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Notch signalling regulates *veinlet* expression and establishes boundaries between veins and interveins in the *Drosophila* wing

Jose F. de Celis[1,*], Sarah Bray[2] and Antonio Garcia-Bellido[3]

[1]Department of Genetics and [2]Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 4TH, UK
[3]Centro de Biologia Molecular, Universidad Autonoma de Madrid, Cantoblanco, Madrid 28049, Spain

*Author for correspondence (e-mail: jdc@mole.bio.cam.ac.uk)

SUMMARY

The veins in the *Drosophila* wing have a characteristic width, which is regulated by the activity of the Notch pathway. The expression of the Notch-ligand Delta is restricted to the developing veins, and coincides with places where Notch transcription is lower. We find that this asymmetrical distribution of ligand and receptor leads to activation of Notch on both sides of each vein within a territory of Delta-expressing cells, and to the establishment of boundary cells that separate the vein from adjacent interveins. In these cells, the expression of the *Enhancer of split* gene *mβ* is activated and the transcription of the vein-promoting gene *veinlet* is repressed, thus restricting vein differentiation. We propose that the establishment of vein thickness utilises a combination of mechanisms that include: (1) independent regulation of *Notch* and *Delta* expression in intervein and vein territories, (2) Notch activation by Delta in cells where Notch and Delta expression overlaps, (3) positive feedback on *Notch* transcription in cells where Notch has been activated and (4) repression of *veinlet* transcription by E(spl)mβ and maintenance of *Delta* expression by veinlet/torpedo activity.

Key words: Notch pathway, vein differentiation, cell interaction, *Drosophila*, *veinlet*, intervein

INTRODUCTION

The wings of Pterigota insects have characteristic structures, the veins, that differentiate in precise two-dimensional patterns (Snodgrass, 1935). In *Drosophila* there are four longitudinal veins formed by proximo-distal stripes of cells that appear more compact and have higher pigmentation than intervein cells. Although veins are classified as dorsal or ventral, depending on the wing surface where they protrude, every vein has both dorsal and ventral components that are specified independently during imaginal development. These come into contact after the disc evaginates and the dorsal and ventral wing surfaces become apposed, when interactions between dorsal and ventral vein territories may contribute to the final differentiation of the vein (Garcia-Bellido, 1977).

The position of the veins is pre-figured in the third instar wing disc by the localised expression of *veinlet* (*ve*) in four dorso-ventral stripes of cells (Sturtevant et al., 1993). *ve* encodes a membrane protein with seven trans-membrane domains (Bier et al., 1990), which facilitates signalling via Torpedo (Top), the *Drosophila* epidermal growth factor receptor homologue (Sturtevant et al., 1993; Price et al., 1989). High levels of Top signalling occur in places of *ve* expression, and this activity appears to direct wing cells into a vein differentiation pathway (Diaz-Benjumea and Garcia-Bellido, 1990a). Vein differentiation continues during pupal development and involves the restriction of specific gene products and cell adhesion proteins to veins or intervein territories (Fristrom et al., 1993; Montagne et al., 1996).

A major element involved in establishing the correct width of both dorsal and ventral vein components is the membrane receptor Notch. Loss-of-function alleles of *Notch* are characterised by vein-thickening phenotypes, whereas *Notch* gain-of-function alleles cause thinner and incomplete veins, both in *Drosophila* and in other species (de Celis and Garcia-Bellido, 1994; Davies et al., 1996). The establishment of vein thickness may be analogous to 'lateral inhibition' in proneural clusters (Artavanis-Tsakonas and Simpson, 1991), since it seems to involve the restriction of cell fate to a limited subset of the cells that have the competence to enter into a particular cell differentiation pathway. However, during vein formation several neighbouring cells acquire the same differentiation state, whereas in proneural clusters only single cells are able to follow a neural fate. The bases of these different outcomes of Notch signalling, namely single cells in proneural clusters and stripes of cells in vein territories, are relevant to understanding the mechanisms that confer versatility to Notch function during development.

The activation of Notch during vein differentiation depends on interactions with Delta (Dl), a transmembrane protein with extracellular EGF-repeats (Kopczynski et al., 1988; Vassin et al., 1987). Analysis of the phenotypes produced by temperature-sensitive alleles of *Notch* and *Delta* indicates that they are required in vein differentiation from the end of larval development until at least 24 hours after puparium formation (hAPF) (Shellenbarger and Mohler, 1978; Parody and Muskavitch, 1993). Thus Notch activity is likely to participate during both

the establishment of veins in the imaginal disc and the maintenance of vein territories throughout pupal development. Intracellular components of Notch signalling include Suppressor of Hairless (Su(H)) and the basic helix-loop-helix (bHLH) proteins encoded by the *Enhancer of split* complex (*E(spl)*) (Knust et al., 1992; Delidakis and Artavanis-Tsakonas, 1992; Schweisguth and Posakony, 1992; Fortini and Artavanis-Tsakonas, 1994). The expression of *E(spl)* genes depends on Notch activity, and involves direct transcriptional activation by Su(H) (Jennings et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995). In the wing blade, one of the seven bHLH proteins of the E(spl) complex (E(spl)mβ) is expressed in a pattern related to vein development, suggesting that it could be involved in mediating the effects of Notch on vein differentiation (de Celis et al., 1996a).

We have studied the expression and function of different components of the Notch pathway in both the wing disc and pupal wing to analyse the mechanism of Notch activation in vein territories. We show that the transcription of *Notch* is higher in broad domains that correspond to the interveins, whereas *Dl* expression is restricted to vein territories. The asymmetry in the distribution of Notch and Delta leads to Notch activation in vein/intervein boundaries and to the separation of vein from interveins. We find that the activities of Notch and the vein-promoting gene *ve* are linked: Ve is required to activate *Dl* expression, and Notch effectively represses *ve* transcription, therefore restricting *ve* to the domain where Notch is not activated. In boundary cells within the domain of *Dl* expression Notch activation results in the accumulation of *E(spl)mβ* mRNA and in an increase in the levels of Notch itself, therefore maintaining the polarity of signalling and the separation of veins from interveins during pupal development.

