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Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc

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SUMMARY

The cells along the dorsoventral boundary of the *Drosophila* wing imaginal disc have distinctive properties and their specification requires Notch activity. Later in development, these cells will form the wing margin, where sensory organs and specialised trichomes appear in a characteristic pattern. We find that Notch is locally activated in these cells, as demonstrated by the restricted expression of the Enhancer of split proteins in dorsal and ventral cells abutting the D/V boundary throughout the third larval instar. Furthermore other genes identified by their involvement in *Notch* signaling during neurogenesis, such as *Delta* and *Suppressor of Hairless*, also participate in Notch function at the dorsoventral boundary. In addition, Serrate, a similar transmembrane protein to Delta, behaves as a ligand required in dorsal cells to activate Notch at the boundary. *Notch* gain-of-function alleles in which Notch activity is not restricted to the dorsoventral boundary cause miss-expression of *cut* and *wingless* and overgrowth of the disc, illustrating the importance of localised Notch activation for wing development.

Key words: *Drosophila*, *Notch*, Dorsal-ventral boundary, Wing, Imaginal disc

INTRODUCTION

The transmembrane protein encoded by *Notch* participates in many cell-fate decisions and mophogenetic events during development, both in *Drosophila* and in other organisms where homologues have now been found (Artavanis-Tsakonas et al., 1995). Most analyses of *Notch* have focused on its role in neurogenesis, where it appears to function as the receptor in a signaling event that leads to a cell being inhibited from adopting the neural fate (Campos-Ortega and Knust, 1990). Other components required for this decision include Delta, another transmembrane protein which is the putative ligand for Notch (Artavanis-Tsakonas et al., 1995), and the Enhancer of split proteins, which are transcription factors (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992) expressed in cells where Notch is active (Jennings et al., 1994). In addition, the Supressor of Hairless [Su(H)] protein has been implicated as a link between the cell-surface Notch receptor and the nucleus (Fortini and Artavanis-Tsakonas, 1994) at least during peripheral nervous system development in *Drosophila* (Schweisguth and Posakony, 1994). However, the pleitropic actions of *Notch* (Shellenbarger and Mohler, 1978) raise the question of whether the same components are involved in all processes where *Notch* is required.

The *Notch* gene was first identified through its effect on the wing margin, loss of one dose of *Notch* results in nicked wings. Subsequent studies have revealed several roles for *Notch* in wing morphogenesis, where it is needed for cell growth and vein differentiation as well as for wing margin formation (Shellenbarger and Mohler, 1978; de Celis and Garcia-Bellido, 1994b). The wing develops from an anlage of a few embryonic cells that proliferate during the larval instars (Cohen, 1993). The wing anlage is subdivided into lineage units called compartments (Garcia-Bellido et al., 1973), and the borders between these compartments have a major influence on the growth and patterning of the disc (Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994). The division into dorsal and ventral compartments occurs during the second larval instar through the activity of the Apterous homeodomain protein, which is expressed throughout the dorsal compartment (Cohen et al., 1992). The dorsal/ventral (D/V) boundary forms at the edge of the *apterous*-expressing cells, and organizes proliferation of both dorsal and ventral cells (Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994), in a process mediated at least in part by the secreted protein Fringe (Irvine and Wieschaus, 1994). The boundary cells acquire a number of specialised characteristics during the third larval instar including expression of several genes required for wing formation, such as *wingless*, *vestigial* and *scalloped* (Williams et al., 1993). Ultimately these cells organise the development of distinctive wing margin structures, including sensory elements and trichomes. The analyses of *Notch* temperature-sensitive alleles (Shellenbarger and Mohler, 1978) and of clones of *Notch* mutant cells (de Celis and Garcia-Bellido, 1994b) indicate that *Notch* is required at the D/V boundary from late second larval instar until pupariation, initially to

allow wing growth and later to allow normal wing margin formation.

To what extent are the pathways involving *Notch* during wing development the same as during neurogenesis? The *Enhancer of split* complex [*E(spl)*-C], for example, is required downstream of Notch during neurogenesis (Lieber et al., 1993; Jennings et al., 1994), but there is no evidence to link these genes with wing morphogenesis, as no wing scalloping phenotype is observed when the dose of the complex is reduced. Here we have examined whether the genetic components required for *Notch* activity during neurogensis also operate in wing morphogenesis. Using clonal analysis and molecular markers we find that Notch is locally activated in dorsal and ventral cells at the D/V boundary through the action of two ligands, Delta and Serrate. Both *Su(H)* and *E(spl)-C* genes are required to mediate the actions of *Notch* in these cells. *Notch* activity at the D/V boundary is critical for wing growth and margin development and it is necessary to restrict and maintain the expression of genes such as *wingless* and *cut* to the cells at this boundary.

MATERIALS AND METHODS

Genetic strains

We have used the loss-of-function alleles $l(1)N^3$, $l(1)N^{\beta}$ (a gift from M. Young), nd^3, Ser^{RX82} Ser^{RX106}, Ser^{RX107} (Thomas et al., 1991), $Ser^{rev2-11}$(Fleming et al., 1990) Dl^{M1}, Dl^{M3} (de Celis and Garcia-Bellido, 1994a), Dl^{RF}, Dl^{6B} (Parody and Muskavitch, 1993) $Df(3R)E(spl)^{RA7.1}$ (Schrons et al., 1992), $Su(H)^{AR9}$ and $Su(H)^1$ (Schweisguth and Posakony, 1992), and the *Notch* gain-of-function alleles Ax^{M1}, Ax^{59d}, Ax^{28} and Ax^{16172} (Lindsley and Zimm, 1992). The cell markers used for clonal analyses were *forked* (f^{36a}) and *bald* (bld^1) (Lindsley and Zimm, 1992). We used two transgenes carrying the *f* wild-type allele inserted in 32A and 87F (designated P[f$^+$]32A and P[f$^+$]87F, respectively (A. García-Bellido, unpublished). The *Minute* alleles to generate M^+ clones were *M(1)15D, M(2)24F* and *M(3)95A* (Lindsley and Zimm, 1992). The *lacZ* reporter lines used were: *ct*HZ-1 (Jack et al., 1991), *apterous-LacZ* (Cohen et al., 1992) and *wg-LacZ* (Perrimon et al., 1991).

Generation of mitotic recombination clones

Mitotic recombination was induced by X-rays (dose 1000 R; 300 R/minute, 100 kV, 15 mA, 2 mm aluminium filter). Irradiated larvae were timed in hours after egg laying (AEL). Clones were induced at the intervals 48-72 and 72-96 hours AEL. Mutant clones in the X chromosome were generated in flies of genotype N^* $f^{36a}/M(1)15D$, where N^* represent $l(1)N^3$, $l(1)N^{\beta}$ or Ax^{M1} *Notch* alleles. Mutant clones in the 3R chromosomic arm were generated in males of genotypes f^{36a}; *mwh bld* P[f$^+$]*87F/Mutant* (twin clones) and f^{36a};*mwh M(3)95A* P[f$^+$]*87F Mutant* (M^+ clones), where *Mutant* represent Ser^{RX82} Ser^{RX106}, Ser^{RX107}, Dl^{M1}, Dl^{M3} or $Df(3R)E(spl)^{RA7.1}$ alleles. $Su(H)^{AR9}$ clones were induced in flies of genotype f^{36a}; *M(2)24F* P[f$^+$]*37A/*$Su(H)^{AR9}$ (M^+ clones). Mitotic recombination proximal to the f^+ insertion produces homozygous mutant clones labeled with the cell marker *f*.

In the twin experiments to evaluate the proliferative abilities of mutant cells, the ratio between mutant (*f*) and wild-type (*bld*) cells in clones induced at 48-72 h AEL were: Ser^{RX82}: 1.06 (43 clones), Dl^{M3}: 0.76 (8 clones), $Df(3R)E(spl)^{RA7.1}$: 0.56 (24 clones). The ratio between *f* and *bld* cells in twin clones induced at the same age in the control experiment f^{36a};*bld* P[f$^+$]*87F*/+ was 1.17 (20 twin clones). In M^+ experiments, the mean number of cells in clones induced at 72-96 hours AEL were: *f* $M(3)95A^+$ $E(spl)^{RA7.1}$: 260 cells (26 clones), *f* $M(3)95A^+$ Dl^{M3}: 433 cells (25 clones). The mean number of cells in control clones induced at the same time interval in flies of genotype f^{36a}; *P*[f$^+$]*87F M(3)95A*/+ was 995 (39 clones).

