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A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing

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SUMMARY

Short-range interaction between dorsal and ventral cells establishes an organizing center at the dorsal/ventral compartment boundary that controls growth and patterning of the wing. We report here that the dorsal/ventral organiser is built though a hierarchy of regulatory interactions involving the *Notch* and *wingless* signal transduction pathways and the *vestigial* gene. *wingless* and *vestigial* are activated in cells adjacent to the dorsal/ventral boundary by a Notch-dependent signal. *vestigial* is initially expressed under control of an early dorsal/ventral boundary enhancer that does not depend on *wingless* activity. Similarly, activation of *wingless* does not require *vestigial* function, showing that *wingless* and *vestigial* are parallel targets of the *Notch* pathway. Subsequently, *vestigial* is expressed in a broad domain that fills the wing pouch. This second phase of *vestigial* expression depends on Wingless function in cells at the dorsal/ventral boundary. In addition, the Notch and Wingless pathways act synergistically to regulate expression of *cut* in cells at the dorsal/ventral boundary. Thus Wingless can act locally, in combination with Notch, to specify cell fates, as well as at a distance to control *vestigial* expression. These results suggest that secreted Wingless protein mediates both long-range and short-range patterning activities of the dorsal/ventral boundary.

Key words: *Notch*, *wingless*, *vestigial*, *cut*, *Distal-less*, *Suppressor of Hairless*, axis formation, morphogen, signal transduction, limb development, *Drosophila*

INTRODUCTION

Studies on the development of *Drosophila* appendages have indicated that pattern formation in these multicellular fields is controlled by organizing centers located at compartment boundaries (reviewed in Blair, 1995; Brook et al., 1996). The first step in establishing these organizing centers depends on subdivision of the limb into functionally distinct subdomains known as compartments (García-Bellido et al., 1973), by the localized expression of transcription factors (reviewed by Lawrence and Morata, 1994). The homeobox gene *engrailed* and its homologue *invected* specify posterior compartment identity in the wing and the leg (Sanicola et al., 1995; Zecca et al., 1995; Guillen et al., 1995; Tabata et al., 1995). The LIM/homeobox gene *apterous* specifies dorsal compartment in the wing (Diaz-Benjumea and Cohen, 1993; Blair, 1993; Blair et al., 1994).

An asymmetric signaling event between the two compartments is then needed to direct cells near the compartment boundary to adopt a new fate. *engrailed* activates expression of the secreted signaling molecule Hedgehog (Hh) in posterior cells. Hh diffuses to nearby anterior cells, and there activates expression of the secreted signaling molecule Decapentaplegic (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Similarly, *apterous* activates expression of Fringe, a putative secreted protein (Irvine and Wieschaus, 1994), which in turn activates expression of Serrate (Kim et al., 1995), a trans-membrane ligand for Notch (Rebay et al., 1991). Serrate signals through Notch to activate expression of the nuclear protein Vestigial, the homeodomain transcription factor Cut, and the secreted signaling molecule Wingless (Kim et al., 1995; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; de Celis et al., 1996). In the case of the D/V organizer, a second signal is sent from ventral to dorsal cells, leading to the symmetric expression of Vestigial, Cut and Wingless straddling the D/V boundary (Blair, 1993; Williams et al., 1994; Diaz-Benjumea and Cohen, 1995; Ng et al., 1996). This second signal may be mediated by Delta (de Celis et al., 1996; Doherty et al., 1996), another trans-membrane ligand of Notch (Rebay et al., 1991; Fehon et al., 1990).

Activity of *Notch* is critical for wing development (Schellenbarger and Mohler, 1978) and is required in cells abutting the D/V boundary (De Celis and Garcia Bellido, 1994). Clones of cells lacking *Notch* activity, that touch the D/V boundary from one side, lose expression of *wingless* autonomously on that side, but also cause loss of *wingless* expression in wild-type cells on the other side of the D/V boundary (Rulifson and Blair, 1995). This suggests that a Notch-dependent feedback loop maintains the activated state of Notch on both sides of the margin, perhaps through the regulation of *Serrate* and *Delta* expression (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; DeCelis et al., 1996; Doherty et al., 1996).

Finally, localized expression of the signalling molecules in

cells at the boundary transmits the organizing signal to more distant cells. Decapentaplegic mediates the organizing effect of the A/P boundary by specifying cell fates and controlling growth in the wing pouch (Zecca et al., 1995; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995; Nellen et al., 1996; Lecuit et al., 1996). Similalrly, the D/V boundary organizer specifies the location of the wing margin and directs proliferation of surrounding cells (Diaz-Benjumea and Cohen, 1993). Wingless activity is required for the formation of the wing margin (Phillips and Whittle, 1993; Couso et al., 1994). Wingless is also required for proliferation and/or cell survival throughout the wing blade. Ectopic expression of Wingless is sufficient to induce wing margin structures and to cause overproliferation in surrounding cells, suggesting that Wingless mediates the long-range patterning activity of the D/V organizer (Diaz-Benjumea and Cohen, 1995).

In this report, we clarify the regulatory relationships between *Notch*, *wingless* and *vestigial* in establishing the D/V organizer. We show that expression of *wingless* and *vestigial* in the margin are direct and parallel responses to the activation of Notch. In contrast to previous reports (Williams et al., 1993, 1994; Couso et al., 1995), we show that *wingless* is not required for the activation of *vestigial*. Likewise, *wingless* activation does not depend on *vestigial* function at the D/V boundary. Following the initial activation of *vestigial* under control of its boundary-specific early enhancer, the domain of *vestigial* expression spreads throughout the wing pouch (Williams et al., 1993, 1994). We report that expression of *vestigial* in this secondary domain depends on Wingless activity, suggesting that a secondary function of *vestigial* is to mediate the long-range effects of secreted Wingless protein in the wing pouch. We also report that Wingless and Notch cooperate to activate the expression of *cut*, suggesting that the Wingless and Notch pathways interact synergistically in the wing imaginal disc. Taken together, these results illustrate that a hierarchical relationship between *Notch*, *wingless* and *vestigial* patterns the D/V axis of the wing.