MATERIALS AND METHODS

Drosophila strains

The following alleles were used: at the *Notch* locus, the null allele N^{55e11} (Kidd et al., 1983), the loss-of-function alleles fa^{nd} and N^{MLz} (a novel *Notch* loss-of-function allele generated by the insertion of a PlacZ element in *Notch*, data not shown) and the gain-of-function alleles Ax^{28}, Ax^{59b} and Ax^{16172} (Kelley et al., 1987); at the *Dl* locus, the lethal alleles Dl^{M1}, Dl^{M2} and Dl^{M3} (de Celis et al., 1991) and a *Dl* LacZ line Dl^{P1171}; at the *Su(H)* locus, the lethal allele $Su(H)^{AR9}$ (Schweisguth and Posakony, 1994); at *Hairless*, the lethal allele H^2 (Bang and Posakony, 1992) and at the *E(spl)* locus, the deficiency $E(spl)^{b32.2}$ that deletes the seven *E(spl)* bHLH genes (Schrons et al., 1992). As a vein-specific marker we used a *Star*-lacZ line (Heberlein et al., 1993). To analyse the consequences of the ectopic expression of E(spl)mβ protein we used the Gal4 system (Brand and Perrimon, 1993). The UAS line UAS-E(spl)-Mβ (de Celis et al., 1996a) was combined with the GAL4 lines GAL4-179 (a gift from A. Brand) and GAL4-sal (Thomas et al., 1995). The UAS-ve was generated by cloning the coding sequence of *ve* into PUAST (Brand and Perrimon, 1993). The expression patterns of these lines were characterised in third instar disc and in pupal wings by combining each GAL4 line with the reporter UAS line UAS-IMPT (Sweeney et al., 1995).

The expression patterns of *Notch*, *Delta* and *E(spl)mβ* were analysed in mutant discs or pupal wings of the following genotypes: a combination of the viable *veinlet* allele ve^1 (Sturtevant et al., 1993) and the viable *vein* allele vn^1 (Simcox et al., 1996), which results in the elimination of all longitudinal veins (*ve vn*; Diaz-Benjumea and Garcia-Bellido, 1990b); in the transgenic line rho^{30}, which results in ectopic *ve* expression (Noll et al., 1994), and in the *Notch* alleles fa^{nd} and Ax^{16172}. The phenotypic analysis of genetic combinations was carried out in flies raised at 25°C, unless otherwise stated. Wings were mounted in lactic acid/ethanol (1:1) and photographed using a Zeiss axiophot microscope.

Mosaic analysis

Clones were generated by X-ray-induced mitotic recombination. Larvae were irradiated (dose 1000R; 300 R/minute, 100 Kv, 15 mA, 2 mm aluminium filter) 48-72 hours after egg laying. $Minute^+$ clones were scored in males of the following genotypes f^{36a}; $Su(H)^{AR9}/M(2)Z$ $P[f^+]30B$, f^{36a}; $Dl^{M1}M(3)w$ $P[f^+]87$, f^{36a}; Dl^{M3} $M(3)w$ $P[f^+]87$, f^{36a}; $H^2/M(3)w$ $P[f^+]87$ and f^{36a}; $E(spl)^{b32.2}/M(3)w$ $P[f^+]87$. Mitotic recombination proximal to the f^+ insertion produces homozygous mutant cells labelled with the cell marker *forked* (*f*). Clones in Dl^+ background were induced in flies *bld* Dl^{M1}/ *Dp(3;3)bxd110*, Dl^+ and *bld* Dl^{M2}/*Dp(3;3)bxd110*, Dl^+. Clones in *ve vn* background were induced in flies f^{36a}; ve^1 vn^1 Dl^{M1}/ve^1 vn^1 $P[f^+]87F$ and f^{36a}; ve^1 vn^1 $E(spl)^{b32.2}/ve^1$ vn^1 $P[f^+]87F$.

In situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation with digoxigenin-labelled DNA probes in imaginal discs were performed as described previously for both imaginal discs (Cubas et al., 1991) and pupal wings (Sturtevant et al., 1993). In situ hybridisation with digoxigenin-RNA-labelled probes was carried out using the same protocols, but the hybridisation step and washes were at 55°C. The following DNA probes were used: 3 kb *Eco*RI *Dl* cDNA clone (Vassin et al., 1987), 0.7 kb *Hin*dIII/(*Not*I) fragment from c-*mβ*-14a (Delidakis and Artavanis-Tsakonas, 1992), 3 kb *Bgl*II/(*Kpn*I) from a *Notch* cDNA clone (Kidd et al., 1983) and a *ve* RNA probe synthesised from a *ve* cDNA clone (Sturtevant et al., 1993). For *Dl* and *mβ* similar antisense mRNA probes were also synthesised for some experiments.

Immunocytochemistry with the mAb323 antibody to detect E(spl)bHLH expression was performed as described in Jennings et al. (1994). We also used rabbit anti-β-galactosidase (Cappel), mouse monoclonal anti-Notch (Fehon et al., 1991), mouse anti-IMP (Sweeney et al., 1995) and rat-anti Ventral veinless (a gift from J. Casanova and M. Llimargas) antibodies. Secondary antibodies were from Jackson Immunological Laboratories (used at 1/250).

RESULTS

Role of *Notch*-related genes in vein differentiation

Loss-of-function alleles of *Notch* and *Dl* always result in the differentiation of thicker veins, indicating that Notch function is essential for the vein components to acquire their appropriate width. To determine which other elements of the Notch pathway are required to regulate vein differentiation we have analysed the phenotypes produced by lethal alleles of *Dl*, *E(spl)*, *Su(H)* and *H* in clones. We find that lethal alleles of these genes cause vein thickening (*Dl*, *E(spl)* and *Su(H)*) or vein loss (*H*) similar to that observed with *Notch* loss- and gain-of-function alleles respectively. Thus, *Dl*, *E(spl)* and *Su(H)* mutant cells differentiate thicker veins when they appear in vein regions, but they never produce ectopic veins when restricted to intervein territories (Fig. 1A-F,H). The strongest vein-thickening phenotypes are observed when mutant clones extend into two adjacent interveins. Conversely, clones of *H* lethal alleles cause strong phenotypes of vein loss when the clones cover both dorsal and ventral vein component (not shown), and weaker vein-loss phenotypes

