Action of *fat*, *four-jointed*, *dachsous* and *dachs* in distal-to-proximal wing signaling

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Summary

In the *Drosophila* wing, distal cells signal to proximal cells to induce the expression of Wingless, but the basis for this distal-to-proximal signaling is unknown. Here, we show that three genes that act together during the establishment of tissue polarity, *fat*, *four-jointed* and *dachsous*, also influence the expression of Wingless in the proximal wing. *fat* is required cell autonomously by proximal wing cells to repress Wingless expression, and misexpression of Wingless contributes to proximal wing overgrowth in *fat* mutant discs. Four-jointed and Dachsous can influence Wingless expression and Fat localization non-autonomously, consistent with the suggestion that they influence signaling to Fat-expressing cells. We also identify *dachs* as a gene that is genetically required downstream of *fat*, both for its effects on imaginal disc growth and for the expression of Wingless in the proximal wing. Our observations provide important support for the emerging view that Four-jointed, Dachsous and Fat function in an intercellular signaling pathway, identify a normal role for these proteins in signaling interactions that regulate growth and patterning of the proximal wing, and identify Dachs as a candidate downstream effector of a Fat signaling pathway.

Key words: Fat, Cadherin, Limb, Growth, *Drosophila*

Introduction

The wings and notum of the fly develop from clusters of undifferentiated cells in the larva termed wing imaginal discs. The patterning and growth of wing discs is governed by a series of regulatory interactions that have been the subject of intensive study over the last decade (reviewed by Lawrence and Struhl, 1996; Irvine and Rauskolb, 2001; Klein, 2001). Studies of signaling along the AP and DV axes have established paradigms for tissue patterning, and have also been instrumental in the identification of many key components of the Hedgehog, Notch, Wingless (WG; Wnt) and Decapentaplegic (DPP; TGF-β) signaling pathways. More recently, it has become clear that normal wing development is also dependent upon signaling along the proximodistal (PD) axis (Liu et al., 2000; del Álamo Rodriguez et al., 2002; Kolzer et al., 2003), but the identity of the genes that actually effect signaling along this axis remains unknown.

There is a progressive elaboration of patterning along the PD axis over the course of wing development (reviewed by Klein, 2001). During the second larval instar, interactions among the Epidermal Growth Factor Receptor, DPP and WG signaling pathways divide the wing disc into a dorsal region, which will give rise to notum, and a ventral region, from which the wing will arise (Ng et al., 1996; Baonza et al., 2000; Wang et al., 2000; Cavodeassi et al., 2002; Zecca and Struhl, 2002). An initial PD subdivision of the wing is then effected by signaling from the AP and DV compartment boundaries, which promotes the expression of two genes, *scalloped* and *vestigial*, that encode subunits of a heterodimeric transcription factor (SD-VG) in the center of the wing (Kim et al., 1995; Kim et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997; Halder et al., 1998; Klein and Martinez Arias, 1998; Simmonds et al., 1998). This subdivides the wing into distal cells, which give rise to the wing blade, and surrounding cells, which give rise to proximal wing and wing hinge structures (Fig. 1) (Kim et al., 1996; Klein and Martinez Arias, 1998; Azpiazu and Morata, 2000; Casares and Mann, 2000; Liu et al., 2000). The proximal wing is further subdivided into a series of molecularly distinct domains (Fig. 1B). Studies of SD-VG function in the wing led to the realization that the elaboration of this finer pattern depends in part upon signaling from the distal, SD-VG-expressing cells, to more proximal cells (Liu et al., 2000). Thus, mutation of *vg* leads to elimination, not only of the wing blade, where VG is expressed, but also of more proximal tissue (Williams et al., 1991; Williams et al., 1993; Klein and Martinez Arias, 1998; Liu et al., 2000). Conversely, ectopic expression of VG in the proximal wing reorganizes the patterning of surrounding cells (Liu et al., 2000; del Álamo Rodriguez et al., 2002; Kolzer et al., 2003).

A key target of the distal signal is WG, which during early third instar is expressed in a ring of cells that surround the SD-VG-expressing cells (Liu et al., 2000; del Álamo Rodriguez et al., 2002), and which later becomes expressed in a second, more proximal ring (Fig. 1). WG expression in the inner, distal ring within the proximal wing is regulated by an enhancer called *spade-flag* (*spd-fg*), after an allele of *wg* in which this enhancer is deleted (Neumann and Cohen, 1996). Studies of this allele, together with ectopic expression experiments,

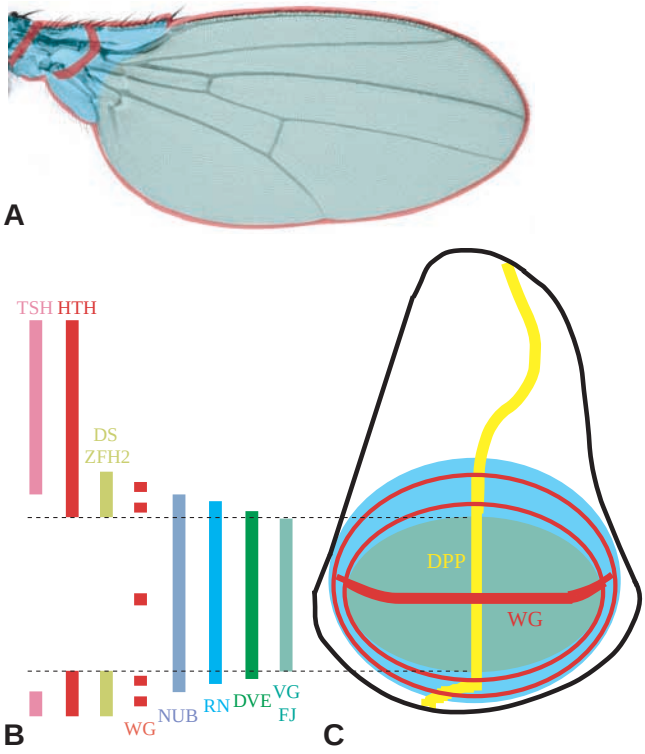

Fig. 1. Proximodistal wing patterning. (A) Adult wing: distal (green), proximal wing and hinge (blue), and WG-expressing cells (red). The boundary between distal and proximal cells is an approximation. We adopt the term proximal wing for the blue region, but note that this entire region is sometimes referred to as the wing hinge. (B) Relative gene expression domains along the proximodistal axis, related to C by the dashed lines. Although for simplicity all genes are shown as having uniform expression levels, some are subject to modulation within their spatial domains (this work) (Williams et al., 1991; Clark et al., 1995; Villano and Katz, 1995; Brodsky and Steller, 1996; Ng et al., 1996; Rieckhof et al., 1997; Azpiazu and Morata, 2000; Casares and Mann, 2000; St Pierre et al., 2002; Wu and Cohen, 2002; Kolzer et al., 2003; Whitworth and Russell, 2003). (C) A wing imaginal disc, shaded as for the adult wing shown in A. Approximate location of DPP-expressing cells (yellow) is also indicated.

revealed that WG is necessary and sufficient to promote growth of the proximal wing (Neumann and Cohen, 1996; Klein and Martinez Arias, 1998). WG also plays a role in proximal wing patterning, as it acts in a positive-feedback loop to maintain expression of Homothorax (HTH) (Azpiazu and Morata, 2000; Casares and Mann, 2000; del Álamo Rodriguez et al., 2002). The *rotund* (*rn*) gene has been identified as an additional target of distal signaling (del Álamo Rodriguez et al., 2002).