MATERIALS AND METHODS

Drosophila stocks

wg^{r0727}, *spd-lacZ*, spd^{fg}, $Df(2L)spd^{hL2}$ and UAS-*dsh* are described in Neumann and Cohen (1996). The *wg-lacZ* used for the detection of *wg* expression in *vg* null discs is described in Kassis et al. (1992). The wg^{IL114} chromosome was used to generate larvae temperature sensitive for *wg* (Treisman and Rubin, 1995). Larvae were raised at 17°C and shifted to 25°C to remove Wg activity. Staging of wg^{IL} larvae was as in Couso et al. (1995). To identify mutant larvae, *vg* and *wg* mutant chromosomes were balanced over the *SM6a-TM6b* compound balancer, which carries the dominant larval marker, *Tubby*. UAS-wg^{+} is described in Lawrence et al. (1995). UAS-Notch(intra) was provided by Laurent Seugnet and Marc Haenlin (described in Doherty et al., 1996). The null allele $vestigial^{83b27R}$ (Williams et al., 1993) and the *vestigial* intron 2 enhancer are described in Williams et al. (1994). The GAL4 driver MS1096 is described in Capdevila and Guerrero (1994). $Su(H)^{SF8}$ *FRT40A* is described in Schweisguth (1995).

Clonal analyses

Su(H) mutant clones were induced using the FLP/FRT technique (Xu and Rubin, 1993), in larvae of genotype *y HSFlp1;* $Su(H)^{SF8}$ *FRT40A*/*N-myc FRT40A. vestigial-lacZ* and wg^{rO727} transgenes were recombined (separately) onto the Su(H) FRT40A chromosome. *cut-lacZ* was introduced using the *ctwHZ1* insertion of the third chromosome (Jack et al., 1991). wg^{cx4} mutant clones were induced in larvae of genotype *y HSFlp1;* wg^{cx4} *FRT40A*/*N-myc FRT40A*. A newly generated insertion of *vestigial-lacZ* was recombined onto the wg^{cx4} *FRT40A* chromosome. Clones were induced by heat shock for 1 hour at 37°C at 36±12 hours larval age. Late *Su(H)* clones marked with *cut-lacZ* were induced at 96±12 hours.

Histochemical methods

Anti-Dll staining is described in Diaz-Benjumea and Cohen (1995); anti-vestigial in (Williams et al., 1993); anti-Wg in Brook and Cohen (1996). For clonal analysis double-labeling was performed using monoclonal antibody 9E11 to the Myc epitope and rabbit anti-β-GAL.

RESULTS

Activity of the *Notch* pathway is cell-autonomously required for the expression of *wingless* and *vestigial* in the D/V organizer

Fig. 1 summarizes the effects of *Notch* activity on the expression of *wingless* (*wg*), *vestigial* and *cut*. Expression of all three genes depends on *Notch* activity. Loss-of-function mutations of *Notch* lead to the loss of *wg* and *cut* expression (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; DeCelis et al., 1996). Notch-dependent activation of *wg, cut* and *vestigial* depends on the activity of *Suppressor of Hairless* [*Su(H)*; Fig. 1B,F,J; see also Couso et al., 1995]. *Su(H)* encodes a DNA-binding protein that is thought to transduce the Notch signal (Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). Consistent with this, *Su(H)*-mutant cells lose expression of the *vestigial* early enhancer, of *wg-lacZ* and of *cut-lacZ* in a cell-autonomous manner (Fig. 2). Clones of *Su(H)*-mutant cells cause loss of wing tissue and scalloping of the wing (Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996), but only if cells at the D/V boundary are mutant (as described previously for *Notch*-mutant clones, de Celis and Garcia Bellido, 1994). These results indicate that *wg, cut* and the early *vestigial* enhancer are targets for activation by a Notch-dependent signal, transduced through Su(H).

To ask if the Notch signal is sufficient to activate these genes, we examined the effects of a ligand-independent activated form of Notch (Struhl et al., 1993). UAS*Notch(intra)* was expressed under control of the GAL4 driver MS1096. MS1096 expresses GAL4 throughout the wing pouch, but more strongly on the dorsal side (Capdevila and Guerrero, 1994; Neumann and Cohen, 1996). MS1096:UAS*Notch(intra)* leads to mis-expression of *wg*, the early *vestigial* enhancer and *cut* (Fig. 1C,G,K; see also Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). These observations are consistent with the finding that *wg* and *cut* are misexpressed in wing discs mutant for gain-of-function alleles of *Notch* (de Celis et al., 1996) and that the early *vestigial* enhancer is misexpressed in discs where Notch is inappropriately activated by misexpression of *Serrate* (Kim et al., 1995).

vestigial expression at the D/V boundary does not depend on *wingless*

wg and *vestigial* are expressed at the D/V boundary beginning in the second instar, under control of the D/V patterning