Other methods

Immunocytochemistry was performed as described in Jennings et al. (1995). The following antibodies were used: mAb323 to detect *E(spl)*bHLH expression (1/3, Jennings et al., 1994), rabbit polyclonal anti-β-galactosidase (1/2000, Cappel) and rabbit polyclonal anti-cut (1/2000, Blochinger et al., 1993). Secondary antibodies were from Jackson laboratories (used at 1/250).

In situ hybridizations to analyze *wg* and *Dl* expression were carried out using digoxygenin-labeled DNA fragments following the protocol of Cubas et al. (1991). These fragments were a 0.8 kb *Eco*RI fragment from the *wg* cDNA clone wgc14 (Baker, 1988b) and a 4 kb *Eco*RI fragment from *Dl* c3.2 cDNA clone (Vassin et al., 1987).

RESULTS

Phenotypes of *Notch* loss-of-function alleles indicate different requirements in dorsal and ventral cells

Clones of cells mutant for *Notch* null alleles result in dramatic scalloping phenotypes when they encompass the edge of the wing, or abut the edge of the wing dorsally or ventrally (de Celis and Garcia-Bellido, 1994b). This involves loss of the sensory organs and trichomes of the wing margin itself, as well as loss of adjacent tissue. Because absence of *Notch* has effects on cell viability and sensory organ development (de Celis and Garcia-Bellido, 1994b), we have extended the clonal analysis of *Notch* mutations using the hypomorphic alleles $l(1)N^{\beta}$ and $l(1)N^3$. These alleles have two characteristics that make them ideal for analyzing *Notch* function at the D/V boundary. First, they do not affect cell viability, since $l(1)N^{\beta}$ and $l(1)N^3$ cells grow to form clones of normal size in the thorax and wing (data not shown). Second they have little effect on differentiation of sensory elements and indeed we find mechanosensory bristles in clones of mutant cells at the wing margin under certain circumstances (see below).

As with *Notch* null alleles, $l(1)N^{\beta}$ and $l(1)N^3$ clones abutting the margin cause extensive wing scalloping, demonstrating that these effects are not simply due to failures in cell proliferation. These phenotypes are extreme when the clones extend across the D/V boundary, i.e. in clones induced before segregation of dorsal and ventral compartments (26 clones, Fig. 1A,C,E). Surprisingly, clones restricted to either the dorsal (16 clones) or ventral (24 clones) compartment have quite different phenotypes. Ventral clones abutting the wing margin are always associated with strong scalloping (Fig. 1B,D), whereas clones in the dorsal compartment differentiate a normal wing margin (Fig. 1F). Since previous analyses using *Notch* null alleles showed that *Notch* is needed in both dorsal and ventral cells (de Celis and Garcia-Bellido, 1994b), our results demonstrate that *Notch* requirements must be different in the cells on either side of the boundary. In addition, in mosaic wings with $l(1)N^{\beta}$ and $l(1)N^3$ clones abutting the margin, the spacing between veins is altered (Fig. 1C-E). Since this is not seen even with large clones in internal regions of the wing (data not shown), it suggests that *Notch* activity specifically at the D/V boundary is also necessary for the normal patterning of the wing.

Asymmetrical requirements for two *Notch* ligands, *Serrate* and *Delta* at the D/V boundary during wing development

The clonal analysis using $l(1)N^{\beta}$ and $l(1)N^{3}$ suggests that there is a different requirement for *Notch* in dorsal and ventral cells at the D/V boundary. Two genes, *Serrate* (*Ser*) and *Delta* (*Dl*), encode transmembrane proteins (Vassin et al., 1987; Fleming et al., 1990; Thomas et al., 1991) that are able to interact with Notch molecularly (Rebay et al., 1991; Lieber et al., 1992). Both are candidate ligands for Notch at the D/V boundary since *Dl* and *Ser* mutations interact with *Notch* alleles (de la Concha et al., 1988; Xu et al., 1990; de Celis et al., 1993). Furthermore, since *Ser* is expressed in dorsal regions of the wing disc (Thomas et al., 1995), its function could relate to the different requirements for *Notch* in ventral cells. To explore the roles of *Dl* and *Ser* in Notch signaling at the D/V boundary, we have carried out mosaic analysis using *Ser* null alleles (Ser^{-}) and *Dl* lethal alleles (Dl^{l}) neither of which have a dominant scalloping phenotype. In some cases, these alleles have been analysed in *Minute*$^{+}$ (M^{+}) conditions, to give the mutant cells a growth advantage (Morata and Ripoll, 1975).

There is no phenotype associated with clones of Ser^{-} cells away from the wing margin: Ser^{-} clones have similar size to their Ser^{+} twins (see Materials and methods), indicating that proliferation of Ser^{-} cells is normal. Ser^{-} and Ser^{-} M^{+} clones do not affect the differentiation of wing veins or of bristles in the thorax (data not shown). In contrast, Ser^{-} and Ser^{-} M^{+} clones which do contact the wing margin cause similar scalloping to *Notch* mutant clones (Speicher et al., 1994, Fig. 2). However, scalloping only occurs when clones are induced before dorsoventral lineage segregation (7 clones) or in clones restricted to the dorsal compartment (25 clones). In these cases, Ser^{-} clones cause the loss of large regions of the wing, and *Ser* mutant cells appear abutting the scalloped interface between the dorsal and ventral surfaces (Fig. 2A,D,E). No phenotype was seen with clones that extend to the wing margin in the ventral compartment (20 clones, Fig. 2B). Weaker scalloping phenotypes are observed in M^{+} dorsal clones induced at later stages (72-96 hours AEL), although some dorsal clones can contact the wing margin (differentiating wing margin bristles) without causing scalloping (5 clones). The behaviour of *Ser* mutant cells in the dorsal compartment contrasts with that of *fringe* mosaics in the same compartment (Irvine and Weischaus, 1994) as we never observe the formation of internal ectopic wing margins associated with the novel confrontation between Ser^{+} and Ser^{-} cells.

The phenotype of *Dl* lethal alleles (Dl^{l}) in clones indicates that *Dl* also is required at the D/V boundary. However, extensive scalloping occurred only when clones included both dorsal and ventral cells (7 clones, Fig. 2C,F). Dl^{l} clones in the ventral compartment give rise to small nicks (Fig. 2G, 7 clones) or loss of margin sensory elements (7 clones). However, dorsal Dl^{l} clones cause only loss of sensory elements without any associated nicking (8 clones). These results suggest either that *Dl* is required principally before D/V compartment separation or that *Dl* can be supplied by cells on either side of the boundary, its provision by ventral cells being more sensitive to reductions in levels.

Requirement for *Su(H)* and *E(spl)* proteins during wing development

Two intracellular components of the Notch signaling pathway during neurogenesis are *Su(H)* and *E(spl)-C* (Muskavitch, 1994). The *Su(H)* product has been proposed as a transducer of the *Notch* signal, since it interacts with the cytoplasmic domain of Notch (Fortini and Artavanis-Tsakonas, 1994). We find that dorsal (7 clones), ventral (6 clones) and dorsoventral (3 clones) M^{+} clones of a *Su(H)* lethal allele that extend to the wing margin produce extensive scalloping (Fig. 3A-D), with the most severe phenotypes produced when clones comprise both dorsal and ventral cells. These scalloping phenotypes are essentially indistinguishable from those seen with *Notch* null alleles, suggesting that *Su(H)* is required for all aspects of *Notch* function at the D/V boundary.

In order to analyse the role of E(spl)-C proteins we have used a deficiency, $E(spl)^{RA7.1}$which removes 4 of the 7 bHLH genes in the complex (*m5*, *m3*, *m7* and *m8*) in addition to removing *groucho* (Schrons et al., 1992). When clones of $E(spl)^{RA7.1}$ mutant cells extend to the margin on the posterior portion of the wing, loss of margin tissue occurs (8 clones), although only to a modest extent compared to the scalloping caused by *Notch* clones (Fig. 3E,F). Clones extending to the margin in the anterior compartment also cause margin loss (6 clones); however, this is accompanied by overgrowth and pattern duplication phenotypes which reflect a function of *gro* that may be independent of E(spl)bHLH and *Notch* function (de Celis and Ruiz-Gomez, 1995). Thus the phenotypes of $Su(H)^{AR9}$ and $E(spl)^{RA7.1}$ clones are consistent with these genes functioning in the *Notch* pathway in its role at the D/V boundary, with the caveat that the phenotype of *E(spl)* clones is weak.

