity on the equatorial boundary of the clone (green ommatidia).

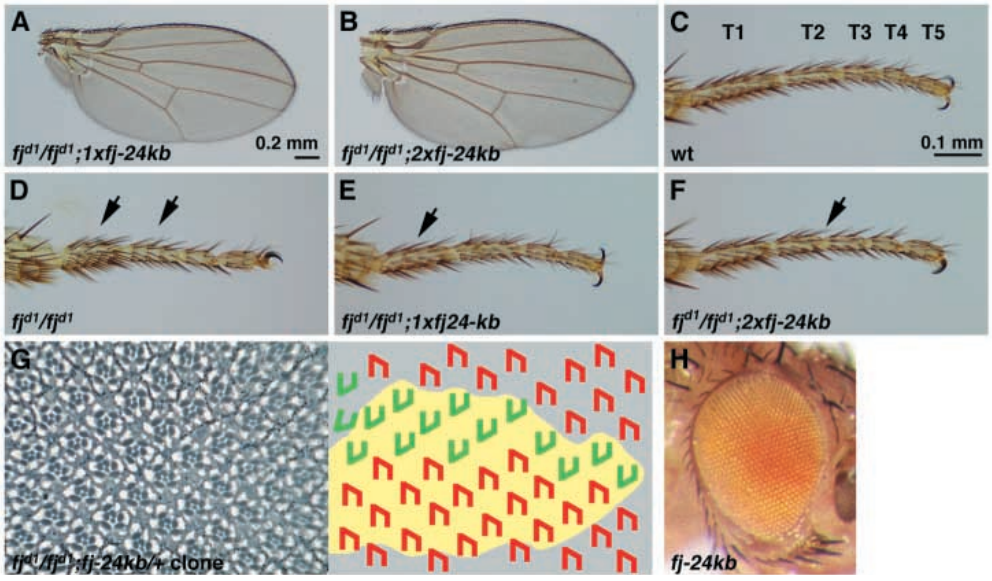

Fig. 5. Rescue of *fj* mutant phenotypes in the wing and leg. (A-F) Wings or tarsal leg segments from females. (C) Wild type; (D) fj^{d1}/fj^{d1}, arrows point to the first tarsal segment, which is shorter than normal, and to the fused second and third tarsal segments; (A,E) fj^{d1}/fj^{d1} carrying one copy of the *fj-24kb* rescue transgene, showing partial rescue of the wing PD patterning defect (A) and partial rescue of the defect in the first tarsal segment (arrow in E); (B,F) fj^{d1}/fj^{d1} carrying two copies of the *fj-24kb* rescue transgene. The wing PD patterning defect is fully rescued (B), and tarsal patterning is largely rescued, including restoration of the T2/T3 joint (arrow in F). Compare female wings with Fig. 4A,B. (G) Adult eye section of a clone of the *fj-24kb* rescue transgene, in a fj^{d1}/fj^{d1} mutant background, which has inverted ommatidia (green) on the polar boundary of the clone (clone marked by loss of pigment and by yellow shading), which phenocopies *fj* mutant clones. (H) Adult eye from a fly carrying the *fj-24kb* transgene, showing a gradient of expression of the *mini-white* gene. This expression pattern is seen in both independent insertions tested.