In this work, we identify Four-jointed (FJ), Dachsous (DS), Fat and Dachs as proteins that influence signaling to proximal wing cells to regulate WG and *rn* expression. FJ is a type II transmembrane protein, which is largely restricted to the Golgi (Villano and Katz, 1995; Brodsky and Steller, 1996; Buckles et al., 2001; Strutt et al., 2004). Null mutations in *fj* do not cause any obvious defects in the proximal wing (Villano and Katz, 1995; Brodsky and Steller, 1996). However, *fj* plays a role in the regulation of tissue polarity, yet acts redundantly with some other factor(s) in this process (Zeidler et al., 1999; Zeidler et al., 2000; Casal et al., 2002). Mutations in *fat* or *ds* can also influence tissue polarity (Adler et al., 1998; Casal et al., 2002; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002; Ma et al., 2003), and both genes encode large protocadherins (Mahoney et al., 1991; Clark et al., 1995). Although the molecular relationships among these proteins are not well understood, genetic studies suggest that *fj* and *ds* act via effects on *fat*, and both *fj* and *ds* can influence Fat localization in genetic mosaics (Strutt and Strutt, 2002; Yang et al., 2002; Ma et al., 2003).

Interestingly, alleles of *fj*, *ds* and *fat*, as well as alleles of another gene, *dachs*, can result in similar defects in wing blade and leg growth (Mohr, 1923; Waddington, 1943). The similar requirements for these genes during both appendage growth and tissue polarity, together with the expression patterns of *fj* and *ds* in the developing wing, led us to investigate their requirements for proximal wing development. We find that all four genes influence the expression of WG in the proximal wing, and genetic experiments suggest a pathway in which FJ and DS act to modulate the activity of Fat, which then regulates transcription via a pathway that includes Dachs. Our observations lend strong support to the hypothesis that FJ, DS and Fat function as components of an intercellular signal transduction pathway, implicate Dachs as a key downstream component of this pathway, and identify a normal role for these genes in proximodistal patterning during *Drosophila* wing development.

Materials and methods

Drosophila stocks and clonal analysis

Mutations used were: fj^{d1} (Brodsky and Steller, 1996), fat^{8} (fat^{fd}) and $fat^{G\text{-}rv}$ (Bryant et al., 1988; Mahoney et al., 1991), ds^{UA071} and ds^{38k} (Clark et al., 1995; Adler et al., 1998), d^{210} and d^{1} (Buckles et al., 2001), $wg^{spd\text{-}fg}$ (Couso et al., 1994), vg^{83b27r} (Williams et al., 1990) and sd^{58} (Campbell et al., 1991). fat^{8} d^{1}, fat^{8} vg^{83b27r}, $fat^{G\text{-}rv}$ $wg^{spd\text{-}fg}$ and fat^{8} $wg^{spd\text{-}fg}$ double mutant chromosomes were generated by meiotic recombination; a fj^{d1} ds^{UA071} chromosome was a gift of D. Strutt (Strutt et al., 2004). UAS and Gal4 transgenes used were: *UAS-vg[49]* (Kim et al., 1996); *UAS-fj[6a.2]* and *UAS-fj[146.3]* (Zeidler et al., 1999); *UAS-GFP*, *AyGal4[17b]* and *AyGal4[5a]* (actin>y+>Gal4) (Ito et al., 1997); and *GS-ds*. *GS-ds* contains an insertion of the UAS-containing Gene Search transposon (Toba et al., 1999), approximately 700 bp upstream of *ds* (flanking sequence includes GTGTACAGTGAAGTGCGGAAAAGAGGTCGAGGGG), and was isolated in a gain-of-function screen for genes that influence cell affinity in the wing (O. Dunaevsky, C. Rauskolb and K.D.I., unpublished). Its influence on *ds* expression was confirmed by in situ hybridization. Reporter genes employed were *spd-lacZ* (Neumann and Cohen, 1996), *fj-lacZ[P1]* (Brodsky and Steller, 1996), *rn-lacZ* (St Pierre et al., 2002) and *ds-lacZ* (Clark et al., 1995). Mutant clones were generated by mitotic recombination, and marked by the absence of a MYC-tagged protein or GFP. They were induced at 24-48 and at 48-72 hours after egg laying (AEL), and then allowed to grow for 48 or 72 hours, using the following stocks:

w; fj^{d1} FRT42D/CyO
w; fat^{8} FRT40A/SM5-TM6b
w; fat^{8} d^{1}FRT40A/SM5-TM6b
w; ds^{UA071} FRT40A/CyO act-GFP
w; d^{1}FRT40A/CyO
w; d^{210}FRT40A/CyO
y w Flp, 2[π-Myc] FRT40A M
y w Flp; 2[π-Myc] FRT40A
y w Flp; Ubi-GFP FRT40A/CyO
y w Flp: FRT42D arm-lacZ

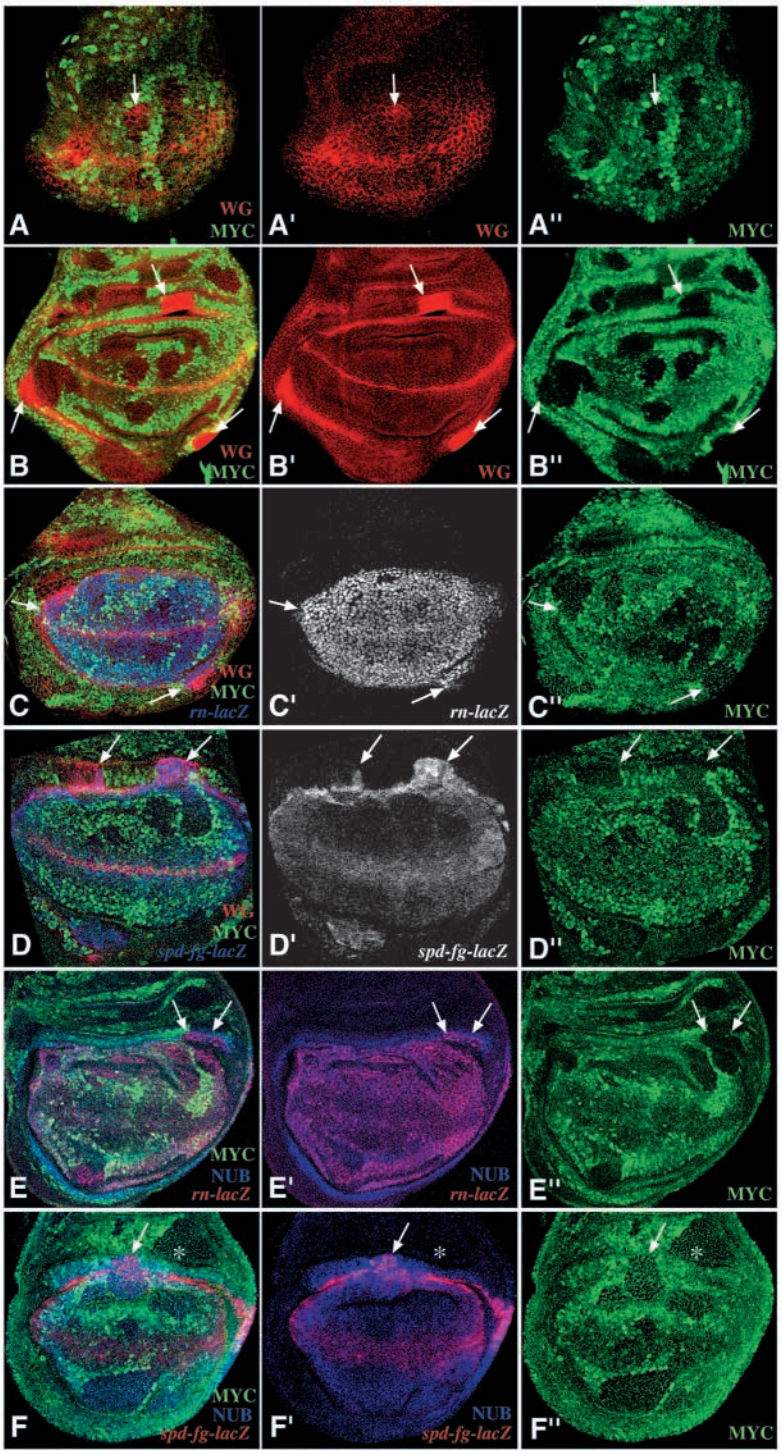

Fig. 2. *fat* mutant clones upregulate targets of distal-to-proximal signalling. In this and subsequent figures, all panels show third instar wing discs, oriented with ventral down and anterior left, and panels marked prime show separate stains of the same disc. Clones of cells mutant for fat^{8} are marked by the absence of MYC (green). Arrows indicate clones with ectopic gene expression. Discs are stained for WG (red), *rn-lacZ* (blue/white in C, red in E), *spd-fg-lacZ* (blue/white in D, red in F), and NUB (blue). (A) Early third instar disc. (B) Late third instar disc. (C) Mid-third instar disc. Ectopic WG expression is associated with an expansion of the *rn* domain. (D) Mid-late third instar disc. The *spd-fg* enhancer and endogenous WG are both ectopically expressed, although differences in subcellular localization result in apparent differences within a focal plane. (E) Late third instar disc with a *fat* clone that extends beyond the NUB domain; arrows here point to the edges of the clone where it extends proximally; *rn* is induced only within NUB-expressing cells. (F) Mid-third instar disc with a *fat* clone in the NUB domain with *spd-fg-lacZ* expression (arrow), and a clone just proximal to this without *spd-fg-lacZ* expression (asterisk).