*E(spl)*bHLH proteins are expressed at the D/V boundary in the wing disc

The expression of E(spl)bHLH proteins provides a cellular assay for Notch activity during neurogenesis (Jennings et al., 1994). Therefore we have analyzed the distribution of these proteins to define the extent of Notch activation associated with the D/V boundary. Using an antibody (mAb323), which recognises at least 5 of the bHLH proteins encoded by the complex (Jennings et al., 1994), we find that E(spl)bHLH proteins are restricted to a row of cells along the D/V boundary (Fig. 4). This line of E(spl)bHLH expression, which is 1-3 cells wide, is detected from late in the second instar (data not shown) and persists throughout the third instar. This expression appears to follow the entire D/V compartment boundary (Fig. 4A-D) and is distinct from E(spl)bHLH expression associated with developing sensory elements at the wing margin (Jennings et al., 1995). To confirm that the cells expressing *E(spl)*bHLH proteins do correlate with the D/V boundary, we used the expression of *apterous* as a marker of dorsal cells (Blair et al., 1994). We find that *E(spl)*bHLH proteins are present in the cells immediately on each side of the boundary being detected in both dorsal (Apterous^{+}) and ventral (Apterous^{-}) cells (Fig. 4E-G).

Genes of the Notch pathway are required for D/V boundary expression of *E(spl)*bHLH proteins

Certain viable alleles of *Notch* lead to dramatic effects on wing morphogenesis. For example, nd^{3}/N^{55e11} flies have wings lacking much of the margin tissue (de Celis and Garcia-Bellido, 1994b). Consistent with *E(spl)* genes being a target of *Notch*, *E(spl)*bHLH protein expression is much reduced in wing discs from at least early third instar nd^{3}/N^{55e11} larvae (Fig. 5B) and expression associated with the D/V boundary and

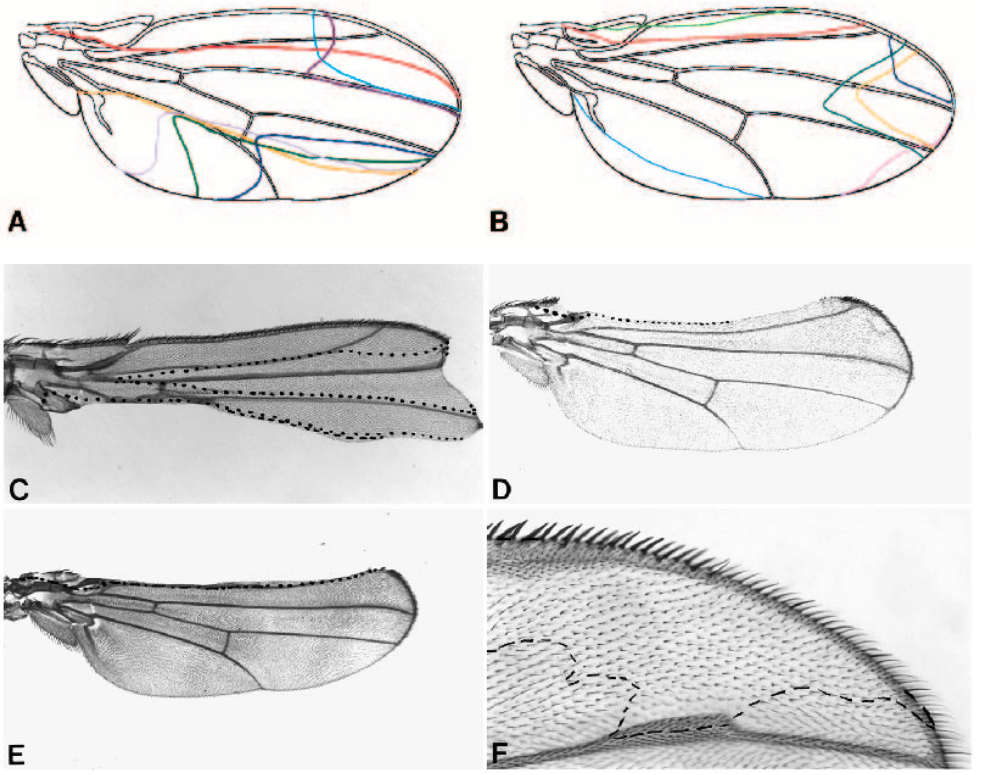

Fig. 1. Phenotype of *l(1)N*^{3} mosaics in the wing margin reveal different requirements for Notch dorsally and ventrally. (A,B) Diagram depicting scalloping caused by dorsoventral (A) and ventral (B) *l(1)N*^{3} *f*^{36a} clones induced at 48-72 hours AEL. Colored lines represent the scalloped wing edge produced by independent clones abutting the margin: the tissue between the colored line and the normal wing margin is lost. The mutant cells extending from the edge of the wing within the wing blade are not represented. (C-F) Examples of different *l(1)N*^{3} *f*^{36a} clones abutting the wing margin: (C) anterior ventral and posterior dorsoventral clone; (D) anterior ventral clone; (E) anterior dorsoventral clone and (F) dorsal clone. The extent of the mutant territories are drawn by dashed lines in dorsal and dotted lines in ventral clones. Note the modifications in the spacing of veins caused by the presence of *l(1)N*^{3} *f*^{36a} clones in the wing margin (C-E) and the differentiation of normal wing margin in a dorsal clone (F)