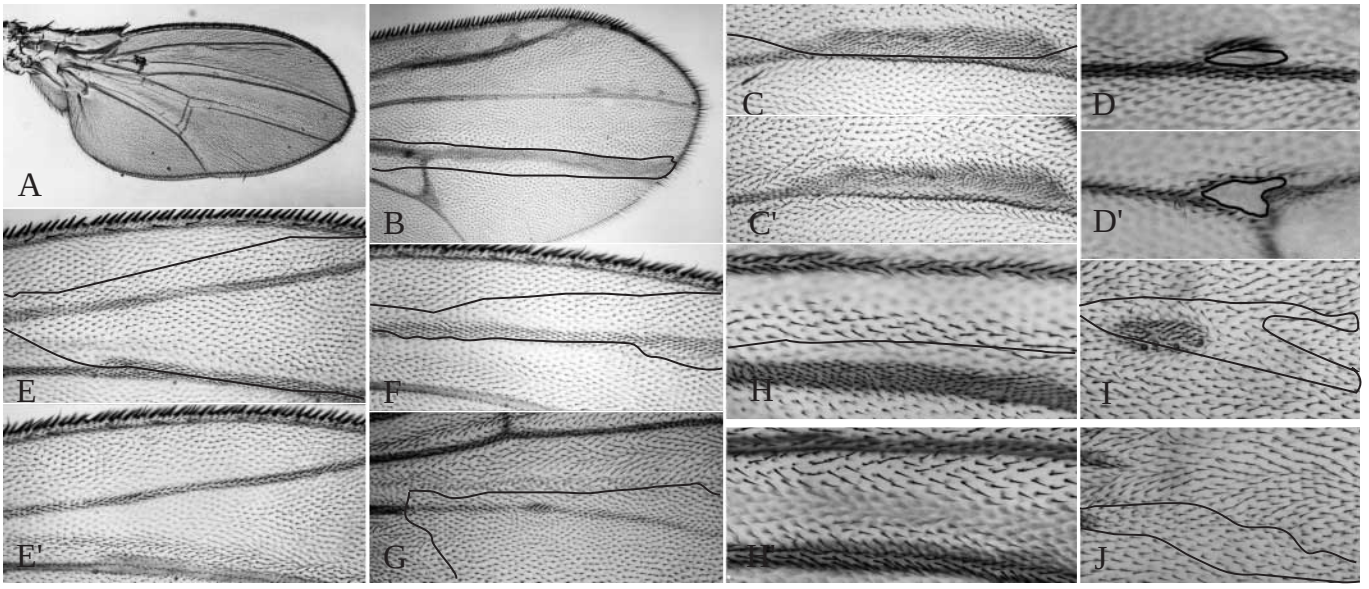

Fig. 1. Examples of vein differentiation defects caused by mutations in Notch pathway genes. (A) Wild-type wing in which dorsal and ventral wing surfaces are not fully apposed, so that dorsal and ventral components of every vein can be distinguished. (B) Large Dl^{M3} clone in the dorsal wing surface covering the position of LIV differentiates a thicker LIV vein. (C-C′) Dorsal Dl^{M3} clone between the veins LII and LIII, which differentiates a thicker dorsal LIII (C), and induces extra-vein differentiation in the ventral component of LIII (C′). (D-D′) Two examples of *bld* Dl^{M1} clones in the dorsal LIII (D) and ventral LIV (D′), which induce vein differentiation in the adjacent one or two wild-type cells. *Dl* mutant cells also differentiate as vein cells in these clones, but due to the cell marker used are not clearly visible in these pictures. (E-E′) Dorsal $Su(H)^{AR9}$ clone encompassing the LII and LIII veins, which causes an autonomous thickening of dorsal LIII (E) but does not affect vein differentiation (LII and LIII) in the ventral surface (E′). (F) Ventral $Su(H)^{AR9}$ clone covering LII, which results in the differentiation of a thicker LII. (G) H^2 clone that reduces vein differentiation in dorsal LV. (H-H′) Ventral $E(spl)^{b32.2}$ clone that causes thickening of the ventral component of LIII (H) but does not affect vein differentiation in the opposite surface (H′). (I) Dorsal Dl^{M1} clone induced in *ve vn* mutant background. Mutant cells differentiate extra-sensilla associated with the remnants of the vein, but most cells in the clone differentiate as intervein cells. (J) Dorsal $E(spl)^{b32.2}$ clone induced in *ve vn* mutant background, which does not differentiate as vein although it covers the territory of LV. Black lines mark the boundaries of mutant clones.

when mutant cells are restricted to only one vein surface (Fig. 1G). These observations indicate that modifications in the activity of the Notch pathway can only alter the differentiation of cells that already have the capability to form veins, as it is the case for *Notch* mutations (de Celis and Garcia-Bellido, 1994). The thicker veins that differentiate in *Su(H)* and *E(spl)* mutant clones are formed by mutant cells that lie within the normal vein region (Fig. 1E,F,H). However all three *Dl* lethal alleles analysed (Dl^{M1}, Dl^{M2} and Dl^{M3}) also cause the differentiation of vein histotype in non-mutant cells abutting the clone when the boundary between mutant and non-mutant cells is close to the normal vein (Fig. 1C). This was analysed in more detail by generating Dl^{M1} and Dl^{M2} clones in a Dl^{+} duplication background, to eliminate any effects of *Dl* haploinsufficiency in neighbouring cells. In these experiments a similar phenotype of vein induction in wild-type cells was seen (Fig. 1D), indicating that Dl is required in vein cells to suppress vein differentiation in adjacent intervein cells.

Some aspects of the phenotypes produced by *Su(H)* and *E(spl)* are milder than those of *Dl* or *Notch* null alleles. Firstly the veins that differentiate in $Su(H)^{AR9}$ and $E(spl)^{b32.2}$ clones are narrower than those formed within comparable clones of *Notch* or *Dl* mutant cells (Table 1). Secondly clones restricted to only one wing surface have no effects in the differentiation of veins in the opposite surface (Table 1). Finally, the sizes of $Su(H)^{AR9}$ and $E(spl)^{b32.2}$ M^{+} clones are comparable to those of control M^{+} clones (data not shown), demonstrating that these alleles do not affect cell viability.

Expression of *Notch*, *Delta* and *E(spl)mβ* is related to vein territories

Mosaic analysis of different genes of the Notch pathway confirms that they participate in a cell interaction mechanism that restricts vein differentiation. The expression of both Notch and Delta proteins in the imaginal disc is modulated in vein versus intervein territories (Fehon et al., 1991; Kooh et al., 1993). Using a vein-specific marker, we confirmed that high levels of Notch protein are associated with intervein regions in late third instar and pupal wing discs (Fig. 2A-C and data not shown). Double staining to compare Notch and E(spl)bHLH proteins with Dl expression reveals that, although the patterns are largely complementary with Dl in regions where Notch and E(spl) are lowest, there is overlap at the vein/intervein boundaries where cells express all three genes (Fig. 2D-I).

