Development 125, 2951-2962 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV1293

Interactions among Delta, Serrate and Fringe modulate Notch activity during *Drosophila* wing development

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Accepted 29 May; published on WWW 9 July 1998

SUMMARY

The Notch signalling pathway plays an important role during the development of the wing primordium, especially of the wing blade and margin. In these processes, the activity of Notch is controlled by the activity of the dorsal specific nuclear protein Apterous, which regulates the expression of the Notch ligand, Serrate, and the Fringe signalling molecule. The other Notch ligand, Delta, also plays a role in the development and patterning of the wing. It has been proposed that Fringe modulates the ability of Serrate and Delta to signal through Notch and thereby restricts Notch signalling to the dorsoventral boundary of the developing wing blade. Here we report the results of experiments aimed at establishing the relationships between Fringe, Serrate and Delta during wing development. We find that Serrate is not required for the initiation of wing development but rather for the expansion and early patterning of the wing primordium. We provide evidence that, at the onset of wing development, Delta is under the control of apterous and might be the Notch ligand in this process. In addition, we find that Fringe function requires Su(H). Our results suggest that Notch signalling during wing development relies on careful balances between positive and dominant negative interactions between Notch ligands, some of which are mediated by Fringe.

Key words: Wing development, Notch signalling, Serrate, Fringe, Delta, Vestigial

INTRODUCTION

The limbs of *Drosophila* provide a useful system to analyze the molecular mechanisms that diversify and pattern groups of cells during development. The wing, for example, is derived from a small population of cells within the wing imaginal disc, which grow during larval life, acquire various identities according to spatial cues and differentiate during pupation. The wing is composed of two well-defined structures: a distal one or blade, which is represented by two large sheets of cells folded on each other and patterned with sensory organs and veins; and a proximal one or hinge, which attaches the blade to the body wall or notum.

A variety of observations indicate that interactions across the dorsoventral (DV) axis mediated by Notch signalling, play an important role in the establishment, growth and patterning of the wing. Loss of function of *Notch*, or of its ligands Delta and Serrate, compromises the development and growth of the wing blade (de Celis and Garcia Bellido, 1994; Couso et al., 1995; de Celis et al., 1996), and the loss of wing of *apterous* (*ap*) mutants can be explained as a result of the loss of expression of the Notch ligand Serrate (Couso et al., 1995; Diaz Benjumea and Cohen, 1995; Joensson and Knust, 1996). On the contrary, ectopic Notch signalling, either through the activation of Notch or through the expression of its ligands Delta and Serrate, triggers extra growth of the normal wing blade (Speicher et al., 1994; Diaz Benjumea and Cohen, 1995; Doherty et al., 1996).

Gain- and loss-of-function experiments suggest that, during the growth of the wing, Notch signalling is restricted to the interface between dorsal and ventral cells (de Celis and Garcia Bellido, 1994; Couso et al., 1995; Diaz Benjumea and Cohen, 1995; Panin et al., 1997; de Celis and Bray, 1997). However, Notch is expressed throughout the developing blade and its ligands, Serrate and Delta, are expressed throughout the dorsal and ventral cells, respectively. This suggests that there must exist factors that restrict Notch signalling to the DV interface. Recently it has been proposed that, in the case of Serrate, the *fringe* (*fng*) gene product performs one such restrictive function (Panin et al., 1997; Fleming et al., 1997). *fng* encodes a secreted molecule and its expression is under control of *ap* (Irvine and Wieschaus, 1994; Panin et al., 1997). Gain- and loss-of-function studies suggest that *fng* can induce *Ser* expression and that it acts as a boundary-determining factor in invertebrates and vertebrates (Kim et al., 1996; Rodriguez-Esteban et al., 1997; Laufer et al., 1997). In addition to promoting *Ser* expression and restricting its activity, it has been proposed that Fringe enhances Delta signalling (Panin et al., 1997). The interactions of Fringe with the Notch ligands Serrate and Delta have been proposed as the basis for the restriction of Notch signalling to the DV boundary (Panin et al., 1997; Fleming et al., 1997).

Most of the interactions between Notch and its ligands have been explored through ectopic expression experiments. Here we probe the relationships between Fringe, Notch and its

ligands, Serrate and Delta, during the initial stages of wing development and wing blade growth. Our results suggest that Serrate and Delta play overlapping but essentially different roles in wing development. In particular Serrate is dispensable for the initiation of Notch signalling, but is essential for a process whereby the wing primordium is expanded along the AP axis and for the maintenance of Notch signalling along the DV boundary. This analysis of Serrate function leads to questions about its relationship to Fringe. We find that Fringe modulates Serrate signalling by acting on Notch and that it requires the activity of the Su(H).

MATERIALS AND METHODS

Drosophila stocks

The following mutations were used in this work: ap^{UGO35} (Cohen et al., 1992), Ser^{RX106} (Speicher et al., 1994), Ser^{94C} (Couso et al., 1995), $Su(H)^{AR9}$ and $Su(H)^{SF8}$ (Schweisguth and Posakony, 1992).

Expression of *vg* at the DV boundary was detected using the *vg* DV boundary enhancer described in Williams et al. (1993) and referred to here as *vgBE* (for Boundary enhancer). To detect the expression of *wg* in the developing discs, we used either a *lacZ* insertion in the *wg* gene on a CyO chromosome (Kassis et al., 1992) or an anti-Wingless antibody kindly provided by S. Cohen, described in Neumann and Cohen (1996). Sensory organ precursors were detected with a *lacZ* insertion, A101, in the neuralized gene (Huang et al., 1991). Delta expression was sometimes detected with a *lacZ* insertion in *Dl* provided by J. F. de Celis.

Ectopic expression of the different genes was achieved through the GAL4/UAS system of Brand and Perrimon (1993). The UAS*fng* and UAS*vg* constructs were kindly provided by Sean Carroll (Kim et al., 1995, 1996); UAS*N* by M. Baylies; UAS*Nintra* and UAS*Dl* by L. Seugnet and M. Haenlin, and UAS*Dl*30B (Doherty et al., 1996), which is the same line used by Panin et al. (1997), and a second UAS*Nintra* by K. Irvine. The UAS*Ser* (Speicher et al., 1994), UASGFP (Yeh et al., 1995) and the UAS*N* UAS*Ser* chromosome (Klein et al., 1997) have been described before.

The expression of the different UAS constructs was driven in the imaginal discs with various GAL4 inserts. *patched*GAL4 (*ptc*GAL4) expresses UAS*X* in a stripe along the AP boundary of the discs (Speicher et al., 1994). In the third instar, *decapentaplegic*GAL4 (*dpp*GAL4) (Wilder and Perrimon, 1995) is expressed in a similar pattern to *ptc*GAL4, although the expression is weaker over the ventral side (Klein and Martinez Arias, 1998). However, in the second instar, the expression ventrally is virtually nonexistent. *scalloped*GAL4 (*sd*GAL4) is expressed in a pattern that is identical to that of *vestigial* and allows the expression of the construct throughout the developing wing (Klein et al., 1997). *klumpfuss*Gal4 (*klu*GAL4) is expressed from early third instar on and covers the neurogenic region of the notum (Klein and Campos-Ortega, 1997). *ms1096*GAL4 is expressed in a dynamic pattern in the wing disc: it is initiated in the late second instar in the dorsal area of the wing blade. Later, during mid third instar, it begins to be expressed in parts of the notum and throughout the blade (Capdevila and Guerrero, 1994; Klein et al., 1997).

Stocks carrying various GAL4 and UAS combinations in wild type and mutants were generated. All stocks were balanced over the SM6a-TM6b compound balancer, which allowed the identification of larvae of the correct genotype because of the dominant larval marker Tb (Lindsley and Zimm, 1992). Details of the stocks and the stocks themselves are available upon request. In the case of *ap* mutations, stocks were established with a CyO balancer carrying a P-*lacZ* insertion in *wg* and the mutant discs were checked for the absence of the *wg* expression pattern. In the case of some experiments involving *ap* mutants, mutant discs were identified by the aberrant morphology of the wing disc and the absence of the CyO*wglacZ* balancer.

Immunohistochemistry and in situ hybridization

The Vestigial antibody is described in Williams et al. (1993) and, together with the ac antibody, was a gift of S. Carroll. The ac antibody is described in Skeath and Carroll (1991). The Enhancer of split antibody was a gift of Sarah Bray and is described in Jennings et al. (1995). The Ser antibody is described in Speicher et al. (1994) and was a gift from E. Knust. The X-Gal staining is described in Ashburner (1989). In situ hybridizations were performed as described in Tautz and Pfeifle (1989). The fluorescence of the green fluorescent protein (GFP), Texas red- and FITC-conjugated antibodies (purchased by Jackson laboratories) were detected by using the appropriated filter sets on a Zeiss Axiophot microscope.