y w hs-FLP;gug^{35} *FRT79E[2A]/TM6c*
w; FRT79E[2A] ubi-GFP:nls
y w sd^{58} *FRT18A/FM7*
2{hs-π:MYC} FRT18A; hs-FLP Sb/TM6b

Clones of cells ectopically expressing genes of interest (Flip-out) were generated by combining transgenes that provide expression under UAS control with transgenes that allow the generation of clones of cells expressing the Gal4 protein (AyGal4) (Ito et al., 1997). Gal4-expressing clones were marked using a *UAS-GFP* transgene. Flip-out clones were induced at 24-48 and 48-72 hours AEL.

Immunostaining

Imaginal discs from third instar larvae were fixed and stained as previously described (Liu et al., 2000), using as primary antibodies: rabbit anti-VG (1:600, S. Carroll, University of Wisconsin-Madison), mouse anti-WG 4D4 (1:1000, Developmental Studies Hybridoma Bank), mouse anti-NUB (1:100, S. Cohen, European Molecular Biology Laboratory), rabbit anti-β-gal (1:2000, ICN), Goat anti-β-gal (1:1000, Biogenesis), mouse anti-β-gal (1:1000, Sigma), rabbit anti-MYC (1:100, Santa Cruz), rat anti-MYC (1:1000, Serotec), rat anti-DS (1:200, M. Simon, Stanford University) and rat anti-Fat (1:100, H. McNeill, Cancer Research UK). For precise timing, larvae were collected in intervals after the second to third instar molt, but in some cases ages were estimated based on the size and morphology of the disc.

Results

fat represses targets of distal-to-proximal signaling

The *fat* gene is expressed throughout the developing wing imaginal disc (Mahoney et al., 1991). However, the expression and phenotypes of *fj* and *ds* (described below), together with prior studies suggesting a functional relationship among these genes, suggested that Fat might have a role in proximal wing development. Indeed, the two known targets of distal signaling, WG and *rn*, are both strongly upregulated within *fat* mutant clones in the proximal wing (Fig. 2). The influence of *fat* on *rn* and WG is strictly cell autonomous. This autonomous action suggests that Fat is not involved in sending a signal to proximal wing cells, but might be regulated by signals from neighboring cells.

WG expression is upregulated within *fat* mutant cells from early third instar, when the distal ring is first discernible (Fig. 2A), and continues to be upregulated throughout larval development (Fig. 2B). A $wg^{spd\text{-}fg}$*-lacZ* reporter line is also activated within *fat* mutant clones, indicating that the

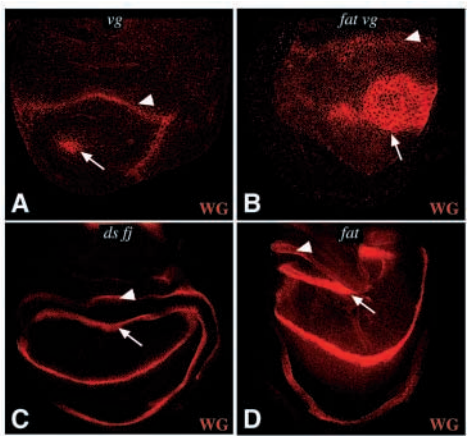

Fig. 3. WG expression in mutant discs. Late third instar discs, stained for WG (red), with inner (arrow) and outer (arrowhead) rings marked. (A) vg^{83b27r}. (B) fat^{8} vg^{83b27r}. (C) fj^{d1} ds^{UA071}. (D) $fat^{8}/fat^{G\text{-}rv}$. Because the disc is more folded (see Fig. 4), WG expression is only partially visible.

regulation of *wg* expression by Fat is effected through the *spd-fg* enhancer (Fig. 2D,F). The induction of WG and *rn* in *fat* mutant clones is not detected in the notum or in the distal wing, and within the proximal wing it is limited to NUB-expressing cells (Fig. 2E,F). Importantly, this temporal and spatial profile of WG regulation by Fat, as well as its action through the *spd-fg* enhancer, match that for the regulation of WG and *rn* by VG (Liu et al., 2000; del Álamo Rodriguez et al., 2002). These observations suggest that the VG-dependent signal might activate WG expression by inhibiting Fat activity.

To further examine this possibility, we analyzed *vg fat* double mutants. *vg* mutant wing discs contain a single ring of WG expression, which, based on the NUB expression domain, appears to correspond to the outer WG ring (Fig. 3A) (Liu et al., 2000). Expression of WG in the inner ring, which normally overlaps NUB, is either not detected (8/14 discs), or is reduced to a small central spot (6/14 discs) in *vg* mutants (Fig. 3A). Importantly, in *vg fat* double mutants, WG expression is always observed in the center of the disc (7/7 discs), and this expression is substantially enlarged (Fig. 3B). The observation that mutation of *fat* can promote WG expression even in the absence of VG is consistent with the hypothesis that Fat is normally repressed downstream of a VG-dependent signal.

Ectopic WG contributes to proximal wing overgrowth in *fat* mutant discs

fat acts as a *Drosophila* tumor suppressor gene, and *fat* mutants die after an extended larval stage, with overgrown imaginal discs (Bryant et al., 1988; Mahoney et al., 1991; Garoia et al., 2000). Although *fat* can influence the growth of most, and perhaps all imaginal cells, examination of *fat* mutant wing discs nonetheless indicates that there is a disproportionate overgrowth of the proximal wing, which is particularly evident in older larvae (Bryant et al., 1988; Garoia et al., 2000) (Fig. 4B,G). The observation that mutation of *fat* results in ectopic WG expression in the proximal wing (Fig. 2), together with the knowledge that ectopic WG expression promotes overproliferation of proximal wing tissue (Neumann and Cohen, 1996), suggested that the disproportionate overgrowth