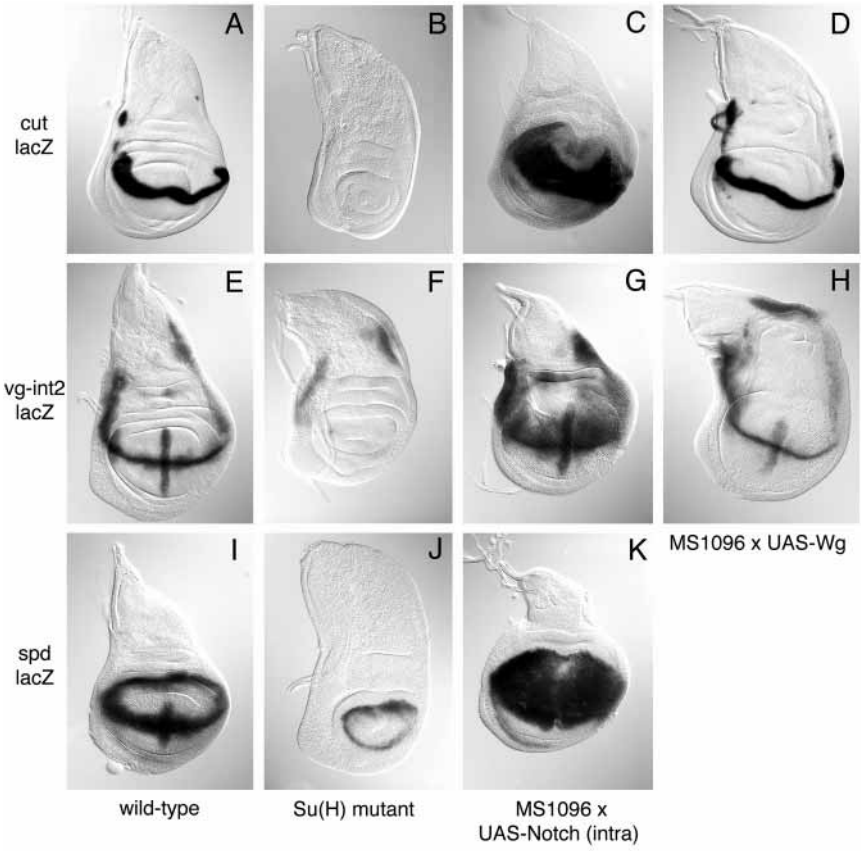

Fig. 1. Notch-dependent expression of *wingless*, *cut* and the early vestigial enhancer. (A,E,I) Wild-type wing discs. (B,F, J) $Su(H)^{AR9}/Su(H)^{SF8}$ mutant wing discs. (C,G,K) wing discs expressing UAS*Notch(intra)* under control of MS1096. (D,H) MS1096:UASwg^{+} wing discs. All discs are mature third instar. (A-D) *cut-lacZ* expression. (E-H) *vestigial* early enhancer-*lacZ* expression. To distinguish the initial activation of *vestigial* expression at the D/V boundary from subsequent expression in the wing blade, we have made use of a boundary-specific early enhancer directing *lacZ* expression (vg-int2 *lacZ*; Williams et al., 1994). (I-K) *wg* enhancer-*lacZ* expression. A specific enhancer from the *wg* gene was used to direct *lacZ* expression in the wing hinge and at the D/V boundary (spd-*lacZ*; Neumann and Cohen, 1996). All three reporter genes are lost from the wing margin in *Su(H)* mutant discs (B,F,J), and are misexpressed in the dorsal compartment when the active form of Notch is misexpressed under MS1096 control (C,G,K). Neither *cut* or *vestigial* are misexpressed in MS1096:UAS*wg* discs (D,H).

system (Couso et al., 1993, 1995; Williams et al., 1993, 1994; Ng et al., 1996). We wished to determine whether *wg* and the early *vestigial* enhancer are independent targets of the Notch pathway, or if they can be placed in a regulatory hierarchy. To ask whether *wg* regulates the early *vestigial* enhancer, we misexpressed a wild-type *wg* cDNA (UASwg^{+}). *wg* activity is required for the formation of wing margin bristles (Phillips and Whittle, 1993; Couso et al., 1993) and ectopic activation of the *wg* pathway is sufficient to induce the formation of margin bristles in the wing blade (Simpson et al., 1988; Blair, 1992; Diaz-Benjumea and Cohen, 1995). In the wild-type wing, margin bristles only form very close to the source of *wg* expression, and so represent a response to high levels of Wg. MS1096:UASwg^{+} induces wing margin bristles throughout the wing blade, indicating that MS1096:UASwg^{+} produces high levels of Wg activity (Neumann and Cohen, 1996). However, MS1096:UASwg^{+} does not cause an expansion of *vestigial-lacZ* expression (Fig. 1H), indicating that Wg activity is not sufficient to direct expression of the early *vestigial* enhancer. Notch activity is sufficient to do so when expressed under the same conditions (Fig. 1G; MS1096: UAS*Notch(intra)*). Similarly, misexpression of Delta, a ligand for Notch on the dorsal side of the disc can direct ectopic expression of the early *vestigial* enhancer on the dorsal side of the disc (Doherty et al., 1996), indicating that failure of ectopic Wg expression to direct expression of the early *vestigial* enhancer cannot be due to the absence of Notch.

These results are incompatible with the proposal by Couso et al. (1995) that ventrally expressed Wg cooperates with dorsally expressed Serrate to induce *vestigial* expression in cells straddling the D/V boundary of the wing disc. If this were the case, one would expect ectopic expression of Wg in dorsal cells (where Serrate is expressed) to direct ectopic expression of *vestigial*. However, this is not what we observe. The proposal that *wg* is required for *vestigial* expression is based on the observation that *vestigial* is lost in discs where *wg* activity is removed during the second instar using the *wg* temperature-sensitive allele (Williams et al., 1993; Couso et al., 1995). However, in the absence of Wg activity during second instar, cells fail to adopt wing identity and remain with the default identity of body wall (Ng et al., 1996; see also Morata and Lawrence, 1977; Couso et al., 1993). We suggest that failure to specify the wing pouch precludes activation of *vestigial* by the D/V system. Because this represents an earlier function of *wg*, it cannot be taken as evidence that *wg* is required to activate *vestigial*.

To clarify whether Wg plays a direct role in *vestigial* expression, we produced clones of cells mutant for the null allele wg^{cx4}. Clones induced before the D/V compartmental restriction is established will remove *wg* on both sides of the D/V boundary, prior to activation of the early *vestigial* enhancer by the D/V patterning system. Such clones do cause extensive non-autonomous loss of wing tissue (Diaz-Benjumea and Cohen, 1995), but do not affect expression of the early *vestigial* enhancer (Fig. 3A,B). We also examined *vestigial-lacZ* expression in $spade^{flag}$ (spd^{fg}) mutant discs. spd^{fg} is a regulatory mutation that reduces *wg* function in the wing hinge and in the wing margin (Neumann and Cohen, 1996). When spd^{fg} is heterozygous with a deficiency that uncovers *wg*, anterior and posterior wing margin structures are lost with 100% penetrance.