RESULTS

Apterous and Serrate are required for overlapping but different functions during wing development

The *apterous* (*ap*) gene of *Drosophila* is expressed in dorsal cells of the wing disc where it controls the expression of the Notch ligand Serrate (Ser) (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995). In the absence of *ap*, the wing blade does not develop, an effect thought to be due to the loss of *Ser* expression (Couso et al., 1995; Fig. 1A,B). Consistent with this, we find that ectopic expression of *Ser*, or of *fringe* (*fng*), which leads to the expression of *Ser* (Kim et al., 1995), rescues the loss of the wing of *ap* mutants (Klein et al., 1998; Fig. 1D,E).

However, the *Ser* and *ap* mutant phenotypes are not identical: while in the absence of *ap* there is no trace of the wing blade, *Ser* mutants bear a small marginless wing blade (Speicher et al., 1994; Joensson and Knust, 1996; Fig. 1C). To explain this difference, we have compared the expression of *wingless* (*wg*) (Fig. 2A-C) and *vestigial* (*vg*) during wing development in these mutants. We monitored the expression of *vg* through the expression of its protein product and through the expression of the *vg* boundary enhancer (*vgBE*), which appears symmetrically across the DV interface at the end of the second instar and reflects early regulatory events in wing development (Williams et al., 1994; Klein and Martinez Arias, 1998) (Fig. 2I). The expression of *vg*, and that of the *vgBE*, decays at the beginning of the third instar in *Ser* mutants, but is never activated in the wing region of *ap* mutants (Fig. 2I-L).

In the wild type, the pattern of *wg* expression evolves from a ventral anterior sector during the second instar, to the complex array of the late third instar through a series of transitional patterns that involve: (1) upregulation at the intersection of the AP and DV axis to outline the wing field (Fig. 2A), (2) spreading of this pattern along the DV interface to define the wing blade (Fig. 2A,B), (3) delimitation of the wing blade and the wing hinge by two concentric rings and (4) establishment of the wing margin through a stripe that bisects the blade (Couso et al., 1993; Klein and Martinez Arias, 1998; Fig. 2C). These transitions are affected in a different manner by *Ser* and *ap* mutants. In *Ser* mutants, the expression of *wg* never spreads along the DV interface and resolves into two rings, which fate map the small wing characteristic of *Ser* mutants (Fig. 2E,G). However, in *ap* mutants, *wg* expression comes to outline a single circle of expression (Williams et al.,

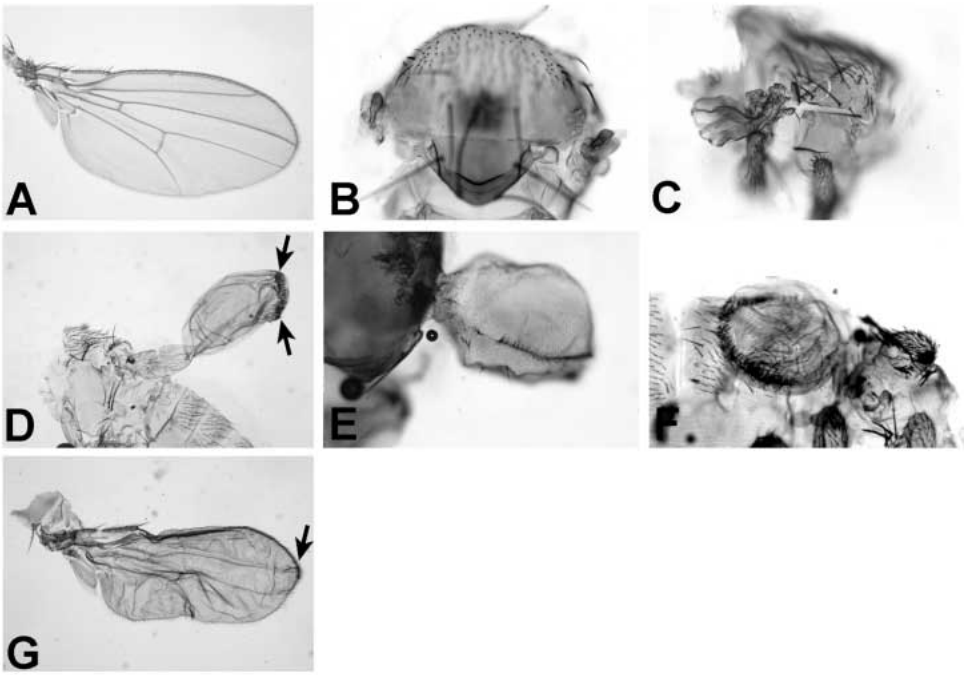

Fig. 1. (A-C) Comparison of the wing mutant phenotypes caused by the loss of function of *apterous* and *Serrate*. (A) Wild-type wing. (B) Thorax of an ap^{UG035} homozygote focussed to show stumps in the position of the wing hinge. (C) Thorax of a Ser^{94c}/Ser^{Rx106} mutant fly, showing the reduced but discernible wing blade. Notice that this mutant phenotype is weaker than that of *ap* (compare with B). (D-F) Rescue of the loss of wing in ap^{UG035} homozygotes by ectopic expression, under the control of *dpp*GAL4 of UAS*Ser* (D), UAS*fng* (E) and UAS*Dl* (F). (G) In contrast to the *ap* mutant situation (D), activation of UAS*Ser* in the wild type is inducing only very few ectopic bristle on the ventral side of the pouch next to the normal margin (arrows). The difference in the capacity of Serrate to induce margin structures suggests that Apterous is suppressing the activity of Serrate.

1993; Ng et al., 1996), which defines proximal hinge structures (Fig. 2H) and the absence of blade characteristic of these mutants. This is a phenotype very similar to that of *vg* null alleles (Fig. 2D).

These results suggest that the phenotype of *ap* mutants cannot be accounted for simply by the absence of *Ser*. Whereas, in *ap* mutants, the development of the wing blade is never initiated, in the absence of *Ser*, this process is initiated normally, but is aborted early on. Furthermore, since the expansion of *wg* expression in the AP direction is required for the establishment of the proper size of the primordium (Klein and Martinez-Arias, 1998), its failure to occur in *Ser* mutants indicates that, in addition to its role in the establishment of the wing margin, *Ser* is required to define the proper size of the wing primordium.

Apterous is required for the expression of *Delta* during wing development

The initial stages of the development of the wing blade require Notch signalling and lead to the activation of *vgBE* at the interface between dorsal and ventral cells (Williams et al., 1994; Kim et al., 1996). The results presented above show that *Ser* is not involved in this event and therefore suggest that there ought to be another Notch ligand, under the control of *ap*, that is responsible for the activation of the *vgBE*. The product of the *Delta* (*Dl*) gene is a good candidate for this function.

Loss of *Dl* function affects the development of the wing, in particular at the wing margin (de Celis et al., 1995; Doherty et al., 1996; Michelli et al., 1997). During the second instar, *Dl* is expressed throughout the wing disc, but it is slightly upregulated over the ventral region (Fig. 3B,C; Doherty et al., 1996; de Celis and Bray, 1997) and shortly afterwards, its pattern of expression is identical to that of *vgBE*, i.e. a 2- to 3-cell-wide stripe that straddles the DV interface (compare Fig. 3A,E and B,F and also de Celis and Bray 1997). Furthermore, the expression of *Delta* is similar to that of the *vgBE* in *Ser* and *ap* mutant discs: in *ap* mutant wing discs, expression of *Dl* is lost at the time when the wing primordium is induced (Fig. 3C,G), whereas in *Ser* mutants expression is detected until early third instar (Fig. 3H). This suggests that Delta might be the activating ligand for the Notch-dependent expression of the *vgBE*, which operates in the absence of *Ser* . Consistent with this possibility, we found that ectopic expression of *Dl* can rescue the loss of wing blade tissue and of wing margin characteristic of *ap* and *Ser* mutants (Figs 1F, 3I).

Reciprocal interactions between Serrate and Delta induce and refine Notch activity at the DV interface

After the establishment and expansion of the wing primordium, there is a new requirement for Notch signalling in the growth and patterning of the wing blade. In this process, both Serrate and Delta act as ligands for Notch and, as in earlier stages, have different patterns of gene expression (de Celis and Bray, 1997; Miccheli et al., 1997), which suggests that they might have different functions. However, we find that ectopic expression of either *Dl* or *Ser* will rescue the loss of wing tissue and of wing margin characteristic of *ap* mutants, and this raises the question of why are there two different ligands to achieve the activation of Notch in these early stages of wing development. One possibility is that these ligands elicit qualitatively different responses. Ectopic expression of Delta can rescue the loss of wing tissue and wing margin characteristic of *Ser* mutants (Fig. 3I), which rules out this possibility and indicates that the main molecular event required for the formation of the wing blade, is the activation of Notch. This is confirmed by the observation that the Nintra construct, which provides constitutive Notch signalling, also rescues the loss of wing tissue and margin of *Ser* mutants (Fig. 3J).