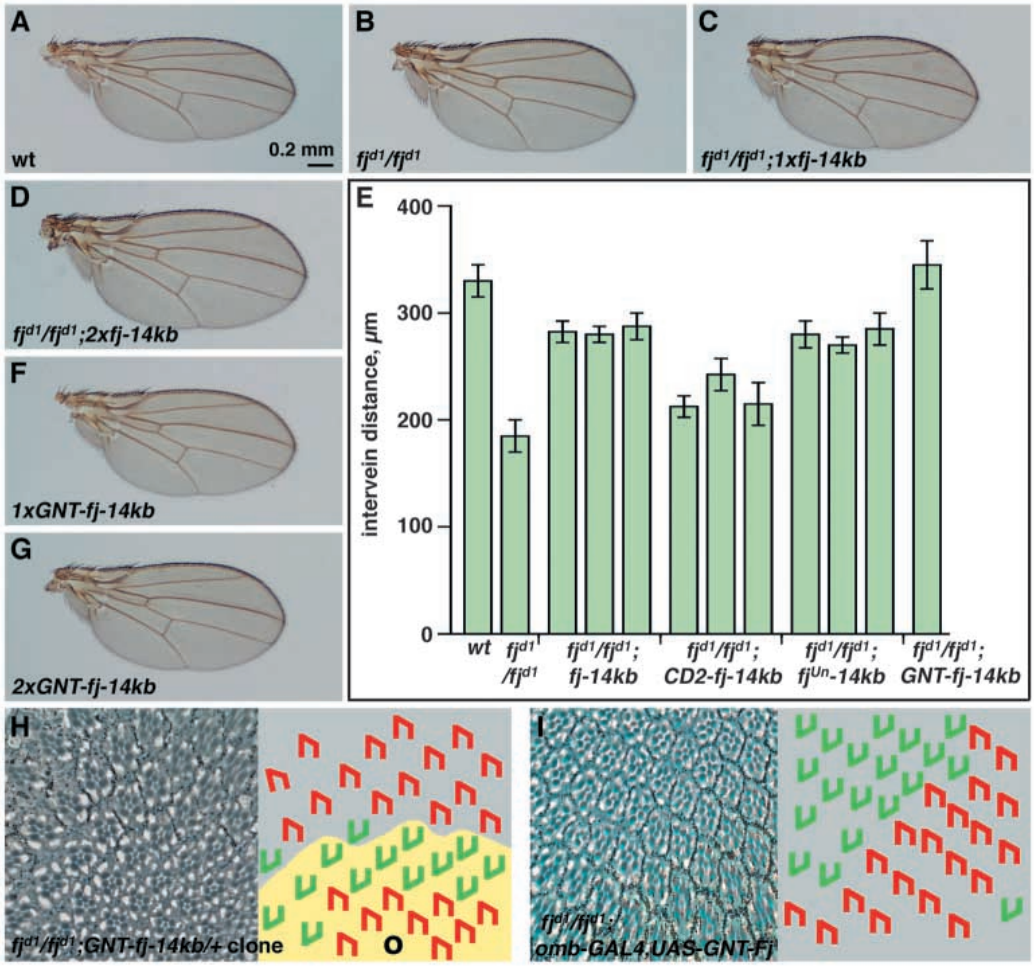

Fig. 6. Rescue of *fj* mutant phenotypes by Fj variants. (A-D) Male adult wings. (A) Wild type; (B) fj^{d1}/fj^{d1}; (C) fj^{d1}/fj^{d1} carrying one copy of the *fj-14kb* rescue transgene; (D) fj^{d1}/fj^{d1} carrying two copies of the *fj-14kb* rescue transgene. One and two copies of the *fj-14kb* transgene give identical partial rescue of the wing PD patterning defect. (E) Graph showing rescue of the intervein phenotype by modified Fj proteins expressed in the fj-14kb rescue transgene. Results are shown for three independent insertions of each variant form of Fj (one insertion of *GNT-fj-14kb*), and are an average of the intervein distance in wings from at least six flies; the vertical bars are the standard deviation between wings. *t*-tests carried out between pairs of constructs show that the difference in rescue between any *fj-14kb* insertion and any *CD2-fj-14kb* or *GNT-fj-14kb* insertion is >99% significant. (F,G) Wings of males carrying 1 (F) or 2 (G) copies of a *GNT-fj-14kb* transgene, in a wild-type background, showing a PD patterning phenotype caused by excess *fj* activity. (H) Adult eye section of a clone of a *GNT-fj-14kb* transgene, in a fj^{d1}/fj^{d1} mutant background. Clone marked by loss of pigment and by yellow shading. The circle indicates an ommatidium with a missing photoreceptor. (I) Ventral eye section, *omb-GAL4* driving expression of *GNT-fj*, in a fj^{d1}/fj^{d1} mutant background.