This correlates with an absence of detectable Wg protein in the anterior and posterior margin (Fig. 4A, B, though Wg is expressed in the distal margin). The spd^{fg}/Df^{hL2} phenotype can be rescued by expressing UASwg^{+} or UASdsh^{+} under control of the early *vestigial* enhancer, demonstrating that the sole defect in these wings is loss of *wg* activity (Fig. 5). Removing Wg activity from the anterior and posterior wing margins compromises Distal-less and *cut* expression in these regions (Fig. 4C-F), but has no effect on expression of the *vestigial* enhancer (Fig. 4G,H). Taken together with the observation that Wg is not sufficient to direct ectopic expression of *vestigial*, these results indicate that the early *vestigial* enhancer does not depend on Wg signaling. Therefore the early expression of *vestigial* is likely to be regulated by the *Notch* pathway alone.

wingless expression does not depend on an early function of *vestigial*

To ask whether *wg* expression at the D/V boundary depends on the prior activation of *vestigial*, we examined *wg-lacZ* expression in wing discs homozygous mutant for the *vestigial* null allele (vg^{83b27R}). In the absence of *vestigial* function, *wg* is expressed at the D/V boundary though the pattern of expression is not entirely normal even in early stages (Fig. 6B). However, *wg* is rapidly lost in older *vestigial* mutant discs (Fig. 6D). It is not possible to determine if this represents a requirement for *vestigial* in the maintenance of *wg* expression, or if the loss of *wg* is an indirect consequence of the extensive cell death that takes place in *vestigial* mutant wing discs (Fristrom, 1968; James and Bryant, 1981). These results indicate that activity of *vestigial* is not required for initiation of *wg* expression at the D/V boundary, and that *wg* and *vestigial* are parallel targets of the Notch pathway in these cells.

Short-range activity of the D/V organizer: *Notch* and *wingless* cooperate to activate *cut*

The short-range patterning activities

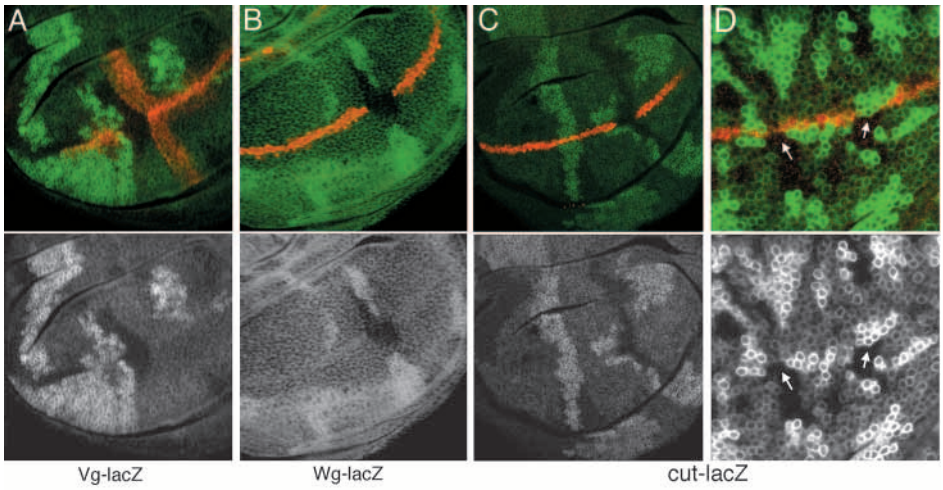

Fig. 2. Cell-autonomous loss of *vestigial*, *wingless* and *cut* expression in *Su(H)* mutant clones. (A) *vestigial-lacZ* expression (red). *Su(H)* mutant cells are visualized by the absence of N-myc expression (green in top panel, shown separately below). Bright green cells are twin spots carrying two copies of the N-myc transgene). (B) *wg-lacZ*-expressing cells (red). *Su(H)* mutant cells are visualized by the absence of N-myc expression (green). (C,D) *cut-lacZ-expressing* cells (red). Note the absence of *cut* expression even in very small clones of *su(H)* mutant cells (arrows in D). Both clones in D are ventral only. The clone on the left has autonomously lost *cut* expression, while the clone on the right has both autonomously lost *cut* expression and caused non-autonomous loss of *cut* expression in wild-type cells on the dorsal side of the D/V boundary (as described for *Notch* mutant clones with respect to *wg* expression, Rulifson and Blair, 1995). Lower panel, N-myc single channel image.