Ectopic expression of *Dl* can lead to the ectopic expression of *Ser* (Doherty et al., 1996; Fig. 4A,C), and ectopic expression of *Ser* leads to ectopic expression of *Dl* (Panin et al., 1997; Fig. 4J). These effects, as well as the normal patterns of expression

of *Ser* and *Dl*, require *Su(H)* (Klein et al., 1997; Fig. 5), which shows that all these effects are mediated by the activation of Notch.

In the course of these experiments, we observed that *Dl* can induce itself (Fig. 4L) even in the absence of *Ser* (data not shown) and that it elicits responses in both dorsal and ventral cells (Fig. 4A-D). This result is different from the one reported by Panin et al. (1997) and prompted us to repeat the experiments with the same UAS*Dl* lines used in previous experiments. In our hands, ectopic expression with UAS*Dl*30B (used in Panin et al., 1997) also elicits responses in dorsal and ventral cells (Fig. 6D; unpublished data). In all cases, whereas the inductions of targets of Notch signalling like *vgBE*, *Enhancer of split (spl) (E(spl))* and *wingless* (*wg*) is similar in dorsal and ventral cells of the wing blade (Fig. 4B,D,I), the induction of *Ser* (Fig. 4A,C) and *cut* (Doherty et al., 1996; unpublished data) is stronger in the dorsal half.

We considered that one reason for our observations might be the proposed enhancement of Delta signalling mediated by Fringe (Panin et al., 1997), since from mid third instar *fng* is expressed in ventral cells (Irvine and Wieschaus, 1994). However, the different sensitivities of the dorsal and ventral cell populations, as well as the ability of Delta to signal ventrally, are observed in early third instar, when the expression of *fng* is restricted to the dorsal side (Fig. 4A,B). Furthermore, expression of *Delta* can induce expression of *Ser* even in an *ap* mutant in which *fng* is not expressed (Fig. 4K).

These results suggest that there are intrinsic differences between dorsal and ventral cells that are independent of Fringe (see also Klein et al., 1998). To test this further, we have induced gene expression with Nintra, whose activity is not subject to extracellular influences and which reflects constitutive Notch signalling. In these experiments we observe the same patterns of responses as those elicited by Delta (Fig. 4E-H). This result shows that the differential responsiveness of dorsal and ventral cells are intrinsic to the cells of the disc and are not created by the activity of Fringe.

Since in the wild type, *Dl* is expressed in dorsal and ventral cells at the beginning of the third instar (de Celis and Bray, 1997 and data not shown), this means that, at the time of the definition of the DV interface, both *Ser* and *Dl* are

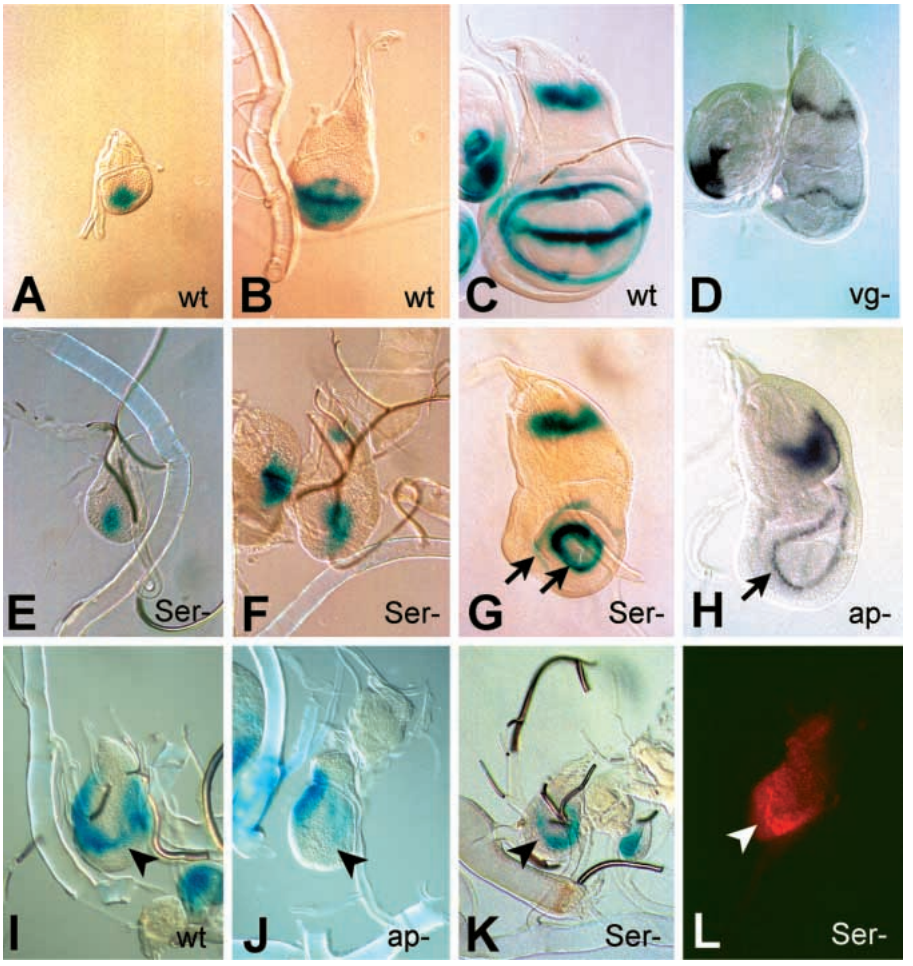

Fig. 2. Patterns of expression of *wg* and *vg* in *Ser* and *ap* mutant wing discs. Ventral to the bottom, anterior to the left. (A-C) Evolution of the *wg* pattern of expression during late second and third larval instar, visualized through a *lacZ* in the *wg* gene. During the second instar, *wg* is initially expressed in a wedge-like sector from which expression expands in anteroposterior direction at late second instar (A). After a short period in early third instar in which *wg* expression is detectable throughout the region of the wing primordium, the expression becomes restricted to the prospective wing margin and the hinge region (B). At the hinge region, *wg* is visible first in a single and later in a double ring-like domain; expression in the outer, proximal ring is weaker than that in the inner distal one (C). (D) Expression of *wg* in a late third instar vg^{83b27R} mutant disc. The margin expression and part of the expression is lost and expression is restricted to a single ring in the hinge. This phenotype is very similar to that observed in *ap* mutants (compare with H). (E-G) Sequence of *wg* expression in Ser^{94c}/Ser^{Rx106} mutant wing discs through stages comparable to those in A-C. In *Ser* mutants, *wg* expression starts normally (compare A and D), but the expansion does not occur (compare E, F with B,C). Instead the wedge-like expression directly resolves into the two ring-like expression domains (arrows in G) that delimit the hinge region and a very small wing blade. (H) Expression of *wg* in a late third instar ap^{UG035} mutant wing disc. In addition to the loss of *wg* expression in the region of the margin, the stronger circle of expression in the hinge region is lost and only the proximal circle (arrow) is present. The expression *wg* in *ap* and *Ser* mutants reflects the differences observed in the adult animals (see Fig. 1B.C). (I) Expression of the *vestigial* boundary enhancer (*vgBE*) in a wild-type early third instar wing disc. Activity of the enhancer is detected in a U-like pattern along the DV boundary (arrowhead indicates the region of the wing). (J) Expression of the *vgBE* is absent in the area of the wing primordium (arrowhead) in *ap* mutant discs of comparable age to that shown in I. (K) Expression of the *vgBE* is present in *Ser* mutant discs (arrowhead). (L) Expression of the *vg* is also detectable in *Ser* mutants during the early stages of wing development (arrowhead) but is lost in older discs (Couso et al., 1995; unpublished data).