The regulation of *Notch*, *Dl* and *E(spl)* expression occurs at the transcriptional level, *Dl* mRNA being detected in the vein and *Notch* in broad stripes that correspond to the interveins in late third instar discs (Fig. 3A-C). The expression of *Dl* is maintained in pupal wings 24 hAPF (APF) in dorso-ventral stripes 6-8 cells wide, with those cells at vein-intervein boundaries accumulating maximal levels of *Dl* (Fig. 3E,J). In contrast, the expression of *Notch* evolves during pupal development; it is gradually lost from intervein territories during the

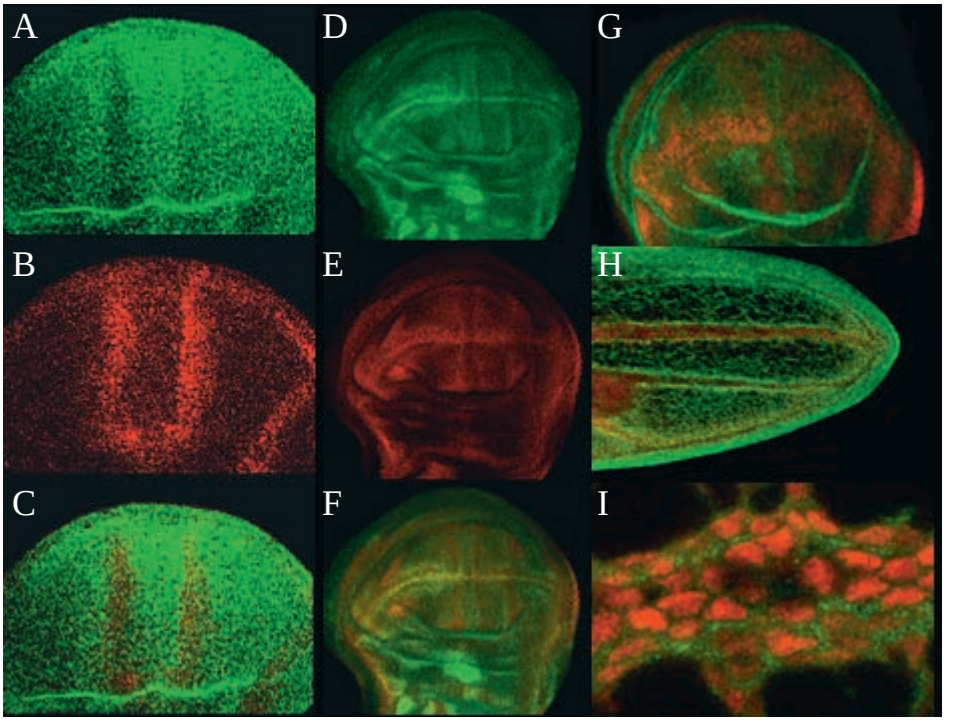

Fig. 2. Expression of Notch and E(spl) in vein territories. (A-C) The distribution of Notch (green, A) relative to a vein marker (Star-LacZ; red, B) was examined in early pupal wing discs using confocal microscopy. The overlay of the two images (C) reveals that the troughs in Notch protein correspond to the position of veins. (D-I) The expression of Delta (Delta-LacZ, red in E) relative to E(spl) (green, D,F) and Notch (green, G-I) proteins was analysed in third instar discs (D-G) and pupal wings (H,I). A single disc is shown in D-F, and the overlay of Dl and E(spl) expression (F) demonstrates that E(spl) is low/absent from the regions where Delta is maximal, but that the cells where E(spl) proteins are at high levels do also express Dl-LacZ (yellow). Similarly, Dl-LacZ (red) is present in places where Notch protein (green) is low in third instar (G) and pupal wings (H). High magnification of the latter demonstrates that Dl-LacZ is expressed in the cells where Notch expression is maximal (I).