However, it should be noted that there are two possible explanations for the higher activity of *GNT-fj-14kb*. The first is that the Golgi-retained protein is itself biochemically more active. The second is that the Golgi-retained protein in some way causes endogenous Fj to be more active, perhaps by promoting its secretion. Two experiments rule out this second possibility. Loss-of-function clones of *GNT-fj-14kb* in the eye give a non-autonomous polarity phenotype, even in a *fj* mutant background (Fig, 6H). Furthermore, overexpression of GNT-Fj using the *omb-GAL4* driver in a *fj* mutant background, still results in strong inversions of ommatidial polarity on the polar boundary of the eye (Fig. 6I). Therefore these results indicate that the activity of *GNT-fj-14kb* is not dependent on endogenous Fj and furthermore that it does not act non-autonomously by promoting secretion of endogenous Fj.

Interaction of *fj* with *ft* and *ds*

Our results show that Fj does not need to be secreted to function, but that it nevertheless acts non-autonomously. Thus, Fj must be acting intracellularly to modify the activity of one or more other proteins that then signal outside the cell. As *ft* and *ds* have similar mutant phenotypes to *fj* in PD patterning and planar polarity, they are good candidates for being targets of Fj activity.

First, we looked for genetic interactions between *fj* and weak allele combinations of *ft* and *ds*. Double mutants of *fj* and *ft* had a wing PD patterning phenotype that was stronger than either of the single mutants (Fig. 6B, Fig. 7A,B). The PD patterning defect of *ds fj* double mutants was harder to assess, as there was no posterior crossvein; but there was no obvious reduction in the length of the total wing compared with either single mutant (Figs 6B, 7C,D). However, we did observe reproducible defects in wing trichome polarity in *ds fj* double mutants. Defects in the polarity of wing trichomes are normally only observed in *fj* mutant clones, but not in *fj* homozygous animals (Zeidler et al., 2000) (Fig. 7E). Similarly, the weak *ds* combination ds^{1}/ds^{UA071} does not show significant polarity defects (Fig. 7F). However, in ds^{1} fj^{d1}/ds^{UA071} fj^{d1} double mutants, reproducible hair polarity swirls occur in one region of the wing, between veins 2 and 3, above the anterior crossvein (compare Fig. 7E-G). In addition, fj^{d1}/fj^{d1} or ds^{1}/ds^{UA071} mutants give only rare planar polarity defects in the eye, in which less than 1% of ommatidia are inverted (Zeidler et al., 2000; Strutt and Strutt, 2002) (Fig. 7H,I), while in the double mutant 16% of ommatidia are inverted (Fig. 7J). These synergistic interactions are consistent with the hypothesis that *fj* acts in a common pathway with *ds* and *ft*.