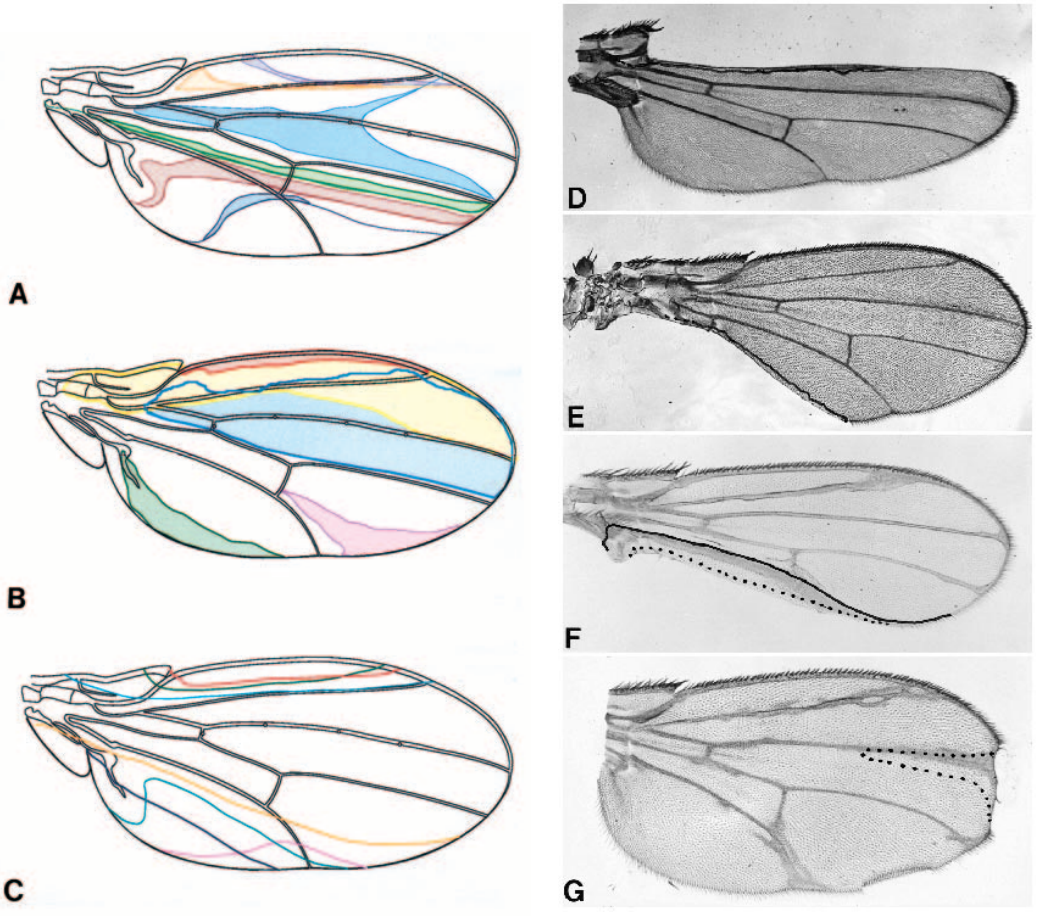

Fig. 2. Asymmetrical requirements for *Ser* and *Dl* at the D/V boundary. (A,B) Diagram depicting *Ser*$^{-}$ *M*$^{+}$ clones in the dorsal (A) or ventral (B) wing blade induced at 72-96 hours AEL. All clones are represented as colored domains bounded by solid lines. (A) Dorsal clones cause scalloped margins. The line closest to the wing margin represents the scalloped edge, the wing territory between this line and the normal margin is lost. (B) Ventral clones form a normal wing margin. (C) Plot of dorsoventral *Dl*^{M1} *M*$^{+}$ clones induced at 48-72 hours AEL with the scalloped edge of each mutant clone represented by a different colored line. (D,E) Examples of *Ser M*$^{+}$ dorsal clones induced at 48-72 hours AEL affecting the anterior (D) and posterior (E) wing margin. (F) Dorsoventral *Dl*^{M1} *M*$^{+}$ clone induced at 48-72 hours AEL (G) Ventral *Dl*^{M1} *M*$^{+}$ clone induced at 72-96 hours AEL Solid lines represent dorsal and dotted lines ventral internal borders of the clones.

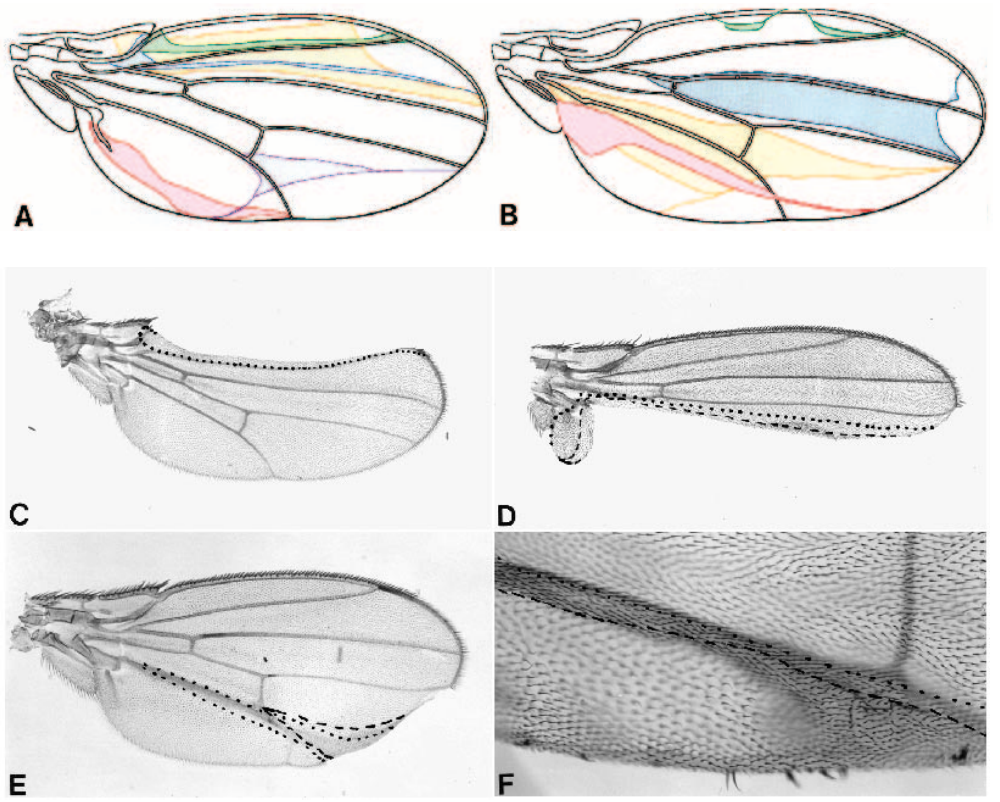

Fig. 3. *Su(H)* and *E(spl)* complex are involved in *Notch* signaling at the D/V boundary. (A,B) Plot of dorsal (A) and ventral (B) $Su(H)^{AR9}M^{+}$ clones induced at 72-96 hours AEL and abutting the wing margin. Colored areas surrounded by solid lines represent the extent of the mutant territories. The scalloped wing edge formed in these clones is shown by the colored lines that intersect the wing margin, the territory between the line and the wing margin is lost. (C,D) Examples of $Su(H)^{AR9}M^{+}$ clones abutting the wing margin in the ventral compartment (C) or spanning both dorsal and ventral compartments (D). (E,F) Examples of dorsoventral $E(spl)^{RA7.1}$ clones that reach the posterior wing margin, (F) Higher magnification picture of a dorsoventral $E(spl)^{RA7.1}$ clone in which the reduced size of the region between the LV vein and the scalloped posterior wing margin can be seen along with mutant posterior hairs. The borders of clones are marked as in Fig. 1.

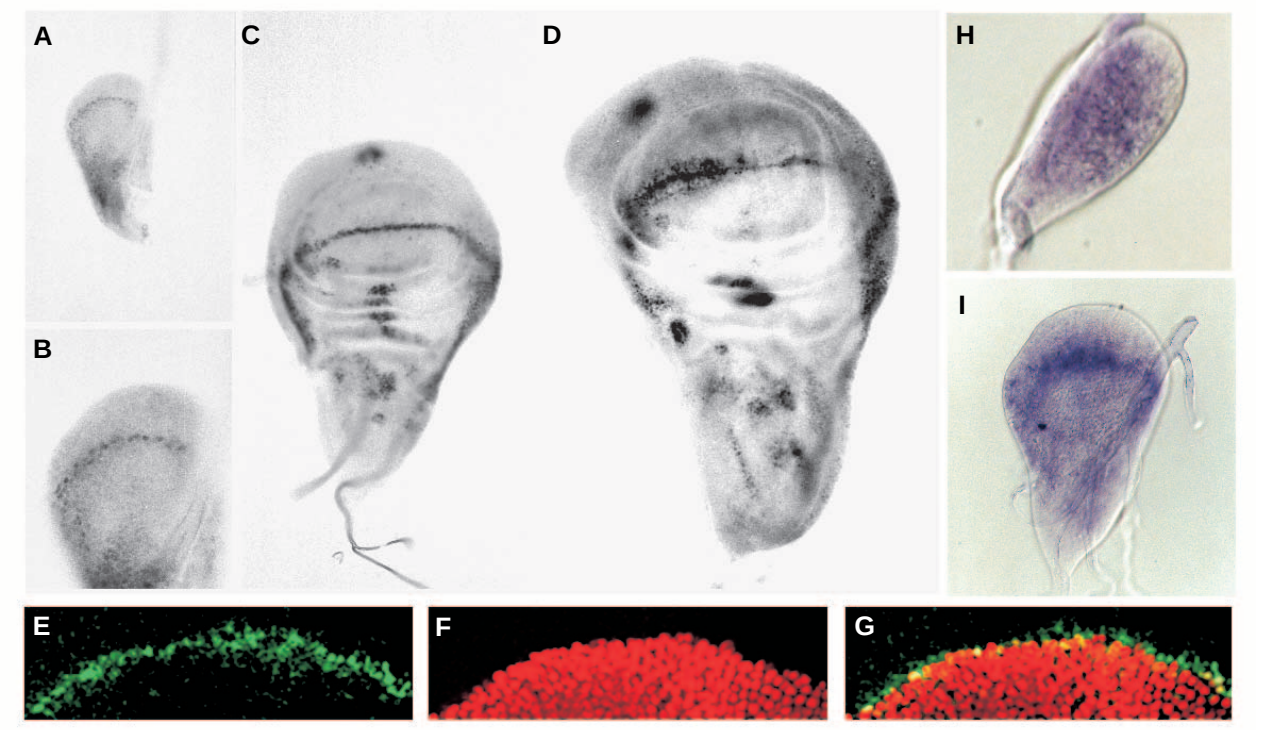

Fig. 4. E(spl)bHLH proteins are detected in two cells along the D/V boundary and Dl expression is up regulated at the D/V boundary. (A-D) Expression of E(spl)bHLH proteins was detected in wing imaginal discs of different ages using mAb323. (A,B) Early third instar, (C) mid third instar (D) late third instar. In all cases, mAb323 immunoreactivity is detected in a line of cells that extends along the edges of the notal region and across the wing pouch. All discs are at the same magnification except B which is 2× magnification of A. (E-G) Confocal images of the dorsal ventral boundary in the posterior region of a mid-third instar wing disc double labeled with mAb323 (green) and anti-β-galactosidase (red) antibodies in a *apterous-lacZ* line. In G, some nuclei contain both E(spl)bHLH and β-galactosidase (yellow) and others contain E(spl)bHLH only (green). (H,I) In situ hybridisation to detect *Dl* mRNA in wing discs of late second instar (H) and early third instar (I). Magnification in I is the same as in B and magnification in H is 2× that in I.