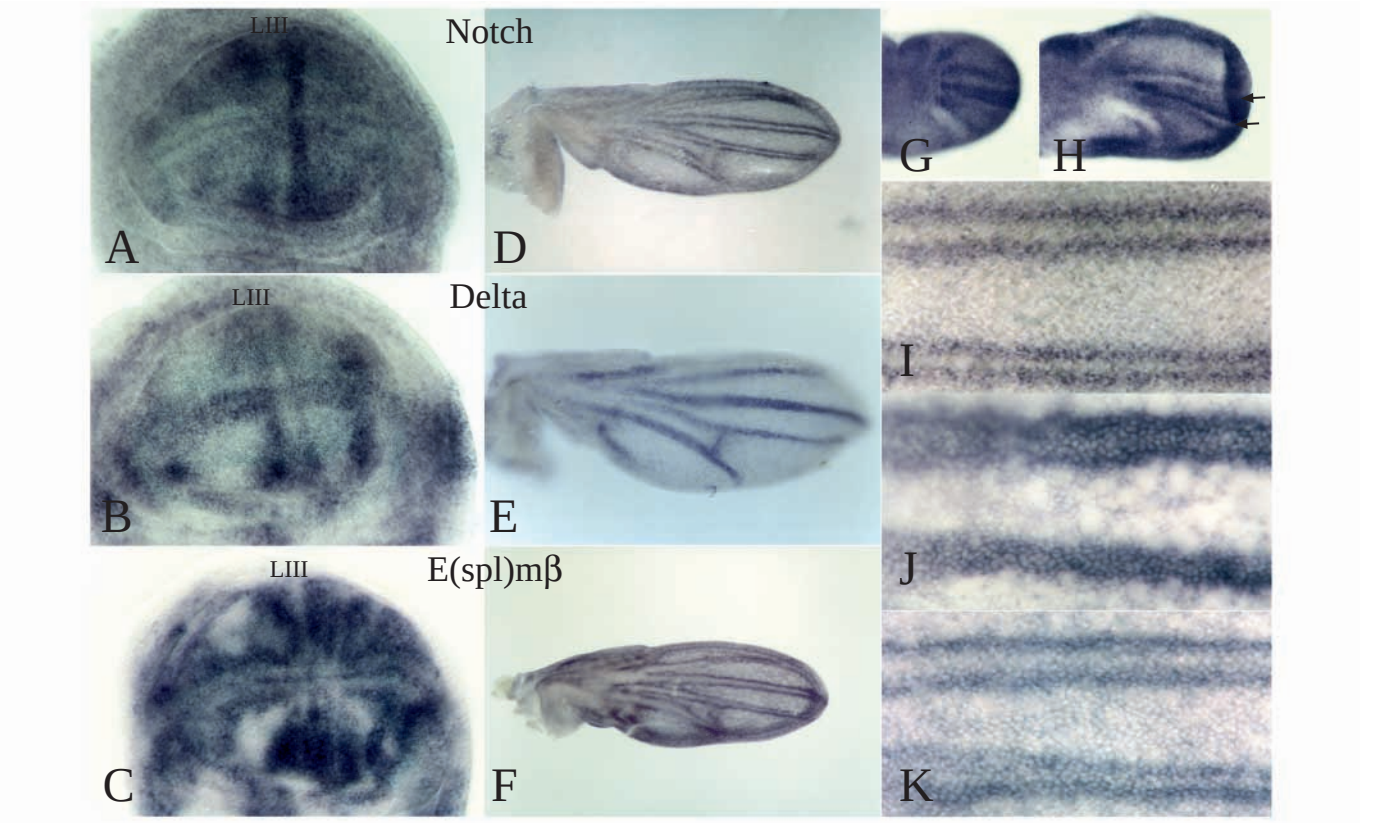

Fig. 3. Expression of *Notch*, *Dl* and *E(spl)mß* in wing discs and pupal wings. (A-C) *Notch* (A), *Dl* (B) and *E(spl)mβ* (C) transcripts are detected in the wing blade region of third instar discs in a pattern that relates to the developing veins (LIII is marked). (D-F) Pupal wings 24-28 hAPF showing *Notch* (D), *Dl* (E) and *E(spl)mβ* (F) expression patterns. (G-H) Pupal wings 5-10 hAPF revealing the transition in *Notch* expression from broad domains typical of the imaginal disc (G) to the preferential accumulation in vein/intervein boundaries (H). Arrows mark LIII and LIV veins. (I-K) High magnification of veins LIII and LIV in pupal wings 24-28 hAPF revealing *Notch* (I), *Dl* (J) and *E(spl)mβ* (K) expression. A comparison between the number of cells expressing *Dl* and the number of cells between *Notch* and *E(spl)mβ* stripes reveals that the cells with maximal *Dl* expression correspond to those where *Notch* and *E(spl)mβ* transcripts accumulate. In A-E and G-I, DNA probes were used; in J-K the probes were antisense RNA.

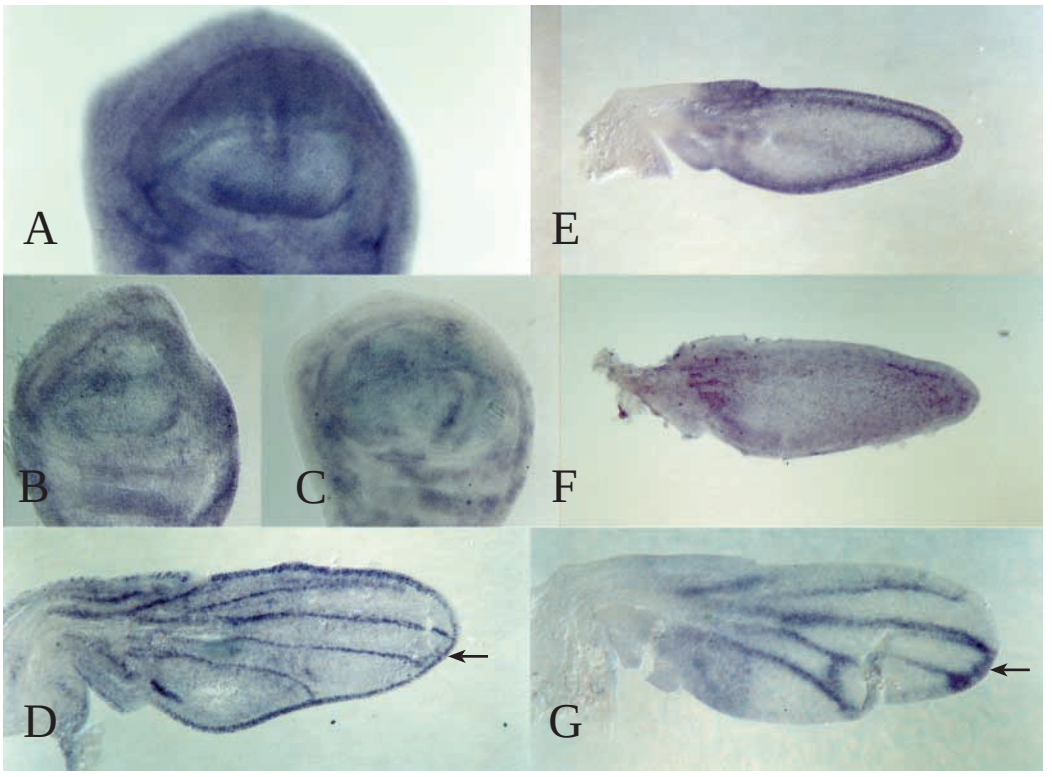

Fig. 4. Expression patterns of *Notch*, *Dl*, and *E(spl)* in *ve vn* discs and pupal wings. (A-C) *Notch* (A), *Dl* (B) and *E(spl)mβ* (C) expression in third instar *ve vn* discs. The preferential accumulation of *Notch* RNA in presumptive intervein territories is clearly present (A), whereas *Dl* (B) and *E(spl)mβ* (C) expression associated with the vein is absent. Expression of *Dl* and *E(spl)mβ* in the presumptive wing margin is still detected. (D) Expression of *ve* in 24-28 hAPF rho^{30} pupal wings. Weak ectopic expression of *ve* in the veins LII, distal LIII/LIV (arrow) and posterior crossvein is detected. (E-F) All expression of *Notch* (E) and *Dl* (F) related with longitudinal veins is absent in 24-28 hAPF *ve vn* mutant pupal wings. G) *Dl* expression in rho^{30} pupal wings 24-28 hAPF. Ectopic *Dl* expression develops in places where *ve* is abnormally expressed (e.g. LIII/LIV, arrow).

first 12 hAPF (Fig. 3G-H), becoming restricted in pupal wings 24 hAPF to stripes of 2-3 cells wide localised at the vein-intervein boundaries (Fig. 3D,I). At this stage, the cells that accumulate high levels of *Notch* correspond to those in which *Dl* expression is maximal (Fig. 2H-I; Fig. 3I,J). The expression of *E(spl)mβ* is detected in the same positions as *Notch* in pupal wings (Fig. 3F,K) indicating that Notch activation is maintained at vein/intervein boundaries during pupal development. The expression patterns detected in 24 hAPF pupal wings are maintained at later stages (e.g. 30-35 hAPF, data not shown).