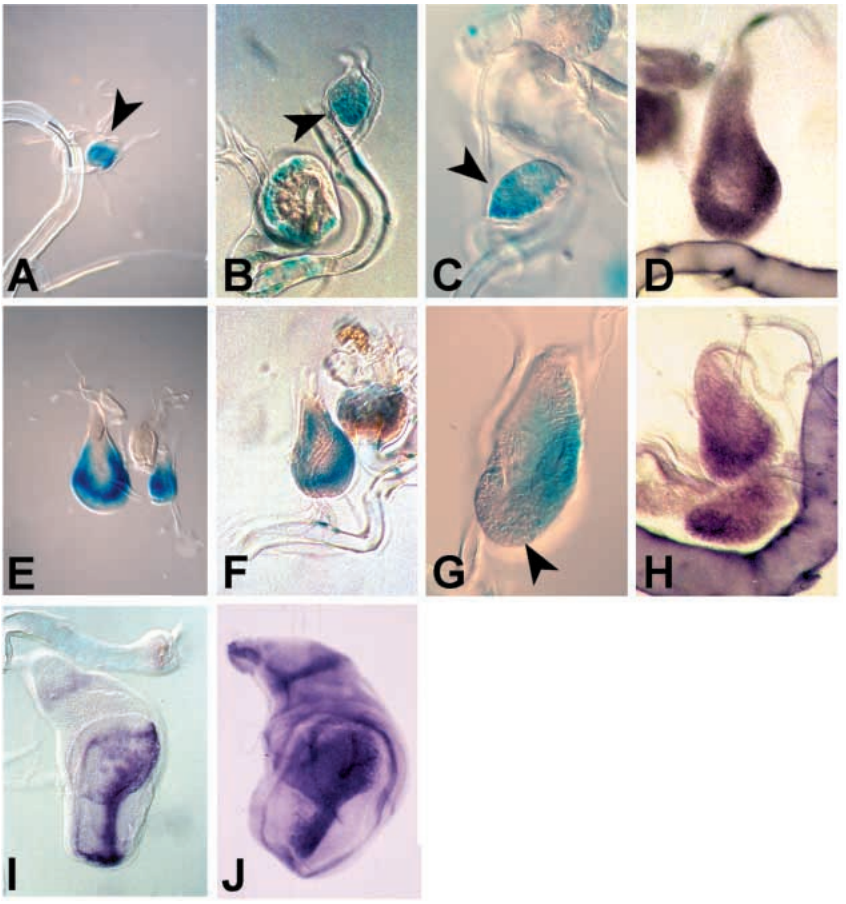

Fig. 3. Comparison of the patterns of expression of *Dl* and the *vgBE* during the early stages of wing development in wild-type and mutant wing discs. Ventral to the bottom. Arrows indicate wing discs. (A,E) Expression of the *vgBE* in second (A) and early third instar (E) wing discs. The activity of the *vgBE* is identical to that of the *vg* gene during this period (Kim et al., 1996; T. K. and A. M. A., unpublished results). (B,F) Expression of *Dl* in second (B) and early third instar (F) wing discs, revealed with a *lacZ* insertion in the *Dl* gene. The expression is very similar to that of the *vgBE*. (C,G) Expression of *Dl* in ap^{UG035} mutant wing discs. As with *vgBE* expression, the expression of *Dl* is unaffected in the early second instar (C), but is lost in later stages (G). This pattern of expression is similar to that described for *vg* expression in *ap* mutant wing discs (Williams et al., 1993). (D) Expression of *Dl* detected by in situ hybridization of a DIG-labelled *Dl*-DNA probe. The pattern is identical to that observed with the *Dl* enhancer trap line used in A-C and E-G. (H) Detection of *Dl* transcript accumulation in an early third instar *Ser* mutant wing disc. (I) Expression of *wg* transcripts in a Ser^{94c}/Ser^{Rx106} mutant disc in which UAS*Dl* is expressed under the control of *dpp*GAL4. The wing area is strongly enlarged as indicated by the enlarged circular *wg* domain in the hinge region. Further strong induction of *wg* expression is detectable in a broad stripe across the wing blade (compare with Fig. 2G). (J) A similar rescue is observed when UASNintra is expressed in *Ser* mutants under those conditions.

coexpressed in dorsal cells. Furthermore, many of the genes that are expressed at the DV interface in the middle of the third instar under the influence of Notch have a symmetric distribution around this boundary (Williams et al., 1994; Miccheli et al., 1997), and this raises questions about the relative contributions of each of the Notch ligands to the activation of Notch. It might be that, as it has been suggested, the function of Serrate in dorsal cells is to allow Delta to signal (de Celis et al., 1996) through a dominant negative effect on Notch (de Celis et al., 1996; de Celis and Bray, 1997; Klein et al., 1997; Miccheli et al., 1997). Delta also has a dominant negative effect on Notch signalling (Miccheli et al., 1997; Fig. 6H) and we observe that this effect is corrected by coexpression of Delta with full-length Notch (Fig. 6I) suggesting that, as is the case with Serrate, the relative abundance of Notch has a crucial influence on the signalling ability of Delta. In this situation, coexpression of both ligands might result in a different degree of Notch activation than would be achieved individually and therefore can be used to fine regulate the activity of Notch.

To test these regulatory interactions further, we have coexpressed *Ser* and *Dl* with *dpp*Gal4 (Fig. 6A-C). We find that this results in a refined pattern of the induced stripes of target gene expression relative to the effects of expression of either *Dl* alone or *Ser* with *Notch* (Fig. 6A,B). Most prominently, the broad stripe of ectopic *wg* expression induced by *Dl* alone in dorsal cells, becomes refined to the region of higher expression at the posterior end of the *dpp* expression domain, where there are the highest levels of *Delta* expression. Therefore, in the presence of Serrate, Delta is able to signal although with a reduced activity, which perhaps reflects a competition between Serrate and Delta for Notch. This experiment also shows that Serrate not only regulates the expression of Delta but, in an indirect manner, its activity.

Notch is a target of Fringe activity

In contrast to Delta, the effects of ectopic expression of *Ser* on wild-type discs are restricted to ventral cells (Speicher et al., 1994; Couso et al., 1995; Diaz Benjumea and Cohen 1995). This has led to the suggestion that there is an inhibitor of Serrate activity in dorsal cells and that this inhibitor is under the control of *ap* (Diaz Benjumea and Cohen, 1995; Kim et al., 1995). Consistent with this proposal, we observe that ectopic expression of *Ser* in *ap* mutants can induce the expression of downstream targets of Notch in ‘dorsal’ cells (Klein et al., 1998; Fig. 1D,G).

A variety of arguments have led to the proposal that the dorsal inhibitor of Serrate function is encoded by the *fng* gene (Panin et al., 1997; Fleming et al., 1997). For example, ectopic expression of *Ser* with *ptc*GAL4 results in the activation of targets of Notch in two parallel stripes in ventral cells of the developing wing blade, and this can be observed as early as the beginning of the third instar (Fig. 4J). When *Ser* is coexpressed with *fng*, the anterior stripe, but not the posterior one, is lost completely in late third instar discs (Panin et al., 1997 and data not shown). Correspondingly the ectopically induced margin

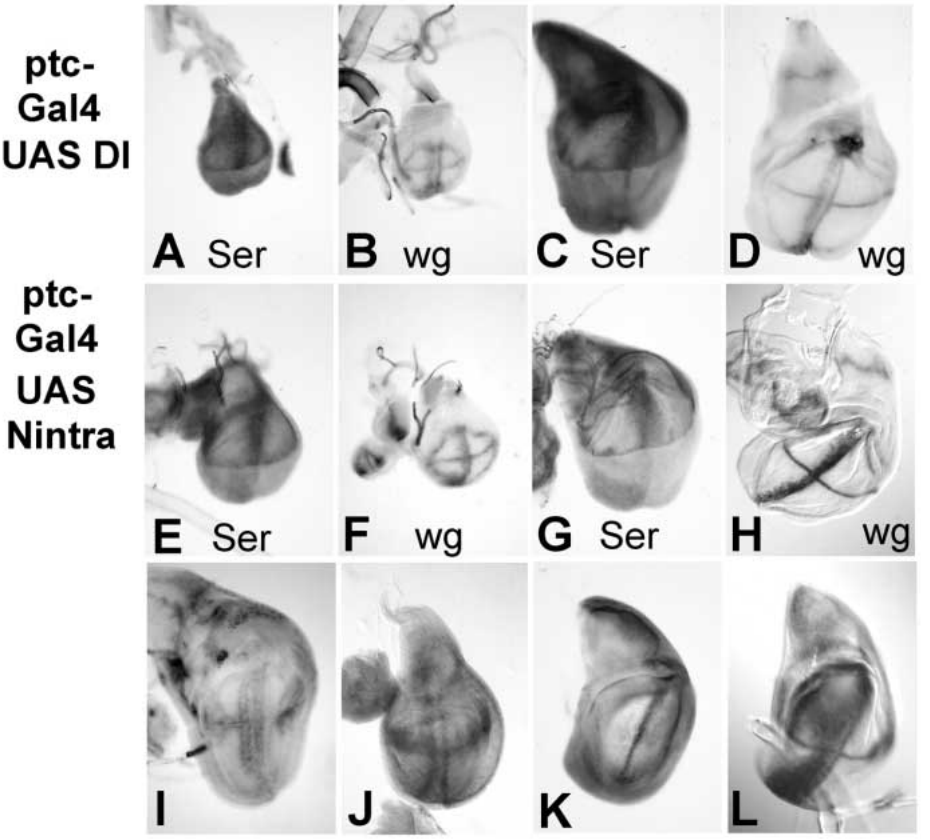

Fig. 4. Effects of ectopic activation of Notch on patterns of gene activity in developing wing discs. Wing discs showing the effects of ectopic expression of *Dl* (A-D,I,K,L), Nintra (E-H) and *Ser* (J). All wing discs are ventral to the bottom and posterior to the right. (A-D) Effects of ectopic expression of Delta in early third (A,B) and late third instar (C,D) discs. Ectopic expression of *Dl* under the control of *ptc*GAL4 results in the ectopic expression of *Ser* in dorsal cells (A) and of *wg* in two parallel stripes in both dorsal and ventral cells (B) during the early third larval instar. In late third larval instar discs, *Ser* expression can be observed in ventral cells, albeit weaker than that elicited in dorsal cells (C), whereas *wg* continues to be expressed in two parallel stripes that span the disc (D). (E-H) Effects of expression of UASNintra under the same conditions as UAS*Dl*. As can be observed, the patterns of expression of *Ser* and *wg* are similar in both cases. (I) Ectopic expression of *Dl* by *ptc*Gal4 leads to the ectopic expression of *E(spl)* symmetrically around the DV boundary. (J) Ectopic expression of UAS*Ser* under the control of *ptc*GAL4 induces expression of *Dl* in two stripes in both dorsal and ventral cells. (K) Expression of Dl under the control of dppGAL4 in an ap^{UG035} mutant wing disc elicits the expression of *Ser*. (L) Expression of *Dl* under the control of *dpp*GAL4 results in the activation of *Dl* expression as revealed by the ectopic expression of a *lacZ* insertion in the *Dl* gene.