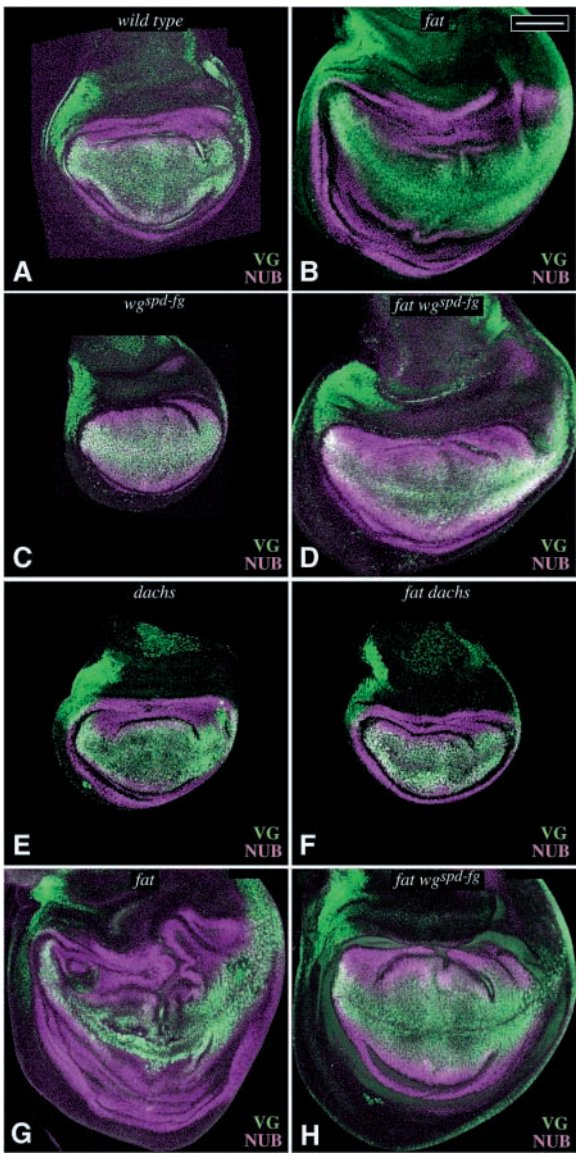

Fig. 4. *wg* and *dachs* are required for overgrowth in *fat* mutant discs. Discs stained for VG (green) and NUB (magenta); discs shown in A-F are at 36-48 hours of third instar, those in G-H are at 48-60 hours. Cells that express only NUB correspond to the distal half of the proximal wing (Fig. 1). (A) Wild-type. (B) $fat^{8}/fat^{G\text{-}rv}$. (C) $wg^{spd\text{-}fg}$. (D) $wg^{spd\text{-}fg}$ $fat^{8}/wg^{spd\text{-}fg}$ $fat^{G\text{-}rv}$. (E) $dachs^{1}$. (F) fat^{8} $dachs^{1}$. (G) $fat^{8}/fat^{G\text{-}rv}$ disc, the overgrowth of the proximal wing is even more pronounced at this age, and the wing becomes highly folded. (H) $wg^{spd\text{-}fg}$ $fat^{8}/wg^{spd\text{-}fg}$ $fat^{G\text{-}rv}$. Wild type and fat^{8} $dachs^{1}$ are not shown at this age, as they begin to pupate. Scale bar in B: 80 μm for A-H.

of the proximal wing in *fat* mutants might be due to ectopic expression of WG. To test this possibility, we recombined *fat* alleles with the $wg^{spd\text{-}fg}$ allele. Indeed, disproportionate overgrowth of the proximal wing disc is suppressed in *fat* mutant animals that also carry $wg^{spd\text{-}fg}$ (Fig. 4D,H; compare with Fig. 4B,G). Thus, two distinct processes contribute to overgrowth in *fat* mutant wing discs: a broad-based process that results in enlargement of the entire disc, and a local upregulation of WG in the proximal wing. This latter process

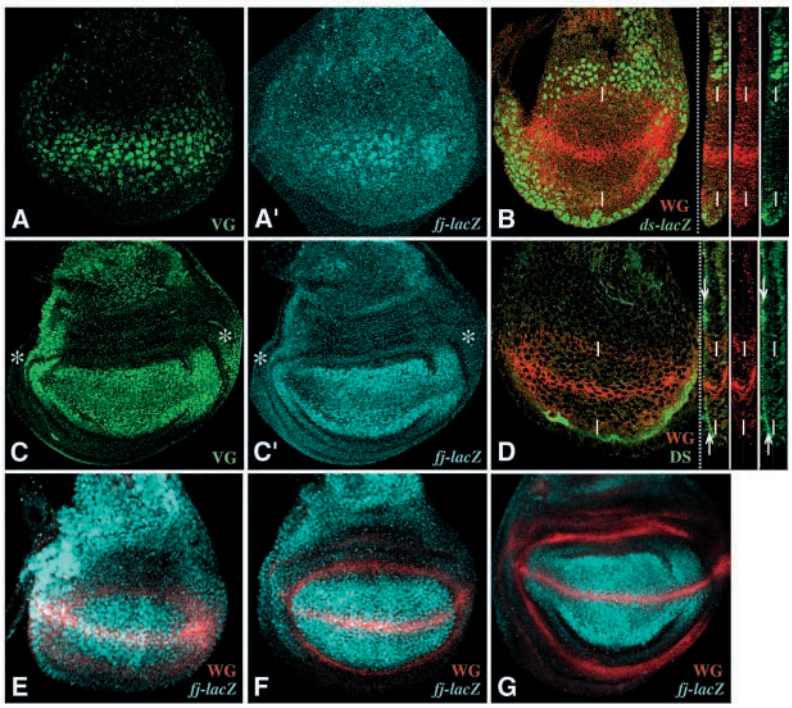

Fig. 5. Expression of *four-jointed* and *dachsous* during wing development. Expression of VG (green), *fj-lacZ* (cyan), *ds-lacZ* (green), DS (green) and WG (red) are shown. (A) Early (0-12 hours of third instar) disc, stained for VG and *fj-lacZ*. (B) Early third instar disc, stained for *ds-lacZ* and WG. White bars identify WG expression in the proximal wing. Images to the right of the dashed line show different channels of vertical sections of the same disc. (C) Mid-late third instar disc (24-36 hours) stained for VG and *fj-lacZ*. Asterisks highlight proximal regions where VG expression remains elavated relative to *fj*. (D) Early third instar disc, stained for DS and WG. DS protein is predominantly apical (arrow). (E-G) Early (E), mid (F) and late (G) third instar discs, stained for WG and *fj-lacZ*.

emphasizes the importance of WG regulation by *fat* to normal wing development.

Expression and regulation of *four-jointed* during wing development

Since SD-VG functions as a transcription factor, its non-autonomous influence on gene expression in the proximal wing presumably results from the regulation of target genes that effect or modulate intercellular signaling. FJ has been reported to be expressed throughout the wing pouch (distal wing primordia) of the wing disc at late third instar (Villano and Katz, 1995; Brodsky and Steller, 1996). In order to investigate FJ as a potential contributor to distal-to-proximal signaling, we first confirmed that its expression is similar to VG throughout the third instar (Fig. 5A,C). The only significant difference observed was that along the DV boundary, expression of VG remains at high levels outside of the distal wing, whereas *fj* expression drops to lower levels (Fig. 5C). At early third instar, WG expression is directly adjacent to *fj* and VG, but as the wing grows they separate (Fig. 5E-G) (Klein and Martinez Arias, 1998; del Álamo Rodriguez et al., 2002; Kolzer et al., 2003). Because SD-VG is required for the growth and viability of wing cells, clones of cells that are mutant for null alleles of *sd* or *vg* fail to proliferate and die (Kim et al., 1996; Liu et al., 2000). However, in clones of cells mutant for a hypomorphic allele of *sd*, sd^{58}, a reduction in *fj* expression is detectable (Fig. 6A).

To confirm that SD-VG is also sufficient to promote *fj* expression, we examined the consequences of ectopic VG expression. Indeed, VG-expressing clones are associated with induction of *fj* expression in the proximal wing (Fig. 6B). However, the induction of *fj* by ectopic VG sometimes occurs in a broader domain that includes cells neighboring the VG-expressing clone. In the eye, FJ has been reported to be able to induce the expression of *fj* in neighboring cells (Zeidler et al., 1999). However, in the wing, ectopic expression of FJ does not result in a detectable induction of *fj* expression (Fig. 7A), nor does ectopic expression of *fj* exert any detectable influence on VG expression (Fig. 7B). Although the mechanism by which FJ becomes induced non-autonomously is not known, our results nonetheless indicate that FJ is a normal downstream target of VG in the distal wing, and that it is expressed in association with ectopic VG in the proximal wing.