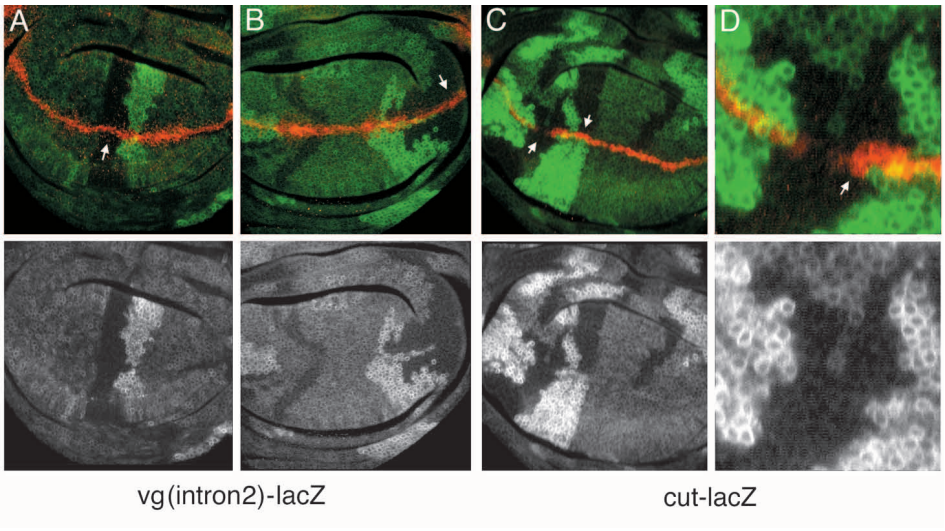

Fig. 3. *vestigial* and *cut* expression in *wg* mutant clones. (A,B) *vestigial-lacZ* (red) is expressed normally in clones of cell mutant for the null allele wg^{cx4} (visualized by the absence of N-myc expression, green in merged image, single channel images shown below). Clones induced prior to activation of the D/V patterning system in the second instar disc can cross the D/V compartment boundary (arrow in A). Arrow in B indicates large clones that remove Wg from the D/V boundary at the lateral edge of the wing pouch. Judging from the shape of the clone, this example may be two separate mutant clones that have merged at the boundary. (C) *cut-lacZ* expression in wg^{cx4} mutant clones. Arrow at right indicates a dorsal clone that meets but does not cross the D/V boundary. *cut* expression is normal. Arrow at left indicates a clone that crosses the D/V boundary, and removes *cut* expression. (D) Detail of clone showing that *cut* is expressed in *wg* mutant cells at the edge of the clone, but expression is reduced in cells more distant from the edge.

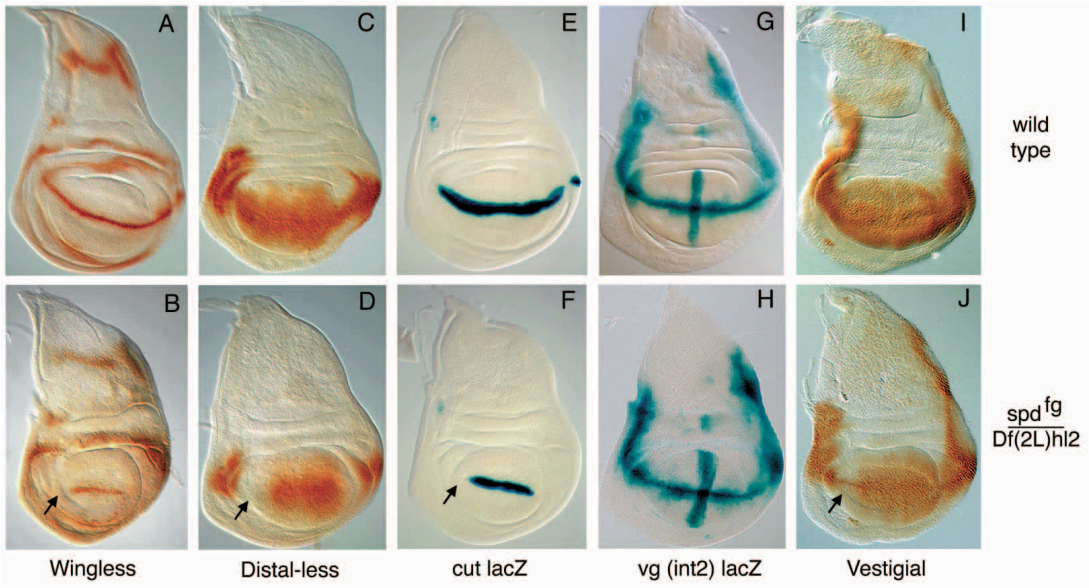

Fig. 4. *wingless* is required for cut, Distal-less and Vestigial expression, but not for the early *vestigial* enhancer. (A,C,E,G,I) Wild-type third instar wing discs. (B,D,F,H,J) *spd^{fg}/Df(2L) hL2* wing discs. (A,B) Wg protein, (C,D) Distal-less protein, (E,F) *cut-lacZ*, (G,H) *vestigial* early enhancer-*lacZ*, (I,J) Vestigial protein. Wg, Distal-less and cut expression are lost from the anterior and posterior margins of *spd* mutant discs (arrows). Expression of the *vestigial* intron 2 enhancer is normal (H), but expression of Vestigial protein is lost from the anterior and posterior regions of the wing pouch (arrow, J) except at the D/V boundary where the early enhancer is expressed.

of the D/V boundary have been attributed to localized expression of *wg* (Phillips and Whittle, 1993; Blair, 1994; Couso et al., 1994; Rulifson and Blair, 1995). Wg activity is required for specification of wing margin sense organs and for *cut* expression. *cut* is expressed in the cells at the D/V boundary but, unlike *wg*, *cut* is first expressed in mid third instar (Blair, 1993, 1994). Removing Wg function during third instar using the temperature sensitive mutant causes loss of *cut* expression (Couso et al., 1994). Fig. 4E,F shows that *cut* expression is lost along the wing margin in *spd^{fg}/Df^{hL2}* discs. To define the requirement for Wg activity more precisely, we examined *cut-lacZ* expression in clones of *wg*-mutant cells. *wg*-mutant clones that touch the margin from one side only do not affect *cut* expression (Fig. 3C). Clones that cross the D/V boundary lose *cut* expression in the center, but retain *cut* in cells at the lateral edges of the clones (Fig. 3C,D). These data indicate that Wg can activate *cut* non-autonomously over short distances, but only in cells at the D/V boundary (Fig. 3D).