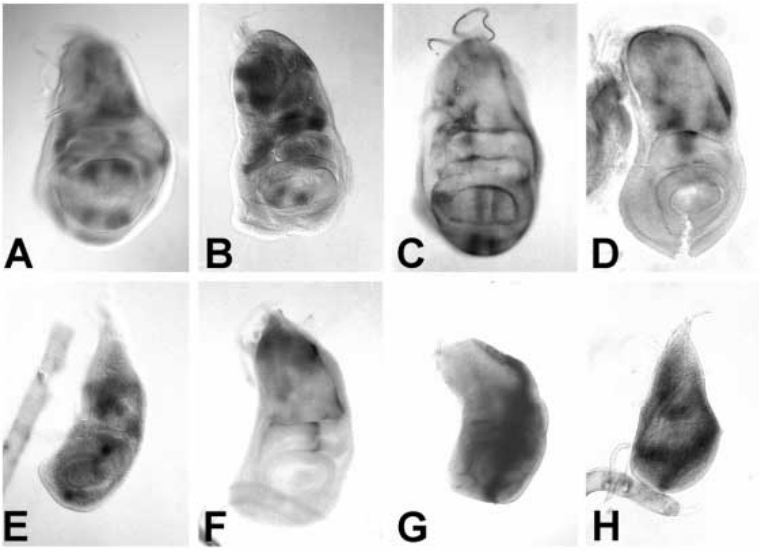

Fig. 5. Dependence of the effects and expression of *fng*, *Ser* and *Dl* on Su(H) in third larval instar wing discs. All discs, with the exception of C, D, F (antibody staining) show RNA expression revealed by in situ hybridization, with ventral to the bottom and posterior to the right. In the third larval instar, *fng* is expressed in a complex pattern that comprises dorsal and ventral cells (Irvine and Wieschaus, 1994) and this is the case in the wild type (A) and in *Su(H)* mutants (B). In the wild type, *Ser* is expressed in both dorsal and ventral cells, in a complex pattern that decorates the wing pouch (C,D) but this pattern is absent in *Su(H)* mutants (D). The same is true of *Dl* (E). In the wild type, ectopic expression of *fng* under the control of *ptc*GAL4 leads to ectopic expression of *Ser* (Kim et al., 1995); however, this is not the case in *Su(H)* mutants (F). (G) Expression of UAS*fng* by *dpp*GAL4 in a *Ser* mutant revealed by in situ hybridization with a *fng* probe. The expression of *fng* is not sufficient to rescue the pouch defect of the mutant (compare with Fig. 2G). Since *fng* is able to recover the pouch in *ap* mutants (Fig. 1E, Klein et al., 1998) and UAS*Dl* that of *Ser* mutants (Fig. 4H) under the same conditions, this result suggests that the activity of *fng* is mediated by *Ser*. (H) Ectopic expression of *Dl* preferentially over the ventral side of an early third instar disc in which UAS*Ser* is expressed under the control of *ptc*GAL4.

structures are reduced to a posterior stripe with characteristics of the posterior compartment (Fig. 7). Since the posterior stripe is a result of nonautonomous signalling by Serrate, this result agrees with the observations that the effects of Fringe are cell autonomous (Panin et al., 1997). Surprisingly, however, we notice that, while Fringe suppresses the function of Serrate cell

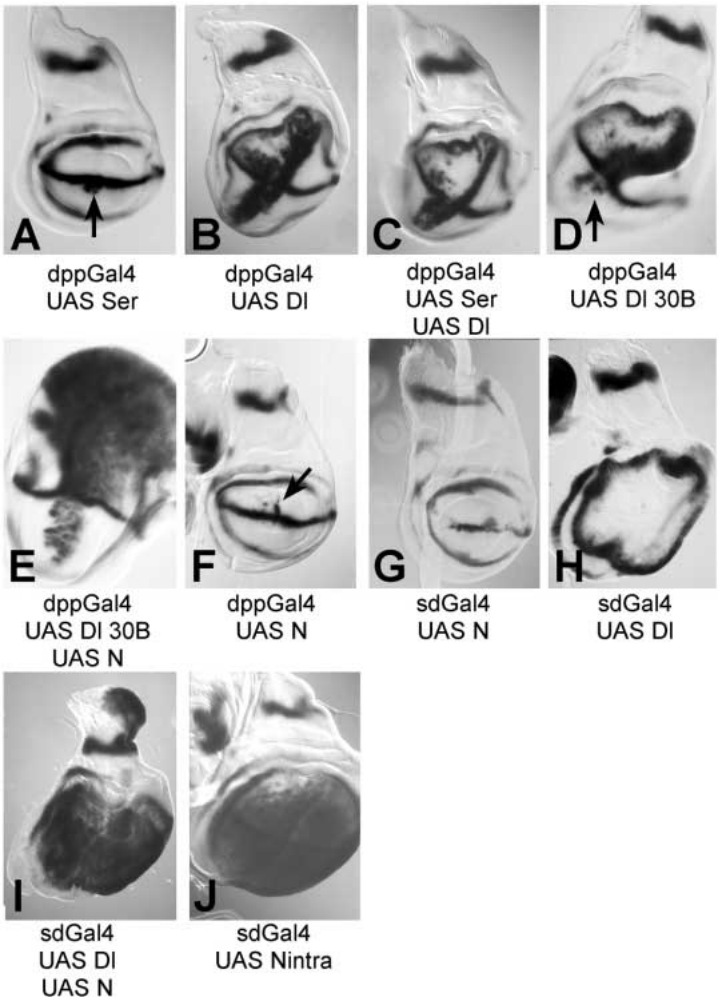

Fig. 6. Effects of ectopic expression of *Ser*, *Dl* or of coexpression of *Dl* with *Notch* and with *Ser*, on wing development. Responses are measured as expression of *wglacZ* in third larval instar wing discs. All discs shown with ventral to the bottom and posterior to the left. (A) Ectopic expression of UAS*Ser* with *dpp*GAL4 leads to a small stripe of ectopic expression of *wg* on the ventral region of the disc (arrow). (B) In contrast, ectopic expression of *Dl* leads to a robust response in dorsal and ventral cells, even though *dpp*GAL4-driven ectopic expression is weaker in ventral cells (see Fig. 4K). (C) The coexpression of *Dl* and *Ser* restricts the expression of *wg* to the posterior region of the *dpp*GAL4 expression domain, where *Dl* is expressed in higher concentration. (D) The effects of ectopic expression of *Dl* in dorsal and ventral cells can be also observed with another line of UAS*Dl* (30B) (arrow). This line appears to trigger lower levels of *Dl* activity (compare with B). (E) Coexpression of *Dl*(30B) with *Notch* leads to an increase in the response as shown by the increased levels of *wglacZ* (compare with D and F). (F) Expression of UASNotch with *dpp*GAL4 leads to weak induction of *wg* expression on the dorsal side of the pouch (arrow). (G) Expression of *Notch* throughout the developing wing, disrupts wing development, perhaps through a dominant negative effect. (H) Global expression of *Dl* throughout the developing wing also has a dominant negative effect, reflected in the absence of *wg* expression along the DV boundary. (I) The dominant negative effect is suppressed if *Notch* is coexpressed with *Dl*. (J) Expression of Nintra throughout the wing leads to a pattern similar to that produced by coexpression of *Dl* and *Notch*, indicating that this pattern is likely to be due to activation of *Notch*.

autonomously, it enhances its signalling ability in a nonautonomous manner (Fig. 7).