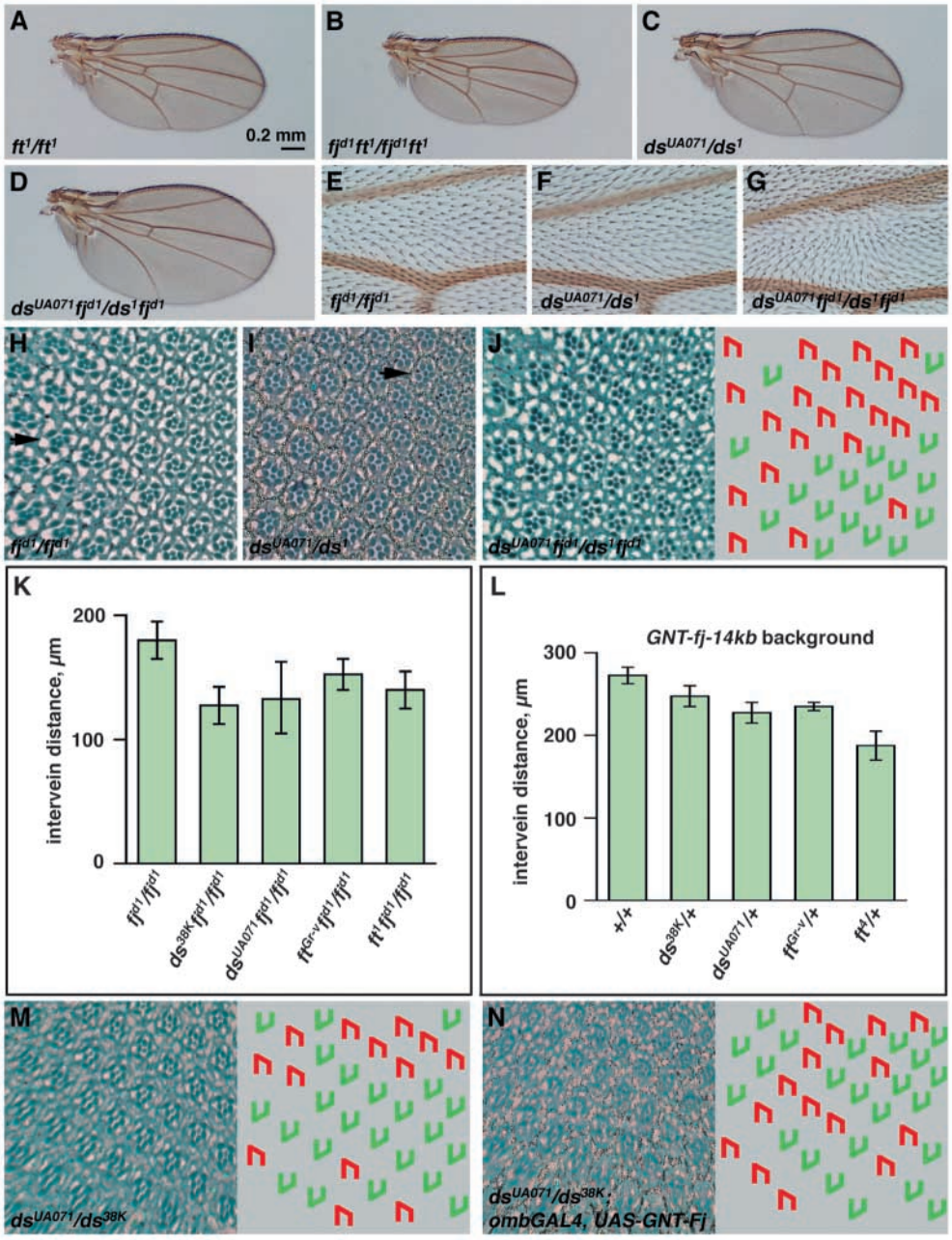

Fig. 7. Genetic interactions between *fj*, *ft* and *ds* in PD and planar polarity patterning. (A-G) Wings from males: (A) ft^{1}/ft^{1}; (B) $fj^{d1}\ ft^{1}/fj^{d1}/ft^{1}$, showing a stronger PD patterning defect than either single mutant; (C) ds^{UA071}/ds^{1}; (D) $ds^{UA071}\ fj^{d1}/ds^{1}\ fj^{d1}$, with no obvious increase in PD defect compared with either single mutant, although the posterior crossvein is always partially or completely missing (the wing is broader than the *ds* single mutant). (E-G) High-magnification images of a proximal region of the wing, between veins 2 and 3, showing the trichomes adopting swirling patterns in the *ds fj* double mutant (G). (H-J) Eye sections of fj^{d1}/fj^{d1} (H), ds^{UA071}/ds^{1} (I) and $ds^{UA071}\ fj^{d1}/ds^{1}\ fj^{d1}$ (J) homozygotes. Only occasional inversions of polarity are seen in the single mutants (arrows in H,I); whereas the double mutant has many DV inversions (J). (K) Graphs showing dominant enhancement by *ds* and *ft* of the fj^{d1}/fj^{d1} wing PD patterning defect (K) or the wing PD patterning defect caused by the *GNT-fj-14kb* rescue transgene (L). All genetic interactions are >99% significant by *t*-tests. (M) Ventral eye section of ds^{UA071}/ds^{38K} females. DV polarity of ommatidia is randomised. (N) Ventral eye section of *omb-GAL4, UAS-GNT-Fj* in a ds^{UA071}/ds^{38K} background. Ommatidial inversions are not seen specifically on the boundary of the eye, and the phenotype resembles ds^{UA071}/ds^{38K} alone.