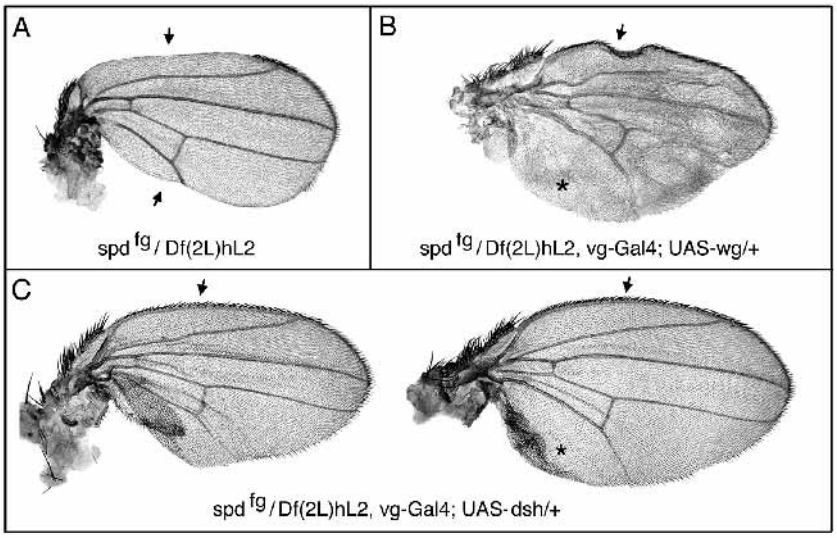

Fig. 5. Rescue of the *spd^{fg}/Df(2L) hL2* wing phenotype by *wg* activity. (A) *spd^{fg}/Df(2L) hL2* mutant wing. Note the loss of anterior and posterior wing margin (arrows), as well as the deletion of wing hinge structures typical of the *spd^{fg}* homozygote wing (see Neumann and Cohen, 1996). (B) Partial rescue of the *spd^{fg}/Df(2L) hL2* mutant phenotype by expression of *wg* cDNA under control of the early vestigial enhancer (vg-Gal4; UASwg). Note the rescue of the anterior wing margin (arrow) and of the posterior wing blade, between vein 5 and the posterior margin (asterisk). Because the combination of Vg-Gal4: UASwg is pupal lethal, the wings were inflated from dissected pupae. The resulting wings appear less flattened and the veins are not as well defined. (C) Partial rescue of the *spd^{fg}/Df(2L) hL2* mutant phenotype by expression of *dsh* cDNA under control of the early vestigial enhancer (vg-Gal4; UASdsh). (Arrows and asterisk as in B) Overexpression of Dsh protein potentiates the response of cells to the Wg signal (Axelrod et al., 1996; Neumann and Cohen, 1996). Note that expression of the vg-Gal4 is significantly broader in the wing hinge region than in the more distal wing margin (see Simmonds et al., 1995). Therefore the relatively extensive rescue of the lateral regions of the hinge may reflect an increases sensitivity to low levels of the Wg signal in these cells. Rescue by Dsh indicates that the *spd^{fg}/Df(2L) hL2* mutant phenotype is likely to be a hypomorphic condition for the wing margin, suggesting that some residual *wg* activity is present early. Note that the vg-GAL4 driver is not expressed in the central region of the wing hinge and so cannot rescue the *spd^{fg}* hinge phenotype.

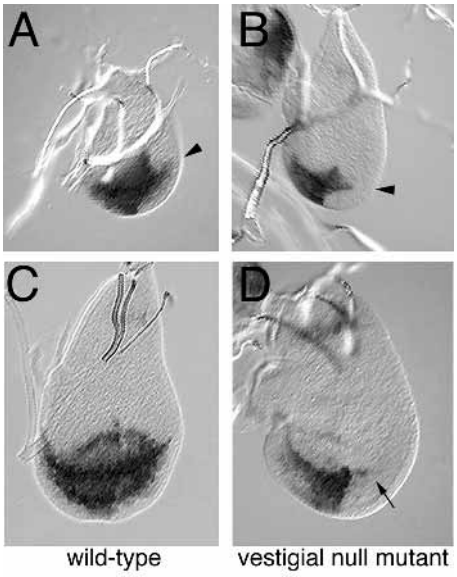

Fig. 6. Vestigial activity is not required for the activation of Wingless. (A-D) *wg-lacZ* expression detected with anti-β-gal antibody. (A) Wild-type late second instar wing disc. (B) vg^{83b27R} (null) mutant late second instar wing disc. Note that the activation of wg along the D/V boundary (arrowhead) appears to be normal. (C) Wild-type early third instar wing disc. (D) vg^{83b27R} (null) mutant early third instar wing disc. The expression of *wg* along the D/V boundary is starting to be lost in patches (arrow). In later discs, no expression of *wg* at the margin can be detected, and *wg* expression in the hinge region is also often lost (data not shown). As the loss of wing tissue in *vestigial* mutants is at least partially due to extensive cell death, this may be an indirect effect.

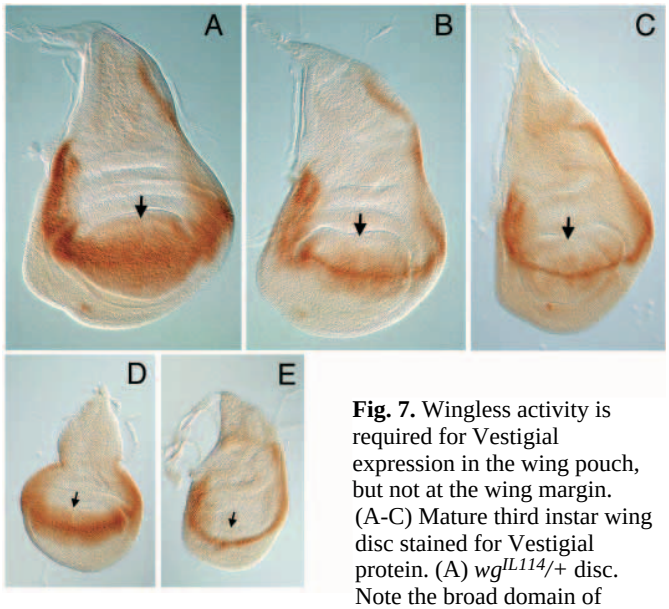

Fig. 7. Wingless activity is required for Vestigial expression in the wing pouch, but not at the wing margin. (A-C) Mature third instar wing disc stained for Vestigial protein. (A) wg^{IL114}/+ disc. Note the broad domain of Vestigial expression filling the wing pouch (arrow). (B,C)wg^{IL114} discs. Wg activity was removed in early third instar. Vestigial expression at the D/V boundary is not affected, but is reduced in the wing pouch (arrows). The disc in C is more severely affected than the disc in B, and may have been slightly younger when Wg was inactivated. (D,E) Mid-third instar wing discs stained for Vestigial. (D) wg^{IL114}/+. Note the high level of Vestigial at the D/V boundary and the lower level in the wing pouch. Expression at the D/V boundary is controlled by the early enhancer. (E) wg^{IL114} disc. Wg activity was removed in late second instar. Vestigial expression at the D/V boundary is not affected, but is lost in the wing pouch.