Fringe is thought to dampen Serrate signalling by affecting its interaction with Notch, but no evidence has been presented to support this suggestion. While testing the effects of ectopic expression of *fng* with various GAL4 lines, we noticed that expression of UAS*fng* throughout the wing blade with *sd*GAL4 (see Materials and Methods), leads to the loss of the whole wing blade in a manner similar to that observed in *Notch* mutants (Fig. 8B). The phenotypes observed are stronger than those of *Ser* mutants, suggesting that Fringe interrupts also the activation of Notch through other ligands like Delta (compare Figs 8B with 2G).

This result mimics the observation that regulatory mutants of *fng*, which lead to early *fng* expression on ventral cells, produce extensive wing notching (Irvine and Wieschaus, 1994). This effect is not reversed if *fng* is coexpressed with *Ser* (Fig. 8G), but it is reversed if *Dl* is coexpressed with *fng* (Fig. 8H) or, interestingly, when *Ser* and *Notch* are coexpressed with *fng* (Fig. 8F). This suggests that increasing the concentration of Notch can titrate the effects of *fng*. Furthermore, the effects of ectopic expression of *fng* are partially suppressed by expression of *Notch* with *fng* and are exaggerated by expressing dominant negative Notch molecules with *fng* (data not shown). Altogether these results strongly suggest that a target of Fringe activity is the Notch molecule itself.

Fringe inhibits Serrate signalling via Notch

The activity of *Fringe* can inhibit Serrate signalling by enhancing the intrinsic dominant negative activity of Serrate over Notch (Klein et al., 1997). Alternatively, it might inhibit Notch signalling via Serrate. To discriminate between these two possibilities, we have taken advantage of the observation that ectopic expression of *Ser* and *Dl* in the wing disc have well-defined effects on the pattern of bristles and veins, and that these phenotypes can be easily interpreted in terms of Notch signalling.

Expression of *Ser* throughout the late wing disc, for example with MS1096GAL4, leads to a strong broadening of the wing veins and a moderate increase in the number of bristles in the notum (Fig. 9F, not shown). Both of these neurogenic phenotypes can be suppressed by coexpressing *Notch* with *Ser*, indicating that they are due to a dominant negative effect of Serrate (Klein et al., 1997 and data not shown). Ectopic expression of *fng* alone in the same pattern results in nicked wings with normal veins and a reduction of bristles in the notum, which is associated with the loss of sensory organ precursors (Fig. 9A-E). Coexpression of *fng* with *Ser* suppresses the extra vein phenotype caused by misexpression

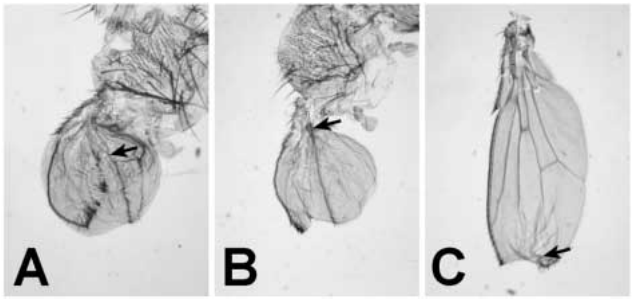

Fig. 7. Consequences of the ectopic expression of UAS*fng* alone and in combination with UAS*Ser* by means of *ptc*Gal4 on wing development. (A) Adult wing resulting from the ectopic expression of *Ser* under the control of *ptc*GAL4. The wings are smaller than wild type and bear two ectopic wing margins. The end of the posterior margin is indicated by an arrow. (B) Adult wing resulting from the ectopic expression of *Ser* and *fng*. Notice that the anterior margin characteristic of *Ser* alone has been suppressed, but the overall shape of the wing is similar to that of *Ser* alone. Furthermore, the formation of the posterior ectopic margin is enhanced and the margin reaches until the hinge (arrow). The analysis of the posterior margin shows that it has the posterior identity, indicating that it is induced in cells next to the *ptc*-expression domain. This suggests that *fng* suppresses the activity of Serrate cell autonomously, but enhances it nonautonomously. (C) Adult wing resulting from the ectopic expression of *fng* under the control of *ptc*GAL4. Apart from a nick and a small ectopic margin (arrow), there are no major changes from the wild type.

of *Ser* and, therefore, supports the notion that Fringe reduces the ability of Serrate to bind Notch. However the loss of bristle phenotype is not altered by the expression of *fng* in *Ser* mutants, which suggests that it is not mediated by an interaction between Fringe and Serrate (Fig. 9B).

The action of Fringe requires the activity of Su(H)

The early pattern of expression of *fng* is under control of *ap*, and ectopic expression of *fng* results in the induction of *Ser* expression, even in the absence of *ap* (data not shown). This has led us to test whether *fng* is also able to induce expression of *Dl* and found that this is the case (Fig. 5H). However, the ability of *fng* to induce *Dl* expression is restricted to the ventral half of the wing disc, just as is its ability to induce *Ser*. Because of the similar phenotypes induced by expression of *Ser* and *fng*, and the ability of *fng* to activate *Ser* expression, we tested whether the outcomes of *fng* expression require the activity of *Ser* by expressing it in *Ser* mutants. *fng* was not able to rescue the wing phenotype caused by an amorphic combination of *Ser* (Fig. 5G), showing that *fng* requires *Ser* for its function in the wing.

In a complementary experiment, we confirmed that the activity of Fringe is not able to rescue the defects caused by *Su(H)* mutants (Fig. 5F). Surprisingly, *fng* was also unable to induce the expression of *Ser* in the wing blade of these mutants. This suggests that the inductive effects of *fng* require *Su(H)* activity. However the wing blade is ill defined in *Su(H)* mutants and, since our results suggest that *fng* operates on the wing blade, the failure of *fng* to activate *Ser* expression in *Su(H)* mutants could be a secondary effect. To circumvent this difficulty, we tested the dependence of *fng* on *Su(H)* in another process that depends on Notch signalling: the formation of the anterior spiracle.

Loss of *Ser* function results in the loss of the anterior spiracle of the larva (Speicher et al., 1994; Fig. 10A,B). The same effect is observed if a dominant negative Notch molecule, or *fng*, is expressed in these cells with the *klu*GAL4 driver (Materials and Methods; Fig. 10C,D,G-I). Expression of *Ser* with *klu*GAL4 can partially rescue the defects in the anterior spiracle of *Ser* mutants (data not shown) indicating that *klu*GAL4 is expressed

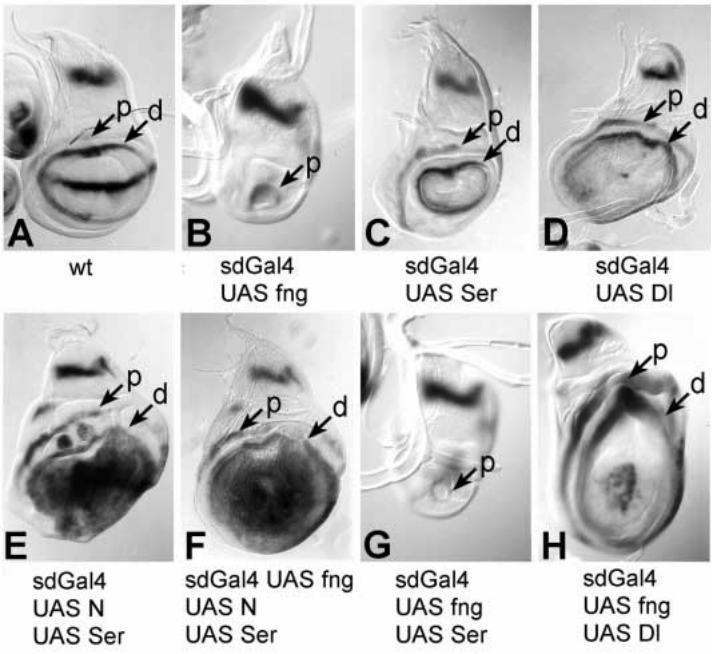

Fig. 8. Effects of global ectopic expression (through *sd*GAL4) of *fng*, alone or together with *Ser* and *Dl* on the development of the wing as revealed by the expression of *wglacZ* in the third larval instar. Ventral to the bottom, anterior to the left. (A) Wild-type wing disc showing the expression of *wg* along the margin and in the proximal (p) and distal (d) rings, which delimit the hinge region. (B) Expression of UAS*fng* results in the loss of a large part of the wing, including blade and parts of the hinge region as indicated by the loss of the *wg* expression along the margin and the distal circular domain in the hinge region. Notice that the phenotype is significantly stronger than that induced by *Ser* loss-of-function mutations (compare with Fig. 2F and see text). (C) Expression of UAS*Ser* results in the loss of the margin and a reduction of the size of the blade. (D) Expression of UAS*Dl* results in the irregular activation of *wg* expression all over the blade and a corresponding increase in the size of the blade. Notice the dominant negative effect highlighted by the absence of *wg* expression in the center of the blade. This effect is suppressed by coexpression of Dl with Notch (see Fig. 6G,H). (E) Coexpression of UAS*Notch* and UAS*Ser* results in an increase in the size of the wing blade and strong activation of *wg* expression throughout the wing blade. Notice the effect of Ser on the dorsal side of the pouch in this context. (F) Coexpression of UAS*Notch* and UAS*Ser* with UAS*fng* suppresses the effects of UAS*fng* in wing development (compare with B). (G) Coexpression of UAS*fng* and UAS*Ser* results in a phenotype identical to that of expression of UAS*fng* alone. (H) Coexpression of UAS*fng* and UAS*Dl* results in a phenotype similar to that of expression of UAS*Dl* alone.