Dominant genetic interactions were also observed between *fj* and *ft/ds*. Removing one copy of either *ft* or *ds* enhances the wing PD patterning phenotype of fj^{d1}/fj^{d1} mutants (Fig. 7K), and enhances the dominant overexpression phenotype caused by the *GNT-fj-14kb* transgene (Fig. 7L). Conversely, removing one copy of *fj* enhances the weak ds^{1}/ds^{UA071} eye polarity phenotype from 0.3% to 8% ±2 inverted ommatidia (data not shown).

We then investigated whether *ds* acts upstream or downstream of *fj*, by testing whether the effects of overexpressing Fj were still retained in a strong *ds* mutant. Overexpressing GNT-Fj using *omb-GAL4* in the eye causes ommatidia on the ventral edge of the eye to be inverted (Fig. 4E), whereas ommatidia in a *ds* mutant eye are randomised in dorsal-ventral polarity (Fig. 7M). Interestingly, overexpressing GNT-Fj in a *ds* background results in a *ds*-like phenotype (Fig. 7N), showing that in this context *ds* is epistatic to *fj*. In addition, the PD patterning defect caused by overexpressing Fj in the wing (Fig. 3E) is less severe in a *ds* mutant background (data not shown), again suggesting that Ds activity is necessary for Fj function. Overall, these results support the conclusion that Fj acts via Ds in both planar polarity and PD patterning.

Discussion

Fj has been proposed to act as a secreted signalling molecule, based on the fact that its C-terminal region can be cleaved and secreted, and that it exhibits non-autonomous functions in mosaic clones. We have tested the functional significance of this cleavage, by making modified forms of Fj that are either poorly cleaved, constitutively secreted or anchored in the Golgi apparatus. Several different assays support the conclusion that

cleavage and secretion of Fj is not essential for its activity in either planar polarity or PD patterning. In both overexpression experiments and rescue assays, Golgi-tethered Fj has significantly more activity than wild-type Fj, while secreted Fj is less active. Furthermore, even though Golgi-tethered Fj is not secreted, it still produces non-autonomous polarity phenotypes similar to or stronger than those of wild-type Fj. Therefore, we propose that secreted Fj is not the active form, and that Fj acts intracellularly. We think it most likely that Fj acts by modulating the activity of other molecules involved in intercellular signalling.

Thus, our results show that rather than acting in an analogous manner to the cleaved type II transmembrane protein Hedgehog, a better model for Fj function may be the type II transmembrane protein Fringe (Fng). Fng is Golgi-localised and acts as a glycosyltransferase enzyme to post-translationally modify the receptor Notch (N) (Brückner et al., 2000; Munro and Freeman, 2000). This renders N more sensitive to its ligand Delta, and less sensitive to the ligand Serrate. In the case of Fj, there are no molecular homologies that give any clues as to a possible enzymatic activity. Consequently, we cannot be certain of the precise location of its function. However, as our results show that if Fj is tethered in the Golgi, it has higher than normal activity, the Golgi seems most likely to be its preferred site of action.

An important question is whether Fj cleavage has any functional significance. As forced retention of Fj in the Golgi causes hyperactivity, it is possible that cleavage and secretion could be a mechanism to downregulate Fj activity during normal development. However, further experiments will be required to determine if the cleavage step is temporally or spatially regulated.