Ectopic *four-jointed* influences targets of the distal-to-proximal signal

If *fj* contributes to signaling from SD-VG-expressing distal cells, then expression of FJ could be sufficient to induce the expression of targets of the distal signal. Importantly then, clones of cells that ectopically express FJ in the proximal wing can induce expression of both WG and *rn* in neighboring cells (Fig. 7). Ectopic *fj* expression also results in downregulation of WG expression within FJ-expressing cells (Fig. 7B-D). Although virtually all FJ-expressing clones (58/60 scored throughout third instar) effect at least some modulation of WG expression, both the non-autonomous induction and the autonomous repression of WG expression by *fj* are weaker than that associated with ectopic VG expression in that: (1) modulation of WG expression by FJ is more tightly restricted, and, in most cases, is only observed in cells that are within or immediately adjacent to the endogenous WG stripe; (2) when ectopic WG is observed more than a couple cells away from the endogenous WG stripe, this ectopic WG is always weaker than endogenous WG; and (3) the repression of endogenous WG within FJ-expressing cells is usually only partial. By contrast, VG completely represses endogenous WG in the distal ring, and often induces a strong ectopic expression of WG several cells away from endogenous WG (Fig. 6B) (Liu et al., 2000; del Álamo Rodriguez et al., 2002). Despite these differences, the *spd-fg* enhancer also responds to FJ, both targets of distal signaling, *rn* and WG, are similarly affected by FJ, and ectopic FJ is only able to modulate WG and *rn* expression within the NUB-expressing cells in the distal half of the proximal wing (Fig. 7). The observations that FJ regulates the same genes, in the same place, and through the same enhancer as VG suggest that FJ contributes to signaling from distal cells. Similar reasoning (above) suggests that the distal signal acts through Fat, and FJ has been suggested to influence Fat in regulating tissue polarity (Strutt and Strutt,

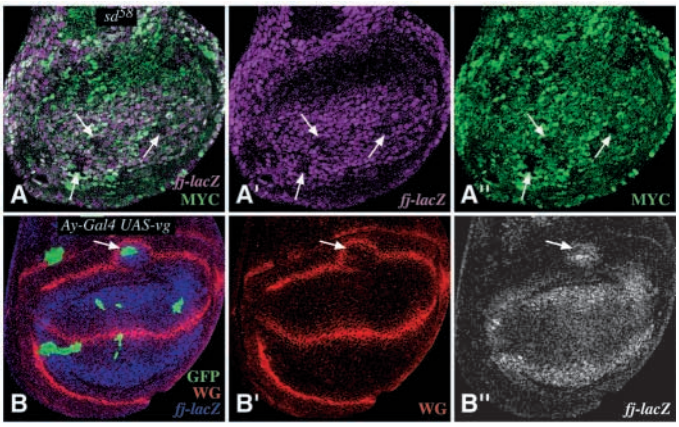

Fig. 6. Scalloped-Vestigial regulates *four-jointed* expression. (A) Mid-third instar disc with sd^{58} clones, marked by the absence of MYC (green), and stained for *fj-lacZ* (magenta). Arrows indicate examples of clones with reduced *fj* expression. (B) Mid-late third instar disc with VG-expressing clones, marked by GFP (green), and stained for *fj-lacZ* (blue/white) and WG (red). Arrow indicates a clone with ectopic *fj*.

2002; Yang et al., 2002; Ma et al., 2003). Together then, these observations imply that FJ influences WG and *rn* expression by modulating Fat activity, and, consistent with this, ectopic FJ expression also modulates Fat protein staining in the developing wing (Fig. 7G).

Mutation of *fj* impairs the initiation of WG expression in the proximal wing

The proximal wing appears normal in *fj* null mutant animals (Villano and Katz, 1995; Brodsky and Steller, 1996). Thus, if FJ contributes to distal signaling, it must do so redundantly. Nonetheless, we considered the possibility that some reduction in WG expression might be detectable in *fj* mutants. In order to enhance our ability to detect subtle changes, WG was examined in *fj* genetic mosaics. In this situation, regions of the disc composed of wild-type cells provide an internal control for normal levels of staining. Importantly, at early to mid third instar, WG expression in the distal ring was reduced in cells adjacent to *fj* mutant distal wing cells (Fig. 8A,B; 10/13 early discs with clones had detectable alterations in WG expression). WG expression was never completely eliminated, consistent with notion that FJ contributes to, but is not absolutely required for, WG expression. The influence of FJ on WG expression depended on the genotype of distal wing cells rather than proximal wing cells (Fig. 8A,B), consistent with the *fj* expression pattern (Fig. 5). Intriguingly, WG expression sometimes (7/32 clone edges) also appeared elevated in mutant cells immediately adjacent to wild-type cells (Fig. 8A). The altered expression of WG indicates that FJ contributes to normal distal signaling, but is not solely responsible for it.

However, by late third instar, *fj* mutant clones are not associated with any noticeable decrease in WG expression (>15 discs) (Fig. 8C). That is, although

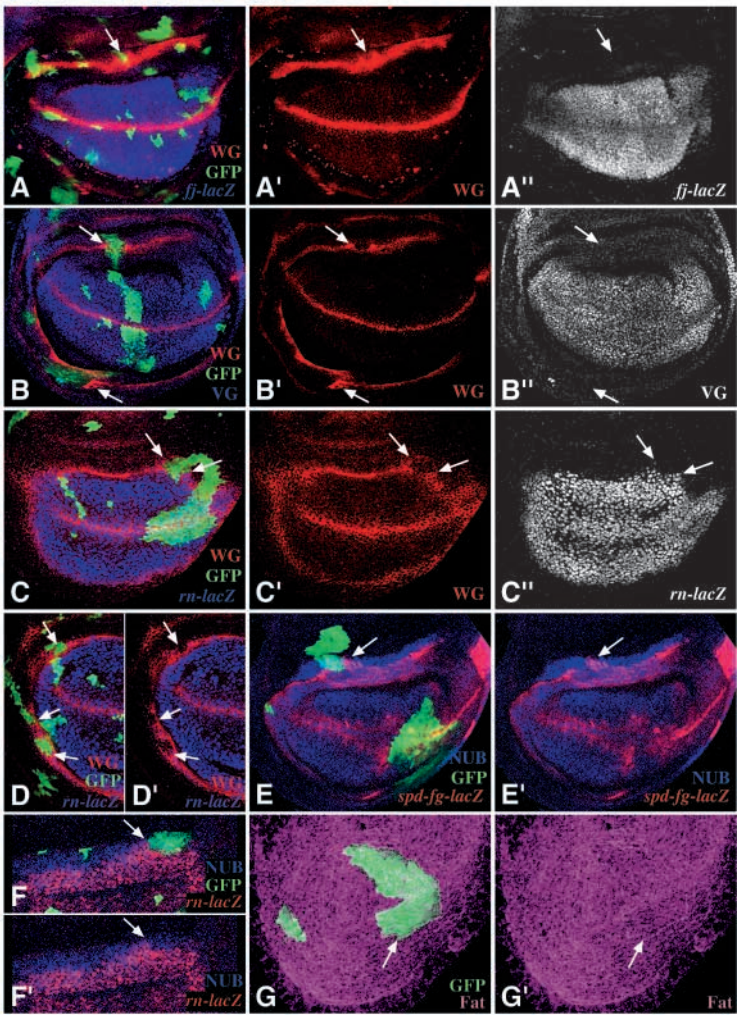

Fig. 7. Four-jointed can influence gene expression in the proximal wing. FJ-expressing clones are marked by GFP (green). (A) Late third instar disc, stained for *fj-lacZ* (blue/white) and WG (red). Arrow points to a clone that induces WG expression in flanking cells in the proximal wing. No induction of *fj* occurs. (B) Mid-late third instar disc, stained for VG (blue/white) and WG (red). Arrows point to clones that induce WG. No induction of VG occurs. (C) Mid-third instar disc, stained for expression of *rn-lacZ* (blue/white) and WG (red). Arrow indicates induction of WG and *rn* flanking a clone. Their expression is also decreased within the FJ-expressing cells. (D) Late third instar disc, stained for rn-*lacZ* (blue) and WG (red). Arrows point to clones that alter WG expression. (E) Mid-late third instar disc, stained for NUB (blue) and *spd-fg-lacZ* (red). *spd-fg-lacZ* is induced only up to the edge of the NUB domain, even though the clone (arrow) extends proximally. Although *spd-fg-lacZ* expression is broader and more diffuse than endogenous WG (Neumann and Cohen, 1996), it does not extend to the edge of NUB expression in the absence of ectopic FJ. (F) Mid-third instar disc, stained for NUB (blue) and *rn-lacZ* (red). *rn-lacZ* is induced only up to the edge of the NUB domain, even though the clone (arrow) extends proximally. (G) Early third instar disc, stained for Fat (magenta). Fat is tightly localized apically; because the disc is not flat this figure is a composite projection of different focal planes to allow visualization of Fat over a broad region. Fat appears to be concentrated along the edge of the clone (arrow).