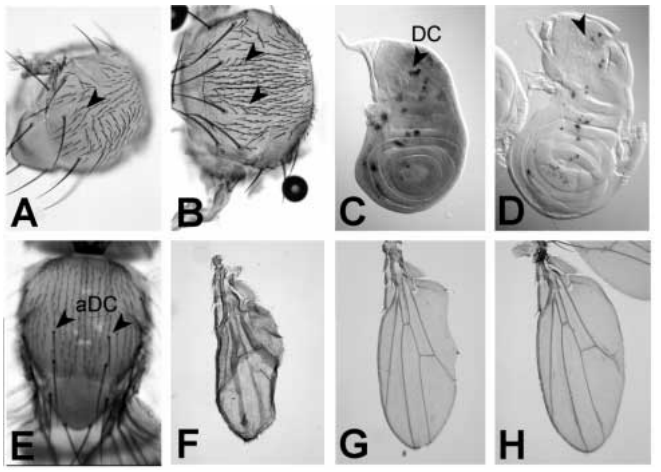

Fig. 9. Effects of the expression of UAS*fng* on bristle development and wing vein patterning. (A) Thorax of an adult fly showing the effects of expression of UAS*fng* under the control of *klumpfuss*GAL4 (*klu*GAL4 see Klein and Campos Ortega, 1997) on the development of bristles. Notice that two of the dorsocentral macrochaetae, the anterior ones, are missing; their positions are indicated by an arrowhead. (B) Thorax of a Ser^{94c}/Ser^{RX106} mutant fly in which UAS*fng* has been expressed with *klu*GAL4. Notice that the dorsocentral bristles are still missing (arrowheads). (C) Wing disc from a fly as in A showing the proneural clusters for the different sense organs as revealed by the expression of Achaete protein. The pattern of Achaete expression is normal. Arrowhead indicates the position of the dorsocentral cluster (DC) from which the anterior dorsocentral bristle will arise. (D) Late third instar wing disc showing the pattern of sensory organ precursors as revealed by neuralized A101 *lacZ*. Arrowhead points to the region of the anterior dorsocentral machrochaete that is missing. (E) Thorax of a wild-type fly showing the normal pattern of bristles. The anterior dorsocentral pair is indicated (aDC). (F-H) Effects of ectopic expression of UAS*fng* and UAS*Ser* with *MS1096*GAL4 which activates gene expression from early third instar onwards in the dorsal half of the wing disc and wing blade. During later phases of the third instar expression is also detectable in the ventral half of the wing blade. (F) Ectopic expression of *Ser* results in a broadening of the wing veins, probably through a titration of Notch signalling during the patterning of the wing veins. (G) Ectopic expression of *fng* results in a nonpenetrant nicking of the wing margin, but has no effect on vein development. (H) Coexpression of *fng* with *Ser* suppresses the dominant negative effects of expression of *Ser* alone on the veins (compare with E).

in the spiracle even in the absence of Notch signalling. In *Su(H)* mutants, the anterior spiracles are present and the ability of *fng* to inhibit anterior spiracle formation is strongly impaired (Fig. 10E,F). Altogether, these results show that some of the activities of Fringe are mediated by Su(H) and suggest that the loss of sensory organ precursors caused by expression of *fng* could be due to the activation of Su(H).

DISCUSSION

The loss of the wing blade characteristic of *ap* mutants is usually ascribed to the loss of *Ser* expression (Diaz Benjumea and Cohen, 1995; Couso et al., 1995). However, *ap* regulates the expression of both *Ser* (Couso et al., 1995) and *Dl* (this work) and, as we have highlighted here, the phenotype of *Ser* mutants cannot account for all the defects of *ap* mutants. Because *Dl* is initially expressed in *Ser* mutants, this leads us to suggest that the *ap* mutant phenotype is a consequence of the loss of both *Dl* and *Ser* expression: whereas *Dl* is likely to be involved in the initial specification of the wing blade, the main function of *Ser* is to maintain Notch activity by maintaining the expression of *Dl* and regulating its signalling ability. A second, and equally important function of *Ser* is to contribute to the spread of *wg* expression, which determines

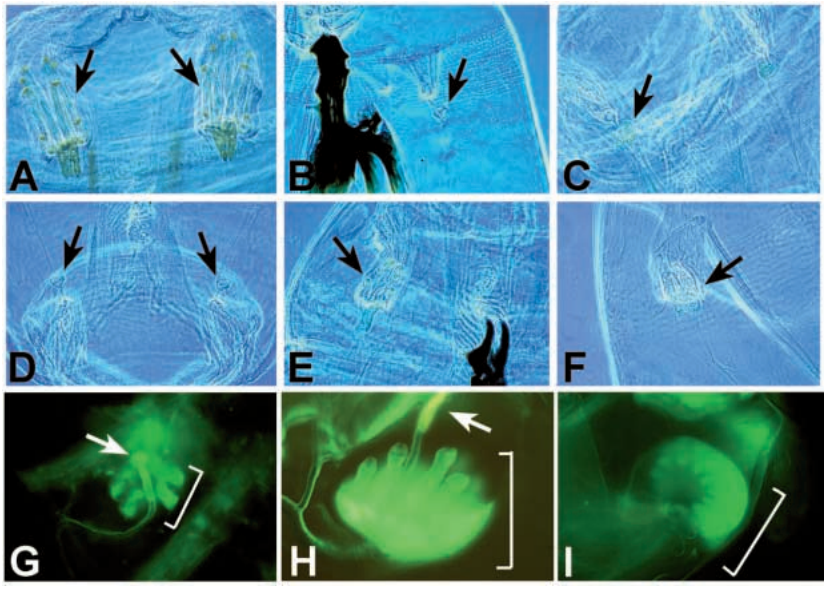

Fig. 10. Effects of ectopic expression of *fng* under the control of *klu*GAL4 on the development of the anterior spiracle. (A) Anterior spiracles of a wild-type larva. Arrows point to the retractable finger-like appendages characteristic of these structures in the third instar. (B) The finger-like structures are lost in *Ser* mutant larvae. (C) The spiracle is also missing following ectopic expression of *Ser*. (D) The development of the anterior spiracle is also impaired through ectopic expression of a dominant negative Notch molecule, UAS*ECN*. The arrows in B, C and D point to the remnant of the anterior spiracles. (E) $Su(H)^{AR9}/Su(H)^{SF8}$ mutant larva showing normal development of the anterior spiracles (arrow). (F) Expression of UAS*fng* in *Su(H)* mutants is not able to suppress the development of the spiracles completely and some small finger-like structures develop (arrow, compare with B). (G-H) Expression of UASGFP under the control of *klu*GAL4 in the anterior spiracle in the early (G) and late second (H) instar of wild type and in a second instar *Su(H)* mutant larva (I). The anlage of the third instar anterior spiracle is highlighted by brackets in G-I. Expression of UAS*Ser* in *Ser* mutants under control of *klu*GAL4 partially recovers the third instar anterior spiracle, indicating, that the expression of *klu*GAL4 is not dependent on *Ser* activity (T. K. and A. M. A., unpublished result).

the extent of the wing blade. In agreement with the suggestion that the *ap* mutant phenotype is a combination of the loss of *Ser* and *Dl* function, clones of cells mutant for either *Dl* or *Ser* generate nicks with a frequency that is increased when clones of cells doubly mutant for the two genes are made (Miccheli et al., 1997).

Restriction of Notch activity to the DV interface

Once the wing primordium has been established, the wing blade begins to grow under the influence of signalling events associated with the DV interface (Neumann and Cohen, 1996; Zecca et al., 1996; Klein and Martinez Arias, 1998). The activity of Notch is important in this process, and therefore understanding the interactions between and functions of its two ligands, Serrate and Delta, is an important step towards understanding this process. In a first instance, these interactions lead to the generation of a source of information at the DV interface that influences cell proliferation and the early patterning stages.