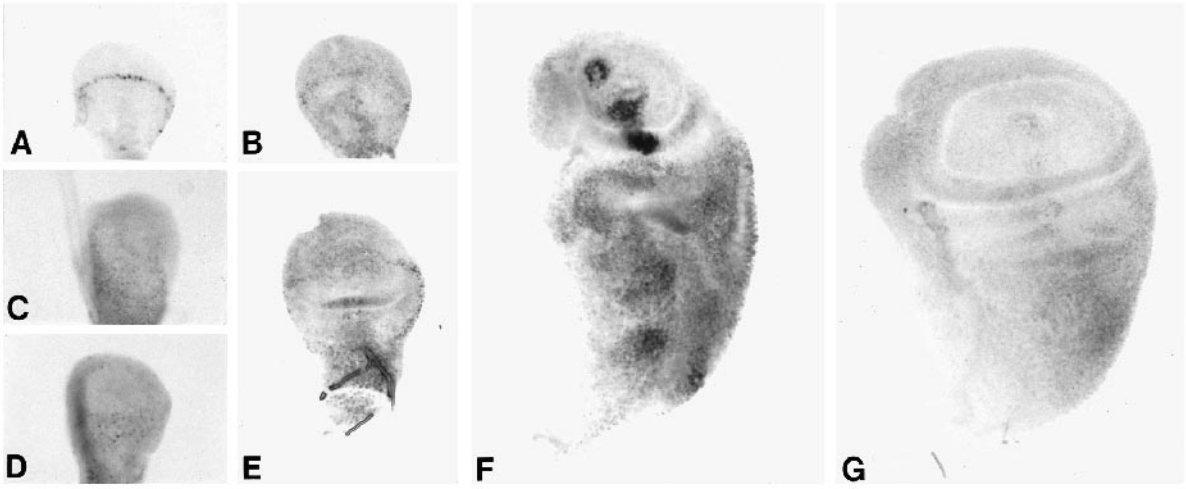

Fig. 5. *Notch*, *Ser*, *Dl* and *Su(H)* are all required for *E(spl)*bHLH expression at the D/V boundary. Wing discs from early (A-D), mid (E) and late (F,G) third instar larvae of different genotypes were stained to detect E(spl)bHLH proteins: (A) wild type, (B) nd^3/N^{55e11}, (C, F) $Ser^{RX106}/Ser^{rev2-11}$ (D,G)$Su(H)^1/Su(H)^{AR9}$ and (E) Dl^{RF}/Dl^{6B}. The latter were raised for 5 hours at the non-permissive temperature prior to fixation. In all cases, expression of *E(spl)*bHLH is decreased along the D/V boundary. In *Ser* mutant discs, E(spl)bHLH expression associated with peripheral nervous system development remains at high levels in late third instar discs (F).

the wing margin is almost completely absent in discs from older nd^3/N^{55e11} larvae (data not shown).

As clonal analyses implicate *Ser*, *Su(H)* and *Dl* in Notch signaling at the D/V boundary, mutations in these genes might be expected to have similar effects on E(spl)bHLH expression. Loss-of-function mutations in either *Ser* or *Su(H)* result in a reduction in the size of the wing disc (Speicher et al., 1994; Schweisguth and Posakony, 1992). *E(spl)*bHLH protein expression along the D/V boundary is absent from *Ser* [$Ser^{RX106}/Ser^{rev2-11}$] and *Su(H)* [$Su(H)^{AR9}/Su(H)^1$] mutant discs of all stages, including early third instar when the discs are still similar in size to wild type (Fig. 5C,D). In *Su(H)* mutant discs, the loss of *E(spl)*bHLH expression extends to other regions including positions where sensory organs develop (Fig. 5G). Conversely, in *Ser* mutant discs, strong expression associated with sensory precursor development is still detected (Fig. 5F) indicating that, in other places where *Notch* is functioning, *Ser* is not required in agreement with the normal differentiation of sensory structures in clones of *Ser* mutant cells.

In order to investigate the effects of *Dl* on *E(spl)*bHLH expression during wing development, we used a combination of temperature-sensitive alleles, Dl^{RF}/Dl^{6B}. After transferring third instar larvae to the non-permissive temperature, the levels of *E(spl)*bHLH proteins are reduced overall and along the D/V boundary the stripe of expression is incomplete (Fig. 5E). These effects on *E(spl)*bHLH proteins are detected in third instar discs after establishment of the D/V boundary, indicating that continuous *Dl* function is required to maintain Notch activity here. Consistent with this *Dl* requirement, in early third instar discs, we detect highest levels of *Dl* mRNA in cells around the D/V boundary (Fig. 4H,I).

Abruptex mutations lead to disc overgrowth, aberrant wing margin structure and ectopic expression of *E(spl)*bHLH proteins

Since mutations which cause a reduction in *Notch* activity at the D/V boundary result in scalloping and loss of E(spl)bHLH expression, mutations that increase *Notch* activity might be expected to have the converse effect. *Abruptex* alleles (*Ax*) of *Notch* have characteristics of *Notch* gain of function (Palka et al., 1990). *Ax* mutations result in complex phenotypes in the wing margin, consisting of absence of sensory organs in proximal regions and clusters of ectopic sensory elements in its distal regions. The latter occur in the proximity of the normal margin (Fig. 6B,C) and develop autonomously in mosaics (Fig. 6D). Many of the *Ax* allelic combinations also result in overgrowth of the wing disc (de Celis and Garcia-Bellido, 1994a).

We have examined *E(spl)*bHLH expression in a series of *Ax* alleles, ranging from weak homozygous viable (Ax^{28} and Ax^{16172}) to pupal lethal (Ax^{59d} and Ax^{M1}) and heteroallelic combinations (Ax^{16172}/Ax^{28}) which show synergism, i.e. produce a more severe phenotype than either allele alone. In almost all *Ax* allelic combinations tested, we detect ectopic expression of *E(spl)*bHLH proteins from early in the third instar. The extent of ectopic expression varies from low levels in weak alleles ($Ax^{28}$and Ax^{16172}, data not shown) to increasingly higher levels in more severe alleles and allelic combinations (Ax^{59d}, Ax^{M1}, Ax^{28}/Ax^{16172}, Fig. 6F-J). The domain of ectopic expression is more extensive in Ax^{M1} and Ax^{28}/Ax^{16172} than in Ax^{59d} and extends asymmetrically around the D/V boundary, with more ectopic expression associated with the dorsal compartment (Fig. 6H-J). The degree of *E(spl)*bHLH ectopic expression in *Ax* mutants correlates with the disc overgrowth defects. Furthermore, the effects of *Ax* mutations on growth are not associated with abnormal separation of dorsal and ventral compartments since the expression of *apterous*, detected using the *apterous-LacZ* line, remains restricted to the dorsal region of Ax^{28}/Ax^{16172}, Ax^{59d} and Ax^{M1} mutant discs (data not shown).