Although Wg is required for *cut* expression, producing high levels of Wg activity in the dorsal wing pouch using MS1096:UASwg^{+} does not cause an expansion of *cut* expression (Fig. 1D). This indicates that Wg is not sufficient to direct *cut* expression, and is consistent with the observation that clones of cells lacking activity of *shaggy/zw3* fail to activate *cut* (Blair, 1994).

These results indicate that Wg signalling can only direct *cut* expression in cells at the D/V boundary. Activity of the Notch pathway may be responsible for generating this competence. Removing activity of the Notch pathway in *Su(H)* clones causes cell-autonomous loss of *cut* expression. Even small *Su(H)* clones of one to two cells lose *cut* expression (Fig. 2D). If the effect of Notch on *cut* were only indirect, via *wg*, then it would be expected that *cut* expression should be rescued non-autonomously in cells near the edge of a *Su(H)* mutant clone, as in *wg* mutant clones (Fig. 3D). However, this is not what we observe. Given that MS1096: UAS*Notch(intra)* directs ectopic *cut* expression (Fig. 1C), we suggest that *Notch* activity is directly required for *cut* expression, and that the *wg* and *Notch* pathways synergize to activate *cut*.

Long-range activity of the D/V organizer: *wingless* directs *vestigial* expression in the wing pouch

The long-range patterning activities of the D/V boundary have been attributed to localized expression of *wg* (Diaz-Benjumea and Cohen, 1995). It has also been suggested that the D/V system works through *vestigial* to control growth of the wing (Williams et al., 1994). Vestigial is expressed in a broad domain throughout the wing, only a subset of which is under control of the early enhancer. Expression of the early enhancer does not depend on Wg activity (Figs 3, 4); however, removing Wg activity in late second instar using a temperature-sensitive mutant leads to almost complete loss of the secondary expression of Vestigial in the wing pouch without affecting expression at the D/V boundary, where the early enhancer is active (Fig. 7D,E). Removing Wg activity in early third instar reduces vestigial expression in the wing pouch (Fig. 7A-C). A comparable result is obtained when Wg is removed locally from the anterior and posterior margins; Vestigial expression is normal at the margin, where the early enhancer is active (Fig. 4H,J), but is reduced in the adjacent regions of the wing pouch (Fig. 4I,J).

These results indicate that localized expression of Wg at the D/V boundary is required for Vestigial expression throughout the wing pouch. Taken together with the observation that clones of cells lacking *sgg* activity show a cell-autonomous increase of Vestigial expression (Blair, 1994), these results suggest that *vestigial* is a direct target of the Wg pathway. Wg does not regulate the early *vestigial* enhancer, but may regulate *vestigial* through the recently described enhancer sequence that drives expression in the rest of the wing pouch (S. Carroll, personal communication). These results suggest that Wg can act over relatively long distances to direct Vestigial expression, and that Vestigial may be an effector through which Wg controls growth of the wing blade.

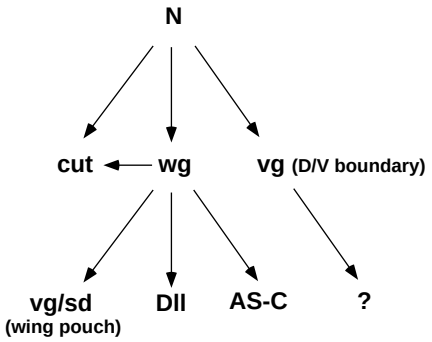

Fig. 8. Schematic model of the genetic hierarchy downstream of Notch that establishes and mediates the function of the D/V organizer. Activated Notch establishes the organizer by directing expression of both *wingless* and the *vestigial* D/V boundary enhancer. Wingless then mediates short-range effects of the organizer by activating *cut* and *achaete-scute complex* (AS-C) expression locally, and also long-range effects of the organizer, by directing *vestigial* expression in a broad domain filling the wing pouch. Notch also has a direct function in the short-range activity of the organizer by synergizing with Wingless to activate *cut*. The late activation of *cut* and the modest scalloping phenotypes of *cut* mutants suggest that it has a local function in wing margin differentiation. Apart from mediating the long-range function of Wingless in the wing pouch, Vestigial also has a distinct and critical function in the cells of the D/V organizer, which is not yet clear.

DISCUSSION

Notch and wingless in the wing margin

Evidence for a negative cross-talk between the *Notch* and *wg* pathways has been presented by Axelrod and coworkers (1996), who suggest that this effect is mediated by Dishevelled (Dsh) protein binding to the intracellular domain of Notch. Dsh is required to transduce the Wg signal in the embryo and in the imaginal discs (Couso et al., 1994; Klingensmith et al., 1994; Noordermeer et al., 1994; Siegfried et al., 1994; Theisen et al., 1994). Increasing the level of Dsh expression can produce phenotypes similar to those caused by ectopic expression of Wg (Yanagawa et al., 1995; Axelrod et al., 1996; Neumann and Cohen, 1996). A reduction in the activity of *Notch* or *Delta* potentiates this effect, while overexpression of the Dsh-binding site in Notch inhibits it, presumably by titrating out Dsh (Axelrod et al., 1996). These observations are consistent with a model in which the Notch and Wg pathways antagonize each other in regulation of the *achaete-scute* complex (*AS-C*) and neural cell fate determination.