Regulation of *Notch* and *Delta* expression in vein territories

The expression of *Dl*, *Notch* and *E(spl)mβ* in the developing veins was studied in several genetic backgrounds in which vein differentiation is altered. In *ve vn* wings all longitudinal veins are absent (Diaz-Benjumea and Garcia-Bellido, 1990b), presumably due to the reduction in Torpedo signalling caused by lower levels of the putative ligand Vn (Simcox et al., 1996; Schnepp et al., 1996) and the absence of Ve (Sturtevant et al., 1993). In the corresponding wing discs expression of *Dl* is eliminated from the veins (Fig. 4B), indicating that the transcriptional activation of *Dl* depends on the previous specification of veins by Top activity. In agreement, ectopic expression of *ve* in pupal wings, as in rho^{30} mutants, leads to ectopic expression of *Dl* in similar regions (Fig. 4F-G). Expression of *Notch* in *ve vn* wing discs appears unchanged, as the two broad

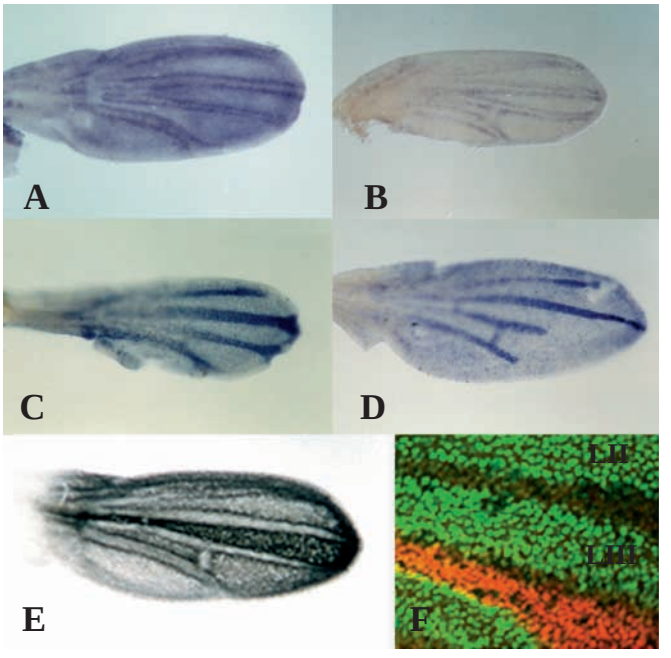

Fig. 5. Expression of *Notch*-related genes in *Notch* mutant pupal wings. (A-D) Expression of *Notch*, *Dl* and *E(spl)mβ* in fa^{nd} and Ax^{16172} pupal wings. The expression of (A) *Notch*, (B) *E(spl)mβ* and (C) *Dl* in fa^{nd} pupal wings 24-28 hAPF follows the pattern of thicker veins that develop in these mutant backgrounds. (D) Expression of *Dl* in Ax^{16172} pupal wings is eliminated in distal stretches of veins LIV and LV, presumably as a consequence of the absence of *ve* in these territories. (E) β-galactosidase expression in N^{MLz} pupal wings occurs in vein-intervein boundaries, and also in the territory between the veins LIII and LIV, due to perdurance of imaginal expression. (F) In fa^{nd}/N^{MLz} the expression of *Notch* (red) in vein intervein boundaries is eliminated, although *Notch* expression between LIII and LIV veins is still present. The developing veins (e.g. LII, LIII) are labelled with a specific antibody against Ventral veinless protein (green).

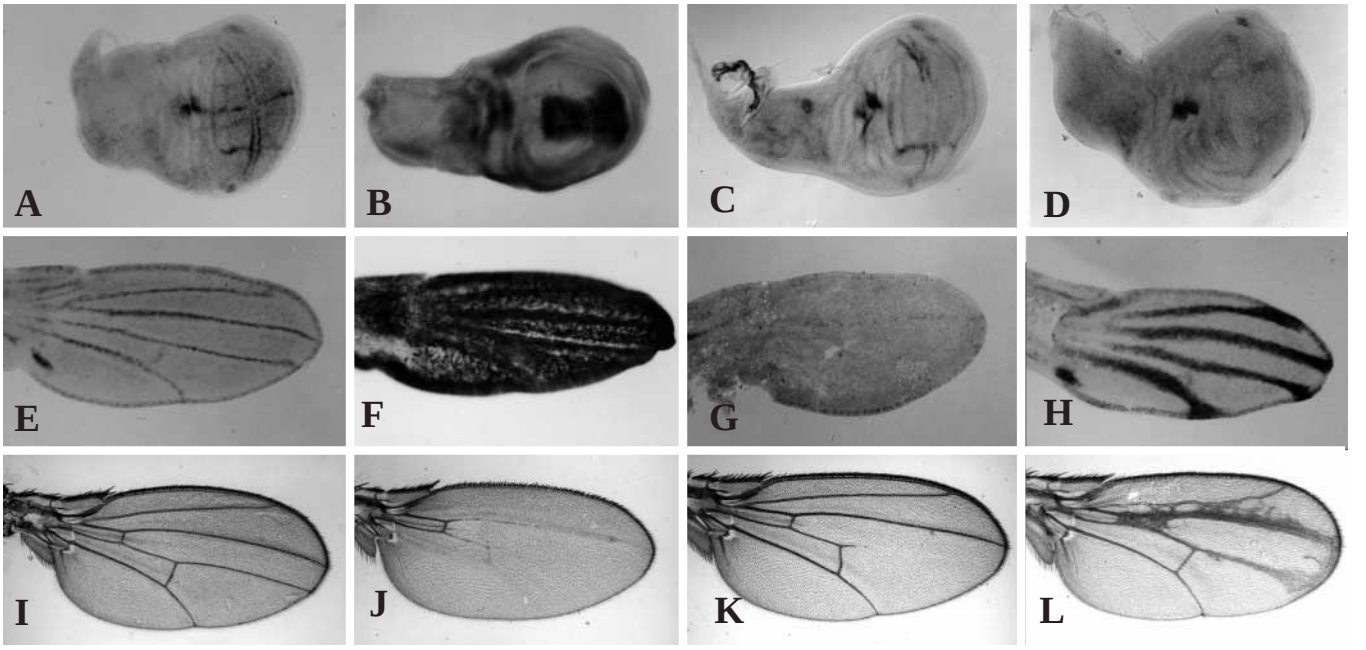

Fig. 6. Effects of modified Notch function on *ve* expression and vein differentiation. (A) Expression of *ve* in wild-type third instar disc. (B) Expression of GAL4-sal in a central domain of the disc that includes the veins LIII and LIV revealed in discs GAL4-sal/UAS-IMP. No further expression is detected 4-6 hAPF (data not shown). C) Ectopic expression of *E(spl)mβ* in the central domain of GAL4-sal/UAS-E(spl)mβ discs eliminates *ve* expression from this territory. (D) Elimination of *ve* expression associated with the longitudinal veins and wing margin in Ax^{59d} third instar disc. Only expression of *ve* associated with the dorsal radius sensillum remains. (E) Wild-type expression of *ve* in pupal wings. (F) Expression of the line GAL4-179 detected in GAL4-179/UAS-IMP pupal wing 24-28 hAPF is generalised but occurs at higher levels in the veins. This line is also expressed in most of the wing blade of the wing disc (not shown). (G-H) Residual expression of *ve* in GAL4-179/UAS-E(spl)mβ pupal wings (G), and expansion of *ve* expression in fa^{nd} pupal wings (H). (I-K) Venation patterns in a wild-type wing (I) compared to a GAL4-179/UAS-E(spl)mβ wing, where most veins are absent (J), and a GAL4-sal/UAS-E(spl)mβ wing (K), where only LIV is truncated. (L) The presence of ectopic *ve* rescues the characteristic vein loss typical of ectopic E(spl)mβ, and results in thicker veins in GAL4-sal/UAS-E(spl)mβ; UAS-ve/+ wings.