Experiments on the ectopic expression of various genes (Panin et al., 1997; deCelis and Bray, 1997; Fleming et al., 1997) and, in some instances of loss of function (Miccheli et al., 1997), have led to the conclusion that, during all these processes, activation of Notch is both a consequence and a cause of the expression of *Ser* and *Dl*. In the wild type, as the wing grows, the patterns of expression of these genes evolve, but initially it is believed that they are expressed in mutually exclusive cell populations and that this leads to the establishment of a feedback loop between their gene products that maintains Notch activity at the DV interface (Panin et al., 1997; Fleming et al., 1997). Our analysis of *Ser* and *Dl* expression in *Su(H)* mutants and of *Dl* expression in *Ser* mutants supports this basic functional interactions and demonstrates that this feedback loop must operate during normal wing development.

In principle, Serrate and Delta can elicit qualitatively identical signals. In the context of both, the patterns of expression that have been described and the feedback loop between Delta and Serrate, this raises the question of why the activity of Notch does not spread throughout the whole wing blade. To account for this possibility, Panin et al. (1997) have proposed that the ability of Delta to activate Notch is restricted to the dorsal half of the developing wing blade, whereas that of Serrate is restricted to the ventral half. This situation will only prevent the spread of Notch activity if, at the very least, the expression of Delta is restricted to the ventral and that of Serrate to the dorsal halves of the wing blade. However, Delta is expressed (and see also de Celis and Bray, 1997), and can signal in both dorsal and ventral cells along the DV boundary even in the presence of functional Serrate. Thus, since Delta can induce *Dl* expression in dorsal cells (this work), the simple feedback loop described above will inevitably result in the progressive spread of Notch activity over the developing wing. This does not happen in normal development and therefore there must be other mechanisms that restrict this signalling event to the DV boundary.

At the moment, it is not clear how this happens, but several factors are known that can influence Notch signalling. For example, at the beginning of the third instar, the wing blade is a thick band of about six to eight cells centered at the DV interface (Kim et al., 1996; unpublished data), and which might have homogeneous Notch activity. As the wing grows, the local nature of the interactions between Notch and its ligands and the fact that the primordium is always the reference for growth would result in a restriction of Notch signalling to the DV interface.

In addition the concentration of Vestigial is crucial for Notch signalling (Klein and Martinez Arias, 1997). Since this is highest at the DV boundary (Williams et al., 1993), it is not difficult to see how Delta and Serrate signalling would be reinforced in this region by Vestigial and thus maintain high levels of Notch activity.

The relative concentration of free Notch to its ligands also must be an important determinant of Notch signalling (Couso et al., 1995; de Celis et al., 1996; de Celis and Bray, 1997; Klein et al., 1997; Miccheli et al., 1997). This means that competitive interactions between Delta and Serrate for Notch will add to the bias defined by the expression of *vg*, the differential sensitivities of the downstream genes and the restrictions associated with the patterns of growth. We have shown that Serrate will dampen Delta signalling dorsally, an effect that might be achieved through the dominant negative activity of Serrate on Notch. This effect of Serrate on Delta would also contribute to ensure that Delta signalling does not spread through the dorsal cells. On the contrary, the dominant negative effect of Delta, coupled to the low sensitivity of ventral cells to express *Ser*, will reduce the ability of spreading on ventral cells.

Altogether these effects could account for the existence of two activating ligands for Notch; one of them, Serrate, with a low dominant negative threshold, which would reduce the concentration of Notch locally and another one, Delta, with a high signalling affinity. In addition, these effects and patterns of expression can account for the observation that dorsal cells are more sensitive to the loss of *Dl* (Doherty et al., 1996; de Celis et al., 1996) than ventral cells (de Celis et al., 1996). While Delta can activate Notch in dorsal cells, Serrate and Delta can both activate Notch in ventral cells.

The activity of Fringe

On the one hand, while our results support the observation that Fringe affects Serrate function, they do not provide any evidence for an stimulatory effect of Fringe on Delta signalling. First, expression of *fng* alone has no effect on the wing veins, which it should if it were to enhance Delta signalling. Secondly expression of *fng* with *sd*GAL4 removes the wing blade in a manner similar to *ap* mutants, which, in the light of our results, suggests that *fng* may also be interfering in the activation of Notch by other ligands, such as Delta, but in a negative rather than a positive manner. Furthermore, a mutation in *fng* causes misexpression of *fng* in the ventral surface of the wing and this leads to deletions of wing tissue (Irvine and Wieschaus, 1994) rather than increases in size as might be expected if Fringe enhanced Delta signalling. Finally, Delta can rescue the loss of wing caused by the loss of *ap* function and, since *fng* is absent in these mutants (Irvine and Wieschaus, 1994), this indicates that Delta can signal appropriately without Fringe. Consistent with this, overexpression of *fng* in the embryo can suppress the antineurogenic effects of Serrate but on its own does not cause antineurogenic phenotypes (Fleming et al., 1997), as might be expected if it enhanced Delta signalling.

Although we have not observed convincing effects of Fng on Delta signalling, there is a possibility that could account for the published reports. It might be that, under conditions of weak Delta activity, coexpression of *fng* leads to the provision of Notch, which Fng would render unable to interact with Serrate and which could then interact with Delta positively.

On the other hand, our results provide further support for a negative role of Fringe in Serrate signalling. Furthermore, whereas previous experiments did not prove that Fringe acts on Serrate or on Notch, we have shown that the target of Fringe is likely to be the Notch molecule itself. A domain within Fringe has weak homology to glycosyltransferases (Yuan et al., 1997). Since Notch is a glycoprotein (Johansen et al., 1989), one way in which Fringe might dampen Serrate signalling is by modifying certain regions of Notch that are involved in binding Serrate. In addition, the observation that many effects of *fng* are not dependent on Serrate, indicate that Fringe can act either on other yet unknown ligands for Notch, or on other receptors that are functionally linked to Notch.

A variety of arguments have led to the suggestion that the effects of Fringe are mediated by boundaries between *fng*-expressing and -nonexpressing cells rather than by the expression of *fng* on some cells and not others (Irvine and Wieschaus, 1994; Kim et al., 1996). Here we have confirmed this by showing that uniform expression of *fng* throughout the wing from early stages of development abolishes the development of the wing.

It has also been suggested that the target of Fringe is the interaction between Notch and its ligands Serrate and Delta and that, while Fringe blocks Serrate signalling, it stimulates Delta signalling (Panin et al., 1997). In this regard, the phenotype that results from expressing *fng* uniformly resembles that of *ap* mutants and is stronger than that of *Ser* mutants. This suggests that, early in development, Fringe can block the activity of Notch ligands other than Serrate, perhaps Delta, since the loss of wing pouch in *ap* mutants can be rescued by the expression of either *Ser* or *Dl*. The expression of *fng*, for example under the control of *dpp*GAL4, can also rescue the loss of wing of *ap* mutants. This is likely to be due to the effects of Fringe on the expression of *Ser* and *Dl*, because it cannot rescue the loss of wing of *Su(H)* or *Ser* mutants, and suggests that the function of Fringe requires Notch signalling.

Since in *ap* mutants the initial pattern of *Dl* expression is unaffected, the rescue of the wing through ectopic expression of *fng* is likely to be exerted by the provision of a boundary of *fng*-expressing and -nonexpressing cells, which allows Delta to operate and establish the feedback loop of Notch activity on its own. The observation that *fng* cannot rescue the loss of wing of *Ser* mutants, in which Delta is also initially expressed, indicates that, although *Ser* is not necessary for the initial interactions across the DV interface, it is necessary for their maintenance.

How does Fringe work? From our experiments, it is clear that Fringe is not involved in the establishment of different dorsoventral sensitivities to Notch signalling within the wing disc, or in the suppression of the spreading of Notch activity away from the DV boundary as recently proposed (Panin et al., 1997). Our results suggest that Fringe is necessary to trigger and establish a stable circuit of Notch signalling at a defined position in the wing disc. This might also be true of other situations in which *fng* is expressed. Fringe can have negative effects on the interaction between Notch and either Serrate or Delta, but it can also stimulate the same interactions. In the second instar, the distribution of Notch ligands might result in the homogeneous and low activity of Notch, the expression of *fng* would break this symmetry at the border with nonexpressing cells and lead to reciprocal Notch signalling, the establishment of a signalling center and its refinement to the DV boundary. This would explain our observation that Notch is the target of Fringe activity and, furthermore, the observation that Su(H) is necessary for the function of Fringe. How Fringe actually achieves this effect on Notch remains unknown and will require a detailed molecular analysis of the interactions of Notch with its ligands and how they trigger Notch activity.

We want to thank J. Kim, K. Irvine, M. Haenlin, L. Seugneut, S. Cohen, E. Knust, J.F. de Celis and S. Carroll for stocks and reagents; S. Rolfe for assistance; S. Bray, J. F. de Celis and K. Brennan for discussions and C. Sharpe for reading the manuscript. This work was funded by The Wellcome Trust (A. M. A. and T. K.), The Newton Trust (T. K.) and the Deutsche Forschungsgemeinschaft (T. K.).

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