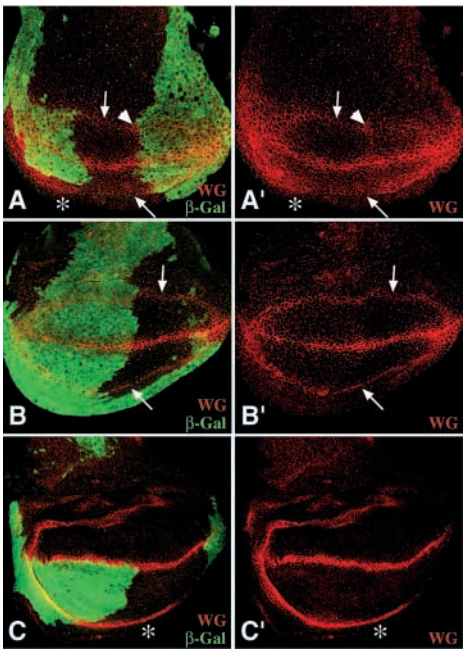

Fig. 8. *four-jointed* influences the initiation of WG expression in the proximal wing. fj^{d1} mutant clones, made using the *Minute* technique and marked by absence of β-galactosidase (green). (A) Early third instar disc. WG expression is reduced within large *fj* mutant clones (arrows). The requirement for *fj* is non-autonomous, as *fj* mutant cells in the proximal wing express WG normally (asterisk). Arrowhead identifies elevated WG expression in cells immediately adjacent to wild-type cells. (B) Mid-third instar disc, arrows point to reduced expression. (C) Mid-late third instar disc, with most tissue mutant. WG expression is no longer noticeably reduced by absence of *fj* (asterisk).

initiation of WG appears impaired, at later stages WG expression recovers. This explains the normal development of the proximal wing in *fj* mutants. This recovery also suggests that WG expression in the hinge is regulated in two phases: an initiation phase that depends on distal signaling, and a later maintenance phase that is independent of distal signaling.

Expression of *dachsous* during wing development

Studies of tissue polarity suggest a close functional relationship among *fj*, *fat* and *ds*. *ds* is expressed preferentially by proximal wing cells (Clark et al., 1995), but low levels have been reported in more distal cells (Strutt and Strutt, 2002; Ma et al., 2003). During third instar, both DS protein expression and *ds* transcription, as detected by a *lacZ* enhancer trap line, appear graded, with the highest levels in proximal wing cells and the lowest levels in distal wing cells (Fig. 5B,D). When the inner ring of WG expression is first detected, at early third instar, it appears on the slope of DS expression, with the highest levels of DS more proximal, and the lowest levels of DS more distal.

dachsous influences WG expression in the proximal wing

Neither *ds* mutant discs (not shown), nor *fj ds* double mutant discs (Fig. 3C), exhibit obvious changes in WG expression, nor do they display the overgrowths of wing tissue observed in *fat* mutants. Nonetheless, clones of cells mutant for a strong *ds* allele, ds^{UA071}, can exert a subtle influence on WG expression. At early third instar, this influence is most often detected as a slight decrease in WG within *ds* mutant cells, and a slight increase in WG in wild-type cells that border the clone (18/37 early to mid third instar clones revealed this effect) (Fig. 9A), although in some cases (8/37) WG expression appeared slightly elevated within mutant cells. At late third instar a slight increase in WG expression is most often (19/35 clones) observed within *ds* mutant cells (Fig. 9B), and a decrease in WG expression is only rarely (3/35 clones) observed. Similarly, at early to mid third instar, ectopic expression of DS was often (12/23 cases) associated with upregulation of WG within DS-expressing cells at the edge of clones (Fig. 9C), although occasionally (4/23 cases) WG was upregulated in neighboring cells (Fig. 9D). At late stages elevation of WG expression in neighboring cells was observed (12/12 cases). Although the influence of DS is complex (see Discussion), its ability to modulate WG expression in the proximal wing is consistent with the suggestion that it can influence Fat activity. It has been reported previously that Fat localization is altered by *ds* mutant clones (Strutt and Strutt, 2002; Ma et al., 2003), and we find that clones of cells ectopically expressing DS can also influence Fat localization (Fig. 9F). To investigate possible interactions between *ds* and *fj*, we also examined clones of cells co-expressing both genes. These are associated with non-autonomous upregulation of WG at all stages (Fig. 9E).

grunge mutations do not affect WG expression in the proximal wing

A transcriptional co-repressor, Grunge (Atro), has been identified that influences tissue polarity and can physically interact with the cytoplasmic domain of Fat (Fanto et al., 2003). To investigate whether it functions in distal-to-proximal signaling, we examined WG expression in gug^{35} mutant clones in the wing. Although the clones exhibited other defects consistent with previously described roles for *gug* (Erkner et al., 2002; Zhang et al., 2002), no influence on WG expression in the proximal wing was detected (Fig. 10A).

dachs is required for the initiation of WG expression in the proximal wing

Although *dachs* has not been reported to influence tissue polarity, hypomorphic alleles of *dachs* can result in wing and leg phenotypes similar to those of *fj* and *ds*, and *dachs* interacts genetically with *fj* (Waddington, 1943; Buckles et al., 2001). To determine whether *dachs* also influences distal-to-proximal signaling, we first attempted to generate clones mutant for a strong allele (d^{210}), but were unable to recover any mutant clones, even when we gave them a growth advantage by using the *Minute* technique. As an alternative, we examined clones mutant for a hypomorphic allele of *dachs*, d^{1}. When examined at early stages of wing development, these clones are always (7/7 clones) associated with a dramatic reduction of WG expression in the proximal wing (Fig. 10B). The reduction in WG expression is cell autonomous, suggesting that *dachs* is required for receiving, rather than sending, the distal signal. Intriguingly however, later in third instar, WG expression partially recovers within *dachs* mutant clones (17 clones, the older the disc the more normal WG staining appears) (Fig. 10C). This recovery suggests again that WG expression in the

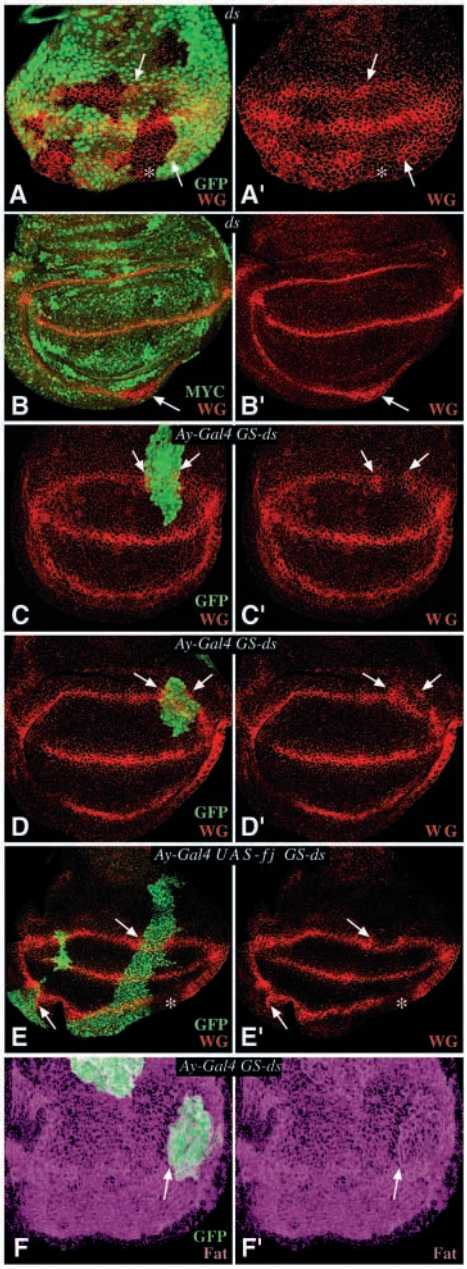

Fig. 9. Dachsous influences WG expression. Discs stained for WG (red), Fat (magenta), MYC (green) or GFP (green). (A) Early third instar disc with ds^{UA071} mutant clones, marked by absence of GFP. In some cases, WG is relatively decreased within clones (asterisk), and relatively increased in flanking wild-type cells (arrows). (B) Late third instar disc with ds^{UA071} mutant clones, marked by the absence of MYC. WG expression is increased within a clone (arrow). (C) Early to mid-third instar disc with clone overexpressing DS, marked by GFP. WG appears elevated in cells at the edge of the clone (arrows), and slightly decreased in more internal cells. (D) Mid-third instar disc with clone overexpressing DS, marked by GFP. Ectopic expression of WG is detectable outside the clone (arrows). (E) Clone overexpressing DS and FJ, marked by GFP. Arrows point to examples of ectopic WG. Asterisk indicates a region where WG expression is out of the plane of focus. (F) Early-mid third instar with clones overexpressing DS, stained for Fat. Image is a composite of projections through different focal planes. Fat appears to accumulate at the clone border (arrow), and to be depleted from neighboring cells.