domains where *Notch* transcripts accumulate at higher levels are still present (Fig. 4A), suggesting that the modulation of *Notch* expression in the wing pouch is independent of the establishment of the veins per se. However expression of *E(spl)mβ* is severely reduced in the wing pouch of *ve vn* discs (Fig. 4C), demonstrating that Notch is not activated in vein/intervein boundaries. The lack of *Dl* and *E(spl)* expression associated with the developing veins in *ve vn* discs is compatible with the observation that clones of *Notch* (de Celis and Garcia-Bellido, 1994), or clones of *Dl* or *E(spl)* (Fig. 1I,J), cannot rescue vein differentiation in *ve vn* mutant wings. In 24 hAPF *ve vn* pupal wings, the expression of *Dl* and *E(spl)mβ* is also absent in the wing blade (Fig. 4F and data not shown). Furthermore, there is no accumulation of *Notch* mRNA in vein/intervein boundaries (Fig. 4E), indicating that this depends on an independent mechanism from that used to establish the initial intervein expression in the disc. We observed a similar failure to accumulate *Notch* in vein/intervein

Table 1. Vein thickness in wild-type and mosaic wings

	LIId*	LIIv†	LIIId*	LIIIv†	LVd*	LVv†
Wild type	1±0 (10)	2.7±0.5	3.1±0.3	1.3±0.3	3±0	2±0.2
l(1)N3	3.3±1.1 (3)	6.6±0.8 (9)	10±1.2 (5)	6.6±1.4 (8)	10±1.5 (3)	7±0.6 (5)
	2.6±0.6	3±0.9	4.2±0.5	4.5±0.5	5±1.2	5±2
N55e11	6±0 (3)	9.1±1.1 (9)	11.4±1.5 (5)	8.5±1.8 (6)	11.8±1.9 (8)	8.2±0.7 (6)
	6.5±0.7	5±0.8	7±1.4	7.8±0.9	6.8±1.1	7.3±1.2
DlM2	5.8±0.7 (6)	9.4±0.9 (5)	9±1.4 (4)	9±1 (3)	9.7±0.9 (4)	9±1.4 (5)
	6.8±0.7	5.5±1	7±1	8±1.5	7.2±1.2	8±0.7
Su(H)AR9	2 (2)	5.5±0.5 (6)	8±1 (7)	5.3±1.5 (3)	6.2±0.9 (4)	5.6±0.5 (3)
	3±0.7	2.2±0.4	2.7±0.5	4±0	3±0	4±1
E(spl)b32.2	1.7±0.5 (4)	4.7±0.5 (6)	9.1±0.9 (7)	6±1.1 (4)	7.3±1.5 (3)	4.7±0.6 (3)
	3±0	1.2±0.4	2.7±0.5	3.2±0.5	3.3±0.3	3±0

*LIId, LIIId, LVd: Dorsal veins LII, LIII and LV, respectively.
†LIIv, LIIIv, LVv: Ventral veins LII, LIII and LV, respectively.
Upper number: mean number of vein cells in the surface where the clone is present; lower number: mean number of vein cells in the opposite wing surface.
Number of veins (wild type) and clones per vein and surface (mutants) analysed are in parentheses.

boundaries when Notch signalling is strongly reduced, as in fa^{nd}/N^{MLz} pupal wings (Fig. 5E-F), suggesting that this late expression of *Notch* depends on Notch signalling.

The expression of *Notch*, *Dl* and *E(spl)mβ* also changes when Notch signalling is modified using other *Notch* alleles (fa^{nd} and Ax^{16172}) in a manner that relates to the final pattern of veins in the mutant wings. In fa^{nd} pupal wings, the expression of *Dl* is detected in broader stripes of cells, and in addition *Notch* and *E(spl)mβ* accumulation are displaced to novel positions that limit the broader mutant veins (Fig. 5A-C). Thus, a reduction in the efficiency of Notch signalling leads to a lateral displacement of the boundary between each vein and their adjacent interveins. In the gain-of-function allele Ax^{16172} we observe that *Notch*, *Dl* and *E(spl)mβ* expression is absent in the regions corresponding to the vein-stretches eliminated by this mutation (Fig. 5D and data not shown), suggesting that the competence to form veins has been suppressed in these regions (see below).

Notch signalling represses *veinlet* expression

The localised expression of *ve* in the veins is a critical component of vein development. In agreement with previous reports (Sturtevant and Bier, 1995), we find that mutations in *Notch* affect *ve* expression. Thus hyper-activation of Notch signalling using strong *Ax* mutations results in the repression of *ve* transcription in the imaginal disc (Fig. 6A,D). Conversely, reductions in Notch signalling, as in fa^{nd} pupal wings, result in an increased number of *ve*-expressing cells in vein territories (Fig. 6H). Taken together, these observations suggest that *ve* is a target of Notch signalling, and a candidate to mediate these effects on *ve* is E(spl)mβ. This was tested using the GAL4 system to direct ectopic expression of this protein in vein territories (Fig. 6B,F). We find that ectopic expression of E(spl)mβ effectively represses *ve* transcription both in discs and in pupal wings (Fig. 6C,G) and consequently changes the vein pattern. Thus all combinations between UAS-E(spl)mβ and GAL4 lines expressed in imaginal and pupal veins result in the elimination of vein stretches (Fig. 6J,K). The strongest phenotypes are observed when *E(spl)* expression is maintained at high levels in the developing veins during both larval and pupal development (GAL4-179/UAS-E(spl)mβ; Fig. 6F,J). Vein suppression by ectopic E(spl)mβ expression in vein territories is rescued by the simultaneous presence of ectopic Ve (Gal4-sal UAS-E(spl)mβ/UAS-ve flies, Fig. 6L), suggesting that most effects of E(spl)mβ on vein formation are exerted through repression of *ve* transcription.

DISCUSSION

Notch activity is required during multiple developmental processes, both in *Drosophila* and in other organisms, where many elements of the pathway have been found to be conserved (Artavanis-Tsakonas et al., 1995). The basis of Notch versatility appears to derive from its ability to regulate different downstream genes in a context-dependent manner, and therefore to understand the relevance of Notch signalling it is important to compare different processes in which Notch is involved. We have studied the mechanism of Notch function during vein formation, where Notch activity appears to separate two populations of cells so that they follow distinct differentiation programs, vein and intervein. This involves defining the extent of vein-competent territories in the imaginal disc and restricting vein differentiation to the central domain of each competent territory during pupal development.