The mouse homologue of Fj (Fjx1) has also been proposed to act as a secreted molecule, on the basis of a hydrophobic stretch at the N terminus that might represent a signal peptide and the presence of predicted signal peptidase cleavage sites (Ashery-Padan et al., 1999). However, we note that the hydophobic region is not at the extreme N terminus and is sufficiently long to be predicted to be a transmembrane domain by the Kyte and Doolittle (Kyte and Doolittle, 1982) and TMHMM (Sonnhammer et al., 1998) algorithms. This structure suggests that Fjx1 may also be a type II transmembrane protein. Consistent with this, in tissue culture experiments, we fail to observe secretion of the C-terminal region of Fjx1 into the medium (data not shown). However, our failure to detect any activity of Fjx1 when overexpressed in flies suggests that there may be a divergence of function between the fly and vertebrate proteins.

In *Drosophila*, the atypical cadherins Ft and Ds are good candidates for being the ultimate targets of *fj* activity. They are required for both planar polarity and PD patterning, and have similar mutant phenotypes to *fj*. In addition, we now show that *fj* interacts genetically with *ds* and *ft* in both planar polarity and PD patterning. Interestingly, *ds fj* double mutants were previously reported to have surprisingly strong phenotypes, which were qualitatively different to those of the single mutants, including duplications or transformations of limb structures (Waddington, 1943). However, we do not see such phenotypes in any of our double mutant combinations, suggesting that the duplications/transformations may be specific to the combination of chromosomes used in Waddington's experiment. Our results instead show that mutations in *fj* enhance the phenotypes of both *ft* and *ds* hypomorphic mutations, suggesting that these genes act in a common pathway.

Epistasis experiments further demonstrate that *ds* is required to mediate *fj* function, and therefore *ds* acts downstream of *fj*; this is in agreement with previous data based on clonal analysis of *ds* and *fj* (Yang et al., 2002). Interestingly, recent experiments have also revealed a role for *fj* in regulating the intracellular distribution of Ds and Ft (Strutt and Strutt, 2002; Ma et al., 2003). In wild-type tissue, Ds and Ft colocalise at apicolateral membranes, and their localisation is mutually dependent. Inside *fj* mutant clones, Ds and Ft localisation is largely unaltered. However, in the row of mutant cells immediately adjacent to wild-type tissue, Ft and Ds preferentially accumulate on the boundary between fj^{+}/fj^{-} cells. In addition, cells inside the *fj* clones appear to be 'rounded-up', suggesting that they prefer to adhere to each other rather than to non-mutant cells (Strutt and Strutt, 2002). Thus, it is thought that *fj* modulates the activity and intermolecular binding properties of Ft and Ds (Strutt and Strutt, 2002; Ma et al., 2003).

An interesting point to note is that both *ds* and *ft* show planar polarity phenotypes as homozygotes (Adler et al., 1998; Yang et al., 2002; Rawls et al., 2002), whereas *fj* only shows polarity phenotypes on the boundaries of mutant clones (Zeidler et al., 1999; Zeidler et al., 2000). The *fj* phenotypes have been explained by models in which *fj* acts redundantly to regulate the production of a gradient, the direction of which determines polarity. Thus, in homozygotes the direction of the gradient is unchanged, and animals show no major defects; but at clone boundaries there is a discontinuity in the direction of the gradient, leading to inversions of polarity. We can now extend this model to suppose that Fj may modulate Ds/Ft activity, but that it does not act as a simple on-off switch; rather Ds/Ft retain some activity even when Fj is not present.

In the absence of a known enzymatic function for Fj, the mechanism by which it might modulate Ft and Ds activity remains uncertain. But we speculate that as Fj acts intracellularly, it is possible that it promotes or mediates the post-translational modification of Ds and/or Ft proteins, and that these molecules mediate the non-autonomous signalling functions of Fj. However, the large size of the Ft and Ds gene products (5147 and 3380 amino acids, respectively) renders the analysis of their post-translational modification highly challenging.

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