Consequences of altered *Notch* activity at the D/V boundary: expression of *cut* and *wingless*

The alterations in E(spl)bHLH expression caused by different

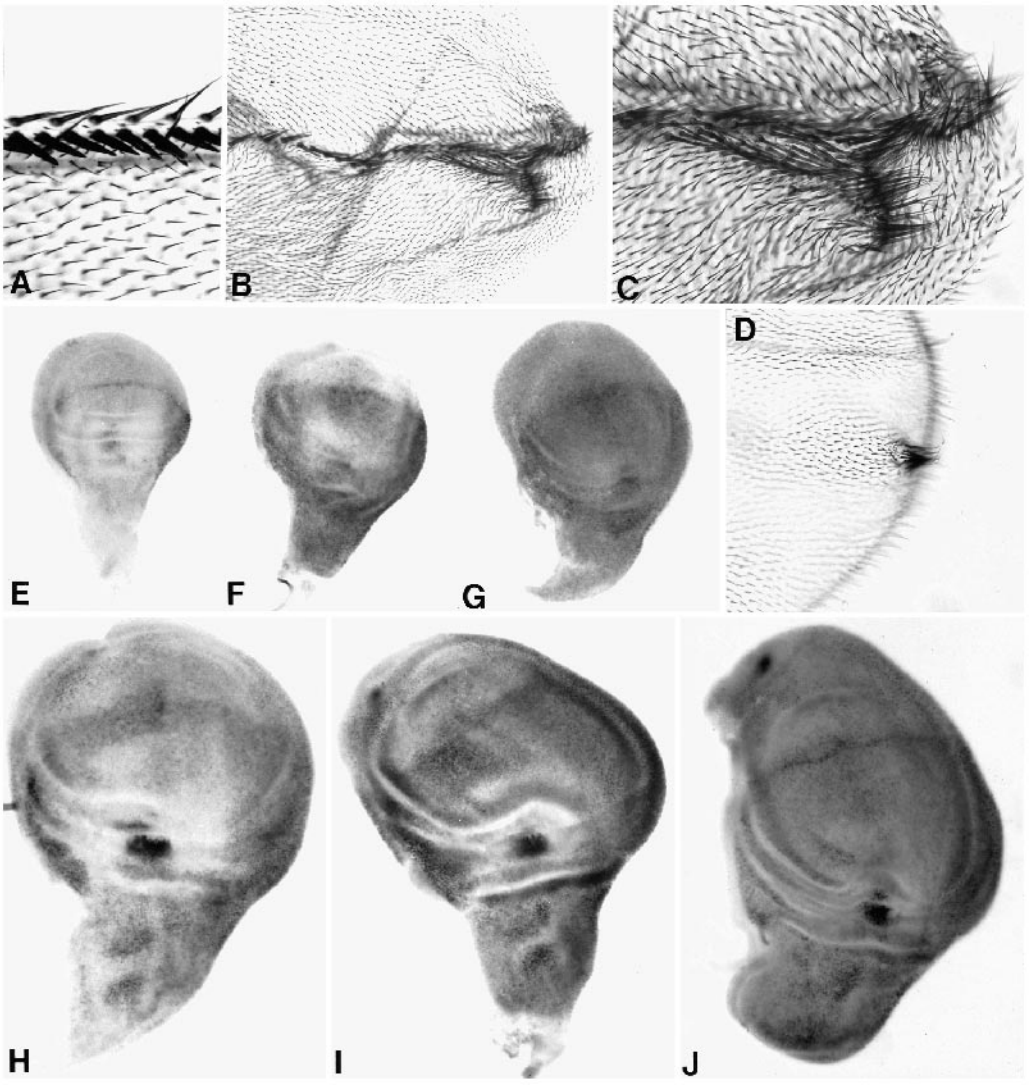

Fig. 6. *Abruptex* alleles of *Notch* lead to ectopic expression of *E(spl)*bHLH proteins and to the differentiation of ectopic wing margin elements. (A) High magnification of the wild-type anterior wing margin, showing the appearance of two rows of trichomes between the dorsal and ventral rows of sensory elements. Cell lineage analysis using *multiple wing hair* as a marker shows that one row belongs to the dorsal compartment and the other to ventral (data not shown). (B,C) Anterior wing margin of Ax^{28}/Ax^{16172} flies, showing the absence of sensory organs in proximal, and the appearance of ectopic sensory organs in distal margin regions, C is higher magnification of B. (D) Differentiation of ectopic sensory elements in an Ax^{M1} f^{36a} clone in the distal wing margin. (E-G) Expression of E(spl)bHLH proteins in wing discs from mid third instar larvae in wild type (E), Ax^{M1} (F) and Ax^{28}/Ax^{16172} (G). (H-J) Expression of E(spl)bHLH proteins in wing discs from later third instar larvae in Ax^{59d} (H), Ax^{M1} (I) and Ax^{28}/Ax^{16172} (J) wing discs. All discs are shown at the same magnification.

Notch alleles indicate abnormal behavior of cells at the D/V boundary. Several genes including *cut* (*ct*) and *wingless* (*wg*) are expressed at the D/V boundary in third instar imaginal discs (Baker, 1988a; Blochinger et al., 1993; Phillips and Whittle, 1993; Couso et al., 1994). We therefore examined the expression of these genes in different *Notch* mutant backgrounds. Modifications in both *wg* and *ct* expression occur in mutant discs that correlate well with the level of *Notch* activity detected by *E(spl)*bHLH expression. In late third instar N^{55e11}/nd^{3} discs, *ct* and *wg* expression is lost in distal and posterior regions of the D/V boundary (Fig. 7B,E). Conversely, in *Ax* mutant discs (Ax^{28}/Ax^{16172}) at the same stage, cells expressing *ct* and *wg* expand in both dorsal and ventral regions around the D/V boundary (Fig. 7C,F). Ectopic expression of both *ct* and *wg* can be detected soon after they appear at the D/V boundary and continues to expand during later stages (Fig. 7H,L and data not shown). This expansion is most dramatic in the dorsal compartment extending as many as 20-30 cells away from the boundary in later third instar discs (Fig. 7I,M). Similar miss-expression has been detected in other *Ax* alleles, e.g. Ax^{M1}(Fig. 7J) indicating that this is a general effect of the aberrant Notch activation in *Ax* mutations.

DISCUSSION

The analysis of Notch function in a wide variety of different developmental processes has led to the hypothesis that activated Notch prevents cells from responding to different cell-fate-promoting signals (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Here we find that Notch is locally activated in the cells at the D/V boundary where it is needed for these cells to co-ordinate wing growth and margin formation. The activity of Notch in maintaining a contiguous stripe of cells at the juxtaposition of the dorsal and ventral compartments does not relate simply to processes where Notch restricts the number of cells that follow a particular developmental pathway, as occurs during neurogenesis. Furthermore, in the cells at the D/V boundary, there is no indication of particular cell-fate promoting signals being antagonised by Notch. In spite of these apparent differences between Notch function in neurogenesis and at the D/V boundary, we find that many of the components of *Notch* action are common to both processes. Thus the phenotype in mosaics of *Dl*, *Su(H)* and *E(spl)-C* are compatible with these genes being required for Notch signaling at the D/V boundary suggesting that the *Notch*