hinge is regulated by distinct initiation and maintenance mechanisms.

dachs is epistatic to *fat*

dachs has recently been found to encode an unconventional myosin (F. Katz, personal communication), and thus is presumably a cytoplasmic protein. The autonomous influence of *dachs* on WG expression, together with its presumed cytoplasmic location, suggested that it might act downstream of *fat*. Since mutation of *fat* and mutation of *dachs* have opposing effects on WG, this possibility could be tested genetically. In d^1 fat^8 double mutant clones, the influence of *dachs* on WG expression is epistatic, as clones in early third instar discs exhibit the same reduction in WG expression that is observed in d^1 mutant clones (12/12 clones) (Fig. 10D). At later stages, WG expression partially recovers (18 clones), but at no time do the clones exhibit significant ectopic WG expression (Fig. 10E). Interestingly, the *dachs* phenotype is also epistatic for the growth effects of *fat*, as the overgrowth phenotype of *fat* mutant discs is partially suppressed in animals that are also heterozygous for d^1 (data not shown), and completely suppressed in animals that are homozygous for d^1 (Fig. 4F).

Discussion

Proximodistal patterning in the wing disc is reflected in a series of concentric domains of gene expression. The initial expression of many of these genes is known or thought to occur in response to WG and DPP, which can act together to promote distal fates and repress proximal fates (reviewed by Mann and Morata, 2000; Klein, 2001). However, important aspects of wing patterning rely on signaling from distal cells to proximal cells. In this work, we have identified a set of genes that influence this process, and provide genetic evidence that in doing so they act as components of an intercellular signal transduction pathway. Studies described here and elsewhere suggest that Fat functions as a key component in this pathway, which is regulated by FJ and DS, and which then modulates transcription via intracellular pathways that include Grunge and/or Dachs (Fig. 11A).

The Fat signaling pathway

The argument that *fj*, *ds* and *fat* function together is supported by the observation that they share common phenotypes in many different processes, including proximodistal growth of legs and wings, tissue polarity, and, as shown here, distal-to-proximal wing signaling. In addition, both FJ and DS can influence Fat protein staining (Strutt and Strutt, 2002; Yang et al., 2002; Ma et al., 2003) (Figs 7, 9). The possibility that they act as components of an intercellular signaling pathway has been suggested based on studies of tissue polarity, but, at the same time, the nature of tissue polarity has complicated attempts to assign distinct roles for these genes in signaling versus receiving cells. Particularly important then, is the identification of transcriptional outputs of Fat signaling. We have identified here two genes, *wg* and *rn*, that are influenced non-

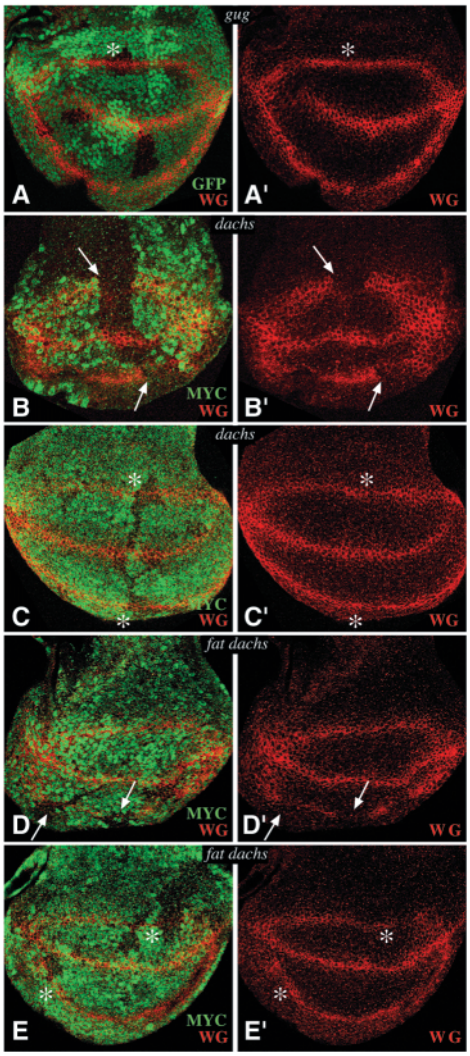

Fig. 10. Dachs is required for distal-to-proximal signalling. Discs stained for WG (red), with mutant clones marked by the absence of MYC or GFP (green). Arrows point to clones with reduced WG, asterisks mark clones with essentially normal WG. (A) *gug*^{35} mutant clones. (B) Early third instar disc with *dachs*^{1} Minute clones. (C) Mid-late third instar disc with *dachs*^{1} clones. (D) Early third instar disc with *fat*^{8} *dachs*^{1} clones. (E) Mid-third instar disc with *fat*^{8} *dachs*^{1} clones. WG expression is still reduced in these clones, but is starting to recover.

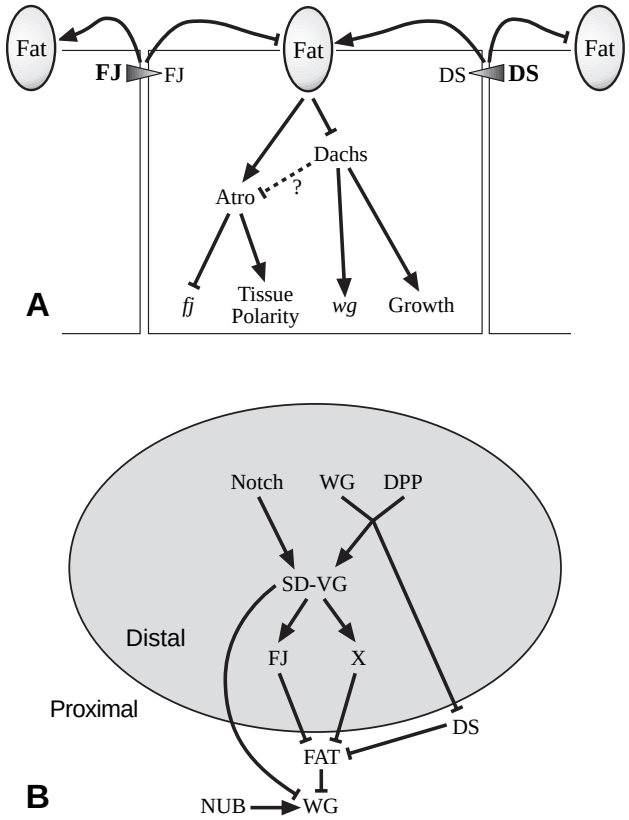

Fig. 11. Models for Fat and distal-to-proximal signalling. (A) Analysis of WG regulation, together with studies of tissue polarity, imply that Fat activity is modulated by the juxtaposition of cells with different levels of FJ or DS activity. Both normal expression patterns and analysis of genetic mosaics imply that at FJ expression borders, Fat is inhibited in cells with less FJ, and activated in cells with more FJ. The effects of DS are more variable, but in some cases Fat is inhibited in cells with more DS, and activated in cells with less DS. Fat functions normally to inhibit WG expression. As Dachs is required for WG, and is epistatic to Fat, the simplest genetic pathway would have Fat antagonizing Dachs activity. Fat regulates some processes via Grunge; however, it is not currently known whether these also require Dachs. (B) SD-VG specifies distal wing fate, and is regulated by Notch, WG and DPP signaling. We hypothesize that FJ acts redundantly with some other gene (X), which would also be regulated by SD-VG, and which would also act through Fat to regulate WG in the proximal wing. We further suggest that DS might be repressed by WG and DPP independently of SD-VG regulation, providing an additional input into Fat signaling. Induction of WG also appears to require NUB, and to be repressed distally.