Establishment of vein territories

A critical component in the formation of veins is the tyrosine kinase receptor Top. Reductions in Top function result in the elimination of veins, whereas ectopic activation of Top produces ectopic veins (Brunner et al., 1994; Diaz-Benjumea and Hafen, 1994), and it has been postulated that the activity of Top is increased in vein regions through localised expression of *ve* (Sturtevant et al., 1993). Alterations in Notch function manifest their effects on *ve* expression in third instar imaginal discs, with reductions in Notch activity resulting in thicker stripes of *ve*-expressing cells, and ectopic activation of the Notch pathway repressing *ve* expression. Furthermore, *ve* activity is required for *Notch*, *Dl* or *E(spl)* mutant cells to differentiate as vein, and ectopic expression of *ve* rescues the vein loss characteristic of ectopic expression of *E(spl)mβ*, indicating that Notch activity during vein differentiation is mediated through regulation of *ve* expression. Although *ve* could be a direct target for the Notch pathway, the fact that *ve* expression does not evolve from initial broad regions to narrower stripes, but is restricted to the veins from the outset, suggests that the early effects of Notch on *ve* are indirect. Thus Notch activity could negatively regulate transcription activators, analogous to the proneural genes, which promote expression of *ve* and therefore high levels of Top activity. Antagonism between Top and Notch signalling has also been noted in the specification of the photoreceptor cell R8 (Baker et al., 1990) and between the homologous pathways during vulval development in *C. elegans* (Horvitz and Sternberg, 1991).

The initial activation of Notch depends on the complementary distribution of Notch and its ligand Dl. Thus in the third instar imaginal disc high levels of *Dl* coincide with the developing veins whereas *Notch* transcription is most prominent in broad domains that separate adjacent veins, with maximal expression between LIII and LIV (Fig. 7A). These complementary expression patterns depend on separate spatial cues, because in discs with reduced Top activity (*ve vn*) there is no accumulation of *Dl* in the developing veins, but the pattern of *Notch* expression is unchanged. The dependence of *Dl* expression on Ve, and the regulation of *ve* by E(spl)mβ, would ensure coordination between Notch activity and *ve* transcription, therefore linking Notch and Top signalling pathways (Fig. 7B).

Notch function during pupal development

The formation of veins of normal width also requires Notch and Dl functions throughout pupal development (Shellenbarger and Mohler, 1978; Parody and Muskavitch, 1993). Notch activity at this stage appears to be restricted to vein/intervein boundaries, as suggested both by the behaviour of cells mutant for *Notch*, *Dl*, *Su(H)* or *E(spl)* and the expression pattern of *E(spl)mβ*. Thus, mutant clones for these genes cause thicker veins but never ectopic veins, and the strongest phenotypes of vein thickening are observed when mutant clones extend into two adjacent interveins, presumably because in these cases both vein/intervein boundaries have failed to form. Similarly, the elimination of vein/intervein boundaries observed in strong

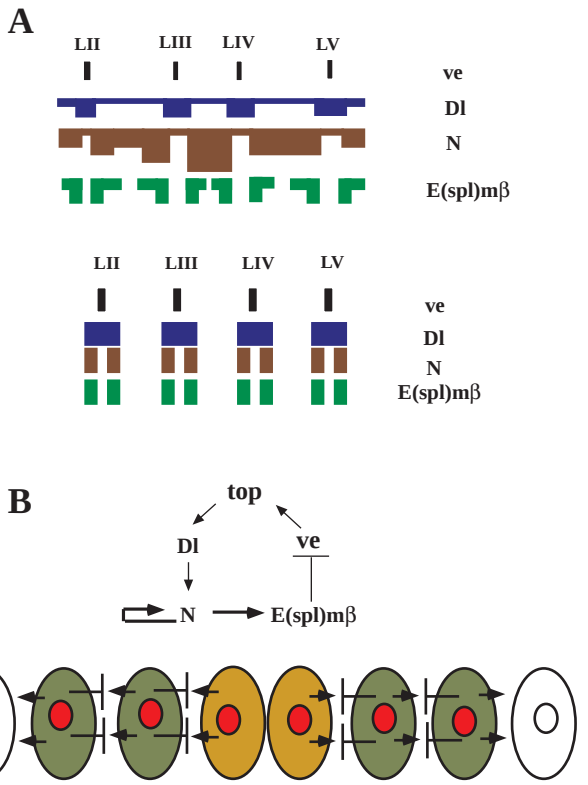

Fig. 7. (A) Transverse sections of the wing blade in the disc (upper panel) and the longitudinal veins LII, LIII, LIV and LV in the pupal wing (lower panel) are represented with the expression of *ve*, *Notch*, *Delta* and *E(spl)mβ* depicted by colour bars. The height of these bars indicates the relative levels of expression in different positions for each gene. (B) Summary of genetic (upper panel) and cellular interactions (lower panel) in the maintenance of vein width during pupal development. Cells are specified as 'pro-vein' due to the restricted expression of the transcription factor Ventral veinless (red nucleus). These cells also express Dl (arrows), whereas Notch expression and activation (as determined by E(spl)mβ expression, green cells) occurs only in 2-3 rows of cells that separate the vein (corresponding to the domain of *ve* expression, brown cells), and the intervein (white cells). Our mosaic and expression results are compatible with a model in which high levels of Dl in vein cells would trigger Notch activation in neighbouring cells. This would allow Dl protein in these cells to interact with Notch in the adjacent row of boundary cells, thus coining *ve* expression to the vein, and consequently limiting the number of cells in which Torpedo is activated. In this model, the extent of Notch activation would depend on Dl expression, which in normal development is limited to the pro-vein, and the relative levels of Dl and Notch, which would influence the efficiency of signalling. Polarity of signalling would be maintained in the receiving cells through a feed-back mechanism that links Notch activation and transcription in pro-vein cells in the vein/intervein boundary.

Notch loss-of-function alleles (fa^{nd}/N^{Mlz}) results in the differentiation of veins whose thickness is comparable to that of *Notch* null clones. After puparium formation *Notch* expression is restricted to vein/intervein boundaries and coincides with that of *E(spl)mβ*, suggesting that the accumulation of Notch here is a consequence of Notch activation. This implies a positive feedback loop on *Notch* transcription that could participate in maintaining the separation between veins and interveins during pupal development. Consistent with this model, we find that strong reductions in the level of Notch activity (fa^{nd}/N^{Mlz}) eliminate the expression of *Notch* in vein/intervein boundaries but do not perturb the early pattern of *Notch* expression, such as that