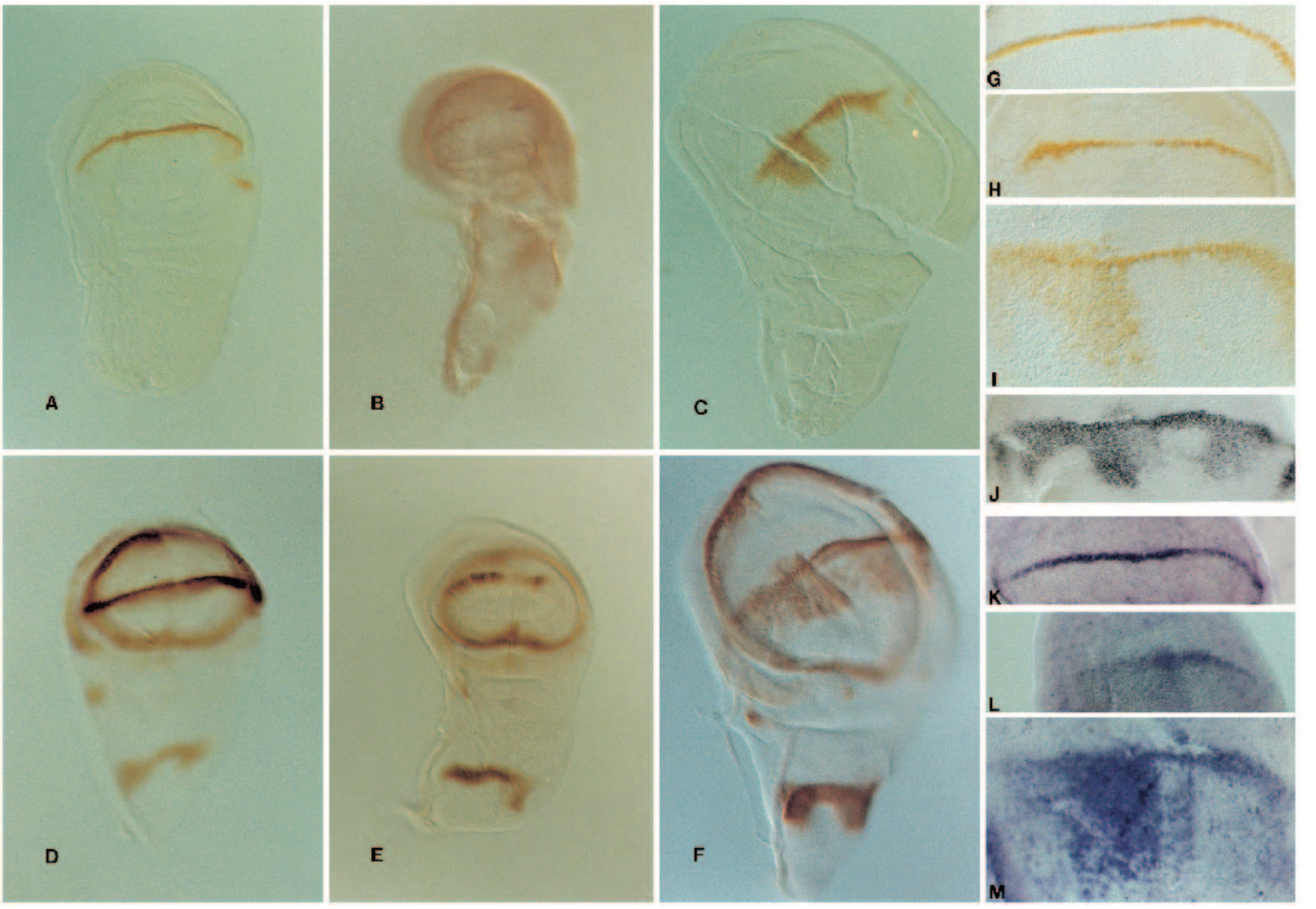

Fig. 7. Expression of *cut* and *wingless* is affected by levels of Notch activity. (A-C) β-galactosidase expression from the *ct-lacZ* line in late third instar wing discs. β-galactosidase expression is restricted to dorsal and ventral cells at the D/V boundary in wild-type discs (A) but is absent or ectopically expressed in N^{55e11}/nd^{3} (B) and Ax^{28}/Ax^{16172} (C) discs, respectively. (D-F) β-galactosidase expression from a *wg-lacZ* reporter gene in wild-type (D), N^{55e11}/nd^{3} (E) and Ax^{28}/Ax^{16172} (F) discs. Expression of *wg-lacZ* is reduced or absent in N^{55e11}/nd^{3} (B) and spreads dorsally and ventraly in Ax^{28}/Ax^{16172} discs. (G-J) *ct* expression pattern detected with an anti-cut antibody in high magnification pictures of wild-type (G) Ax^{28}/Ax^{16172} (H,I) and Ax^{M1} (J) discs of different ages from mid (H) and later (I,J) third instar larvae. Notice that soon after the *ct* stripe is formed, dorsal and ventral cells around the normal stripe ectopically express the *ct* gene. (K-M) *wg* expression detected by in situ hybridisation in high magnification pictures of wild-type (K) and Ax^{28}/Ax^{16172} (L,M) discs at different ages, mid (L) and late (M) third instar. In Ax^{28}/Ax^{1617} discs, *wg* expression expands beyond its normal domain into the dorsal and ventral compartments soon after it is established.

pathway functions as a cassette deployed in different cell fate decisions.

Mechanisms of Notch activation at the D/V boundary

Notch activation at the D/V boundary, as visualized by the pattern of expression of E(spl) proteins, is restricted to the cells immediately either side of the boundary and requires the coordinate activities of two ligands, Ser and Dl. Clonal analysis of *Ser* null alleles shows that Ser is only required in the dorsal cells, where it is presumably involved in interactions with Notch in adjacent ventral cells (Fig. 8A). The lack of similar Notch activation throughout the dorsal compartment, where *Ser* is expressed (Thomas et al., 1995), implies that these cells are unable to respond to the presence of Ser. Two observations suggest that high levels of Ser itself can prevent Notch activation. Ectopic expression of Ser across the D/V boundary causes a loss of margin and adjacent tissue (Thomas et al., 1995), indicating that Ser can suppress Notch activity even in cells that are normally responsive. In the ventral compartment, ectopic *Ser* expression induces the activation of *wg* and *vg*, but only in cells that contain low or no Ser protein themselves (Kim et al., 1995). This negative interaction between Ser and Notch is unlikely to be the only factor preventing Notch activation through the dorsal compartment; novel Ser^{+}/Ser^{-} interfaces generated by removal of *Ser* in dorsal clones do not form margin tissue, unlike similar mosaics with $apterous^{-}$ or $fringe^{-}$ cells (Diaz-Benjumea and Cohen, 1993; Irvine and Wieschaus, 1994). Thus it is probable that the restriction of Notch activation to cells at the D/V boundary also requires other functions activated by *apterous* in the dorsal compartment, for example the expression of the secreted protein encoded by *fringe* (Irvine and Wieschaus, 1994). Such a mechanism is similar to that operative in restricting Hedgehog signaling to anterior cells abutting the anteroposterior compartment boundary. Hedgehog is secreted in all posterior cells but only anterior cells are able to respond, the posterior cells are unable to do so as a conse-