autonomously by FJ and cell autonomously by Fat in the proximal wing. Similarly, expression of *fj* itself is influenced non-autonomously by FJ (Zeidler et al., 1999) and cell autonomously by Fat (Yang et al., 2002) in the eye, and Serrate expression is influenced non-autonomously by FJ (Buckles et al., 2001) and cell autonomously by Fat (E.C. and K.D.I., unpublished) in the leg. The observation that four different genes in three different tissues are each influenced non-autonomously by FJ and cell autonomously by Fat suggests strongly that FJ and Fat have common roles on the sending and receiving sides, respectively, of a broadly deployed intercellular signaling pathway. The identification of a normal developmental event in which one population of cells (distal wing) signals to adjacent cells (proximal wing) via these genes adds further support to this argument, and, at the same time, provides a developmental context for further identifying and characterizing roles of pathway components.

Regulation of Fat activity

The common feature of all of our manipulations of FJ and DS expression is that WG expression, and by inference, Fat activity, can be altered when cells with different levels of FJ or DS are juxtaposed. In the case of FJ, its normal expression pattern, mutant clones and ectopic expression clones are all consistent with the interpretation that juxtaposition of cells

with different levels of FJ is associated with inhibition of Fat in the cells with less FJ and activation of Fat in the cells with more FJ (Fig. 11A). The influence of DS, however, is more variable. Studies of tissue polarity in the eye suggested that DS inhibits Fat activity in DS-expressing cells, and/or promotes Fat activity in neighboring cells (Yang et al., 2002). The predominant effect of DS during early wing development is consistent with this, but its effects in late discs are not. Studies of tissue polarity in the abdomen suggest that the DS gradient might be interpreted differently by anterior versus posterior cells (Casal et al., 2002), and it is possible that a similar phenomena causes the effects of DS to vary during wing development.

The influence of *ds* mutation on gene expression and growth in the wing is much weaker than that of *fat*. It has been suggested that FJ might influence Fat via effects on DS (Yang et al., 2002), and *fj* mutant clones have been observed to influence DS protein staining (Strutt and Strutt, 2002; Ma et al., 2003). Our observations are consistent with the inference that both DS and FJ can regulate Fat activity, but they do not directly address the question of whether FJ acts through DS. They do, however, indicate that even the combined effects of FJ and DS cannot account for FAT regulation, and, assuming that the strongest available alleles are null, other regulators of Fat activity must exist. It is presumably because of the counteracting influence of these other regulators that alterations in FJ and DS expression have relatively weak effects. In addition, according to the hypothesis that Fat activity is influenced by relative rather than absolute levels of its regulators, the effects of FJ or DS could be expected to vary depending upon their temporal and spatial profiles of expression, as well as on the precise shape and location of clones.

Downstream signaling

Our observations, together with those of Fanto et al. (Fanto et al., 2003), imply the existence of at least two intracellular branches of the Fat signaling pathway (Fig. 11A). One branch involves the transcriptional repressor Grunge, influences tissue polarity, certain aspects of cell affinity, and *fj* expression, but does not influence growth or WG expression. An alternative branch does not require Grunge, but does require Dachs. Dachs is implicated as a downstream component of the Fat pathway, based on its cell autonomous influence on Fat-dependent processes, and by genetic epistasis. The determination that it encodes an unconventional myosin (F. Katz, personal communication), and hence presumably a cytoplasmic protein, is consistent with this possibility. It also suggests that it does not itself function as a transcription factor, and hence implies the existence of other components of this branch of the Fat pathway. This Grunge-independent branch influences WG expression in the proximal wing and imaginal disc growth. However, further studies will be required to determine whether Dachs functions solely in Grunge-independent Fat signaling, or whether instead Dachs is required for all Fat signaling.

Distal-to-proximal signaling in the wing

The observations that *fj* expression is regulated by SD-VG, and that *fj* is both necessary and sufficient to modulate the distal ring of WG expression in the proximal wing, suggest that FJ influences the activity of a distal signal, which then acts to influence Fat activity (Fig. 11B). However, the relatively weak effects of *fj* indicate that other factors must also contribute to distal signaling (X in Fig. 11B), just as *fj* functions redundantly with other factors to influence tissue polarity. As DS expression is downregulated in a domain that is broader than the VG expression domain, a direct influence of VG on the DS gradient is unlikely, and the essentially normal appearance of WG expression in the proximal wing in *fj ds* double mutants implies that DS is not a good candidate for Signal X. Rather, we suggest that DS acts in parallel to signaling from VG-expressing cells to modulate Fat activity. This VG-independent effect would account for the remnant of the distal ring that sometimes appears in *vg* null mutants (Fig. 3A) (Liu et al., 2000). Importantly though, the observation that the phenotypes of hypomorphic *dachs* mutant clones on WG expression are more severe than *fj* and *ds* suggests that the hypothesized additional factors also act via the Fat pathway. We also note that the limitation of WG expression to the proximal wing even in *fat* mutant clones implies that *wg* expression both requires NUB, and is actively repressed by distally-expressed genes (Fig. 11B).

The recovery of normal WG expression by later stages in both *fj* and *dachs* mutant clones implies that the maintenance of WG occurs by a distinct mechanism. Prior studies have identified a positive-feedback loop between WG and HTH that is required to maintain their expression (Azpiazu and Morata, 2000; Casares and Mann, 2000; del Álamo Rodriguez et al., 2002). We suggest that once this feedback loop is initiated, Fat signaling is no longer required for WG expression. Moreover, the recovery of normal levels of WG at late stages suggests that this positive-feedback loop can amplify reduced levels of WG to near normal levels.

The distinct consequences of VG expression and FJ expression in clones in the proximal wing suggest that another signal or signals, which are qualitatively distinct from the FJ-dependent signal, is also released from VG-expressing cells. When VG is ectopically expressed, WG is often induced in a ring of expression that completely encircles it (Liu et al., 2000). However, this is not the case for FJ-expressing clones. Both VG- and FJ-expressing clones can activate *rn* and *wg* only within NUB-expressing cells, but VG expression can result in non-autonomous expansion of the NUB domain, and this expansion presumably facilitates the expression of WG by surrounding cells (Liu et al., 2000; del Álamo Rodriguez et al., 2002; Baena-Lopez and Garcia-Bellido, 2003). Another striking difference between VG- and FJ-expressing clones is that in the case of ectopic FJ, enhanced WG expression is only in adjacent cells. By contrast, in the case of VG, WG expression initiates in neighboring cells, but often moves several cells away as the disc grows, resulting in a gap between VG and WG expression. This gap suggests that a repressor of WG expression becomes expressed there, and recent studies have identified Defective proventriculus (DVE) as such a repressor (Kolzer et al., 2003).

Growth regulation by the Fat signaling pathway

In strong *fat* mutants, the wing discs become enlarged and have extra folds and outgrowths in the proximal wing (Bryant et al., 1988; Garoia et al., 2000). The disproportionate overgrowth of the proximal wing is due to upregulation of WG in this region, as demonstrated by its suppression by $wg^{spd\text{-}fg}$ (Fig. 4). At the

same time, clones of cells mutant for *fat* overgrow in other imaginal cells, and *fat* $wg^{spd\text{-}fg}$ discs are still enlarged compared with wild-type discs. Thus, Fat appears to act both by regulating the expression of other signaling pathways (e.g. WG), and via its own, novel growth pathway. The identification of additional components of this pathway will offer new approaches for investigating its profound influence on disc growth.

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