We have presented evidence that the Notch and Wg pathways can also act co-operatively (Fig. 8). The observation that Wg can only activate *cut* in the cells located at the D/V boundary (Fig. 3D), and that *Su(H)* is required cell-autonomously for *cut* expression (Fig. 2D) suggests that activity of both pathways is required to activate *cut* expression. How can Wg and Notch act antagonistically to regulate AS-C expression in cells near the D/V boundary, while simultaneously acting synergistically on *cut* in cells at the D/V boundary? One possibility is that Notch might be required for *cut* expression if Notch were needed for reception of the Wg signal (Couso and Martinez Arias, 1994). This seems unlikely because Notch is not required for reception of the Wg signal in specification of sense organ precursors (Rulifson and Blair, 1995). An alternative explanation is that the observed high levels of the Notch ligands, Serrate and Delta, in cells adjacent to the boundary (Kim et al., 1995; Doherty et al., 1996) may keep Notch in an activated state and make it insensitive to inhibition by Dsh. This could allow simultaneous activity of the *Notch* and *wg* pathways in the cells at the D/V boundary.

vestigial mediates the long-range function of *wingless* in controlling growth of the wing pouch

vestigial activity is critically required for wing development; the wing and the haltere are almost completely lost in *vestigial* mutants (Williams et al., 1993). *vestigial* appears to have two distinct functions. *vestigial* is initially activated by *Notch* at the D/V boundary, and we have shown here that this expression does not depend on Wg activity (Fig. 8). A regulatory mutant that removes the early enhancer required for the activation of *vestigial* at the D/V boundary causes a reduction of the wing that is as severe as that seen in a *vestigial* null mutant (Williams et al., 1994). Because *vestigial* encodes a nuclear protein, Vestigial expression in cells at the D/V boundary is unlikely to have a direct effect on cells at a distance, suggesting that *vestigial* must act upstream of another signal that relays the patterning information of the D/V organizer. We have presented evidence that this function is not mediated by Wingless, suggesting that there may be a second signal in addition to Wingless, which depends on *vestigial* activity at the D/V boundary.

Furthermore, we have presented evidence that the long-range patterning effects of Wingless are mediated at least in part through *vestigial* (Fig. 8). Following its initial activation at the D/V boundary, *vestigial* expression expands to fill much of the wing pouch (Williams et al., 1993). In this phase, Vestigial expression is highest at the D/V boundary, decreasing in a graded manner toward the base of the wing (Blair, 1994). Clones of cells homozygous for a strong *vestigial* allele are very rarely recovered in the distal part of the wing blade, but can be found close to the hinge and the A/P boundary (Simpson et al., 1981), corresponding to regions where expression of Vestigial is low (or absent). These observations suggest that *vestigial* function is required to promote survival and/or proliferation in cells of the wing pouch. In this context, it is interesting to note that mutations in the tumor suppressor gene *giant discs* can suppress the loss of wing tissue in *vestigial* mutants (Agrawal et al., 1995), further suggesting a requirement for *vestigial* in promoting proliferation in the wing pouch. *vestigial* may work together with *scalloped* in promoting wing growth; *scalloped* encodes a predicted transcription factor that is expressed like *vestigial* and mutants produce similar phenotypes (Campbell et al., 1992; Blair, 1992; Williams et al., 1993). Also, *scalloped* expression is elevated cell-autonomously in clones of cells lacking *shaggy* activity (Blair, 1994), suggesting that *scalloped* may also be a direct target of *wingless* in the wing pouch.

Thus *wingless* is required for the late expression of *vestigial*, and possibly *scalloped*, in a broad domain filling most of the wing primordium. These findings are consistent with the observation that removing *wg* activity from the D/V boundary (following specification of the wing primordium in the second instar) can cause loss of large parts of the wing blade (Diaz-Benjumea and Cohen, 1995; see also Fig. 3F in Couso et al.,

1995), and that ectopic expression of Wg can promote outgrowth of the wing, as well as specifying wing margin fate locally (Diaz-Benjumea and Cohen, 1995).

Is wingless a morphogen in the wing?

Data presented here and elsewhere have suggested that the genes *vestigial*, *scalloped* and *Dll*, and the genes of the *AS-C* are direct targets for regulation by the Wg pathway (Fig. 8). *AS-C* genes are expressed immediately adjacent to the wing margin. *Dll* expression straddles the margin and extends some distance into the wing pouch. *vestigial* and *scalloped* expression extend farther. How does Wg specify these different expression domains? One possibility is that it acts in a concentration-dependent manner to activate genes that have a different activation threshold at different distances. This would require that Wg protein diffuses a considerable distance from the wing margin toward the center of the wing pouch. However, most observations made so far have suggested, on one hand, that Wg and Wnt-1 are short-range signaling molecules (van den Heuvel et al., 1989; González et al., 1991; Bejsovec and Martinez-Arias, 1991; Jue et al., 1992; Vincent and Lawrence, 1994), and, on the other hand, that there is a concentration-dependent patterning function of Wg in the embryonic midgut (Hoppler and Bienz, 1995). Another possibility is that the broad expression domains of *vestigial* and *Dll* are generated by signal-dependent cell memory, that is, cells that are close to the source of *wg* early on retain their expression of *Dll* and *vestigial* as they move out of range of the *wg* signal in the growing disc. These two scenarios are not mutually exclusive, and data suggesting that both of these mechanisms are used for the regulation of *optomoter-blind* and *spalt* by secreted Dpp in the wing pouch have been presented (Nellen et al., 1996; Lecuit et al., 1996). It remains to be seen if similar mechanisms are utilized in the control of Wg target genes in the wing pouch.

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Note added in proof

The work referred to as 'Sean Carroll (personal communication)' has been published while this manuscript was under review (**Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B.** (1996). *Nature* **382**, 133-138.) This paper presents similar results on the relationship between Notch, Su(H) and Vestigial and showed that Vestigial function is required for growth of cells in the wing.