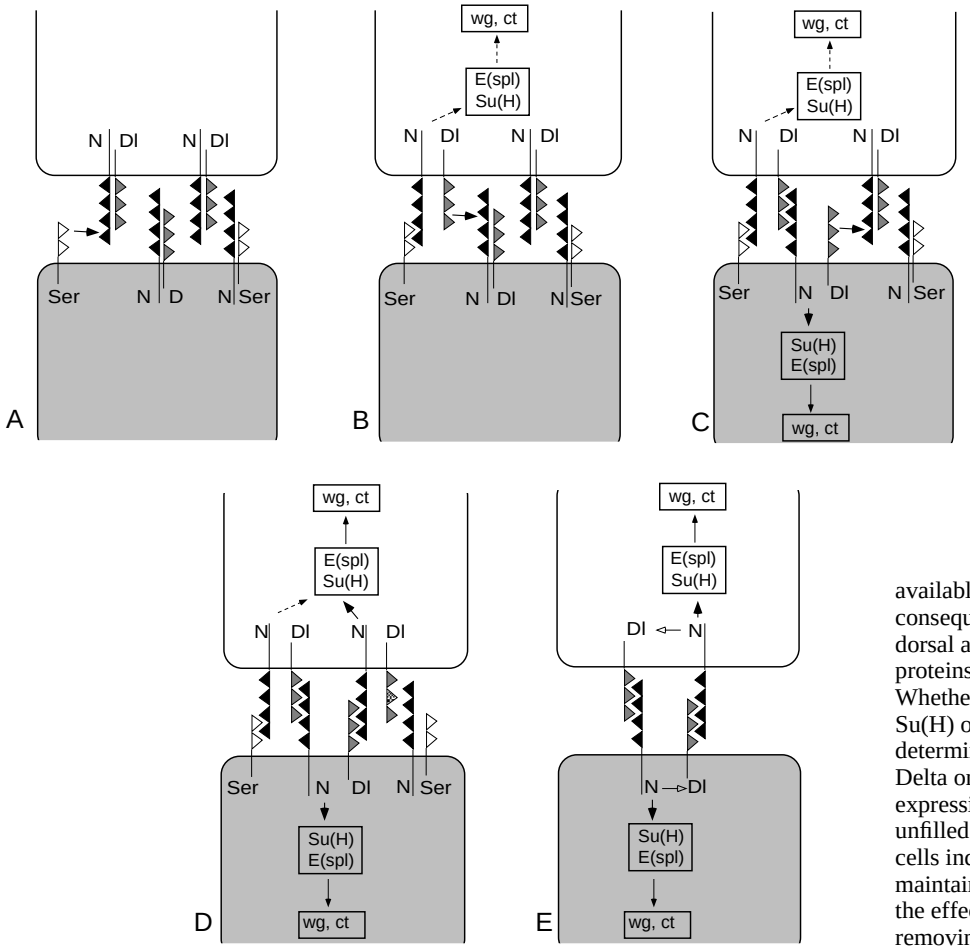

Fig. 8. Mechanisms and consequences of Notch signaling at the D/V boundary. Each panel depicts a ventral and a dorsal cell at the D/V boundary, dorsal cells are shaded to represent Apterous expression. (A) Signaling is initiated by Ser expressed in dorsal cells. (B) As a consequence of Ser interacting with Notch (N), Dl becomes available to interact with Notch on the adjacent cell. We do not know whether Ser interactions with Notch also activate Su(H) and expression of E(spl)bHLH proteins, this possibility is indicated by dashed arrows. (C) The interaction between Dl on ventral cells and Notch in dorsal cells leads to activation of Su(H) and expression of E(spl)bHLH and makes Dl available to signal back to ventral cells. (D) As a consequence of Dl-mediated Notch activation, dorsal and ventral cells express E(spl)bHLH proteins and are competent to express *wg* and *ct*. Whether expression of *wg* or *ct* directly involves Su(H) or E(spl)bHLH proteins remains to be determined. (E) Interactions between Notch and Delta on dorsal and ventral cells maintain expression of E(spl)bHLH and of *wg* and *ct*. The unfilled arrow linking Notch and Delta inside the cells indicates that Notch activation is needed to maintain the capability of signalling, as implied by the effects on both wing surfaces caused by clones removing Notch function from only one surface.

quence of expressing *engrailed* (Tabata and Kornberg, 1994; Sanicola et al., 1995).

The absence of E(spl)bHLH proteins from both dorsal and ventral cells in *Ser* mutant discs indicates that Notch activation in dorsal cells occurs as a secondary consequence of the action of Ser. In addition to Ser, Dl is also involved in Notch activation at the D/V boundary, as shown by the scalloping phenotypes of *Dl* mutant clones that include both dorsal and ventral cells or only ventral cells and by the observation that *Dl* is required to maintain E(spl)bHLH expression during the third instar. Thus, the confrontation between *Ser*-expressing and non-expressing cells at the D/V boundary could have several consequences. First, the presence of Ser could participate in preventing Notch activation in dorsal cells. Second, it could trigger Dl signaling, resulting in Notch activation in dorsal as well as ventral cells abutting the D/V interface (Fig. 8). In addition, the interaction of Ser with Notch in ventral cells could result in direct Notch activation although it is not clear that Ser protein has the capability to activate Notch (Fortini and Artavanis-Tsakonas, 1994) and it is possible that its role is primarily to trigger Dl signalling. Ultimately, it appears that the localized Notch activation depends critically on the relative levels of Ser, Dl and Notch, as genetic combinations where the doses of these genes are altered reveal antagonistic interactions (de la Concha et al., 1988). We have tried to integrate these antagonistic interactions as well as those that result in Notch acivation into the model presented in Fig. 8.

The interaction of Notch in ventral cells with both Ser and Dl is consistent with our finding that certain *Notch* hypomorphic alleles uncover a specific *Notch* requirement in ventral cells. Thus, either these mutant molecules are deficient in the interaction with Ser or the interaction with both ligands necessitates higher concentrations of Notch in ventral cells. Furthermore, the fact that particular Notch alleles and Ser show a restricted requirement in one compartment but affect the development of the D/V boundary as a whole, indicates that normal Notch function in one compartment is essential for the maintenance of Notch activity in the opposite one. This could occur if the potential for signalling to neighbouring cells is linked to Notch activity (Fig. 8). Such a positive feedback mechanism may be important in situations where high levels of Notch activity are maintained within neighbouring cells. This would contrast with sensory mother cell development, where differences in levels of Notch function between neighbouring cells are critical and where Notch activity and Dl signalling appear to be inversely related (Heizler and Simpson, 1991). The localised Notch activity at the D/V boundary is accompanied by an increase in the levels of *Dl* mRNA in this region and ultimately by the accumulation of Notch protein in the receiving dorsal and ventral cells (Muskavitch, 1994). The concentration of ligand and receptor at the D/V boundary may

participate in the maintenance of Notch signalling within this domain.

Alterations in Notch activation caused by *Ax* mutations

The phenotype of *Ax* mutations suggest that these Notch mutant proteins have increased levels of activity (Palka et al., 1990). Here we find that *Ax* mutations cause Notch activation outside the normal domains, as indicated by the ectopic expression of *E(spl)*bHLH proteins, rather than further increasing the amount of Notch activity at its normal sites. Even though the ectopic Notch activation in *Ax* mutants is particularly dramatic along the D/V boundary, it also occurs in other regions of the disc, consistent with the effects of *Ax* mutations on the development of veins and bristles (de Celis and Garcia-Bellido, 1994a). The observation that point mutations in the Notch protein (Kelley et al., 1987) are sufficient to interfere with the mechanism limiting its activation to particular domains, indicates that Notch itself actively participates in this process and that Ax proteins are deficient in a function that prevents Notch activation, possibly through interactions with other proteins. These proteins could either be repressors that modulate Notch response to ligands or factors required for the inactivation of an activated Notch molecule (down-regulation).

Roles of Notch in growth and morphogenesis of the wing

Removal of *Notch* activity at the D/V boundary causes extensive scalloping along the wing margin, associated with modifications to the spacing between veins. Conversely, *Ax* mutations resulting in ectopic *Notch* activity cause overgrowth of the disc and the differentiation of ectopic margin sensory elements. Thus, high levels of Notch at the D/V boundary appear to maintain it as a reference boundary that coordinate the growth of the wing first and the differentiation of the wing margin later. The effects of *Notch* at the D/V boundary are downstream of *apterous* since expression of *apterous* is normal in *Ax* mutant discs. In addition, mutant *Notch* clones respect the D/V lineage restriction, indicating that *Notch* is not required for the clonal segregation between dorsal and ventral cells.

The overall significance of the D/V boundary in wing disc growth is evident from the failure of the wing to form when the boundary is eliminated in *apterous* mutant discs (Williams et al., 1993). The confrontation of *apterous*-expressing and non-expressing cells triggers the increased expression of various genes, such as *wg*, *vg* and *sd* in the D/V boundary in the transition between second and third larval instars (Williams et al., 1993). The localised expression of these genes may involve Notch activity since this is also the time when *Notch* function is required at the D/V boundary, based on the thermosensitive period of N^{ts1} (Shellenbarger and Mohler, 1978) and the expression of *E(spl)*bHLH proteins. Consistent with this, we find that *Notch* alleles with reduced function precipitate a decrease in *wg* expression and conversely *Ax* alleles, which have ectopic *Notch* activity, result in an expansion of *wg* expression. These observations place *wg* downstream of *Notch* in these cells and suggest that some effects of *Notch* mutations on wing morphogenesis are mediated by miss-expression of genes normally restricted to the D/V boundary. This interpretation is compatible with the consequences of ectopic *Ser* expression in ventral cells, namely ectopic activation of *wg* and overgrowth of the ventral surface of the disc (Speicher et al., 1994; Kim et al., 1995).

Although the cellular consequences triggered by Notch signaling at the D/V boundary are not fully understood, it is clear that *Notch* mediates the maintenance of a particular state critical for the co-ordination of growth in the wing. Activation of Notch at this boundary involves the localisation of two ligands, one to the dorsal (Ser) and the other to dorsal and ventral sides of the boundary (Dl). It is possible that the requirement for Notch in other developmental processes involves the establishment/maintenance of boundaries. For example the lack of *Notch1* during mouse development results in somite defects (Conlon et al., 1995) which could reflect a role of *Notch* at the boundary between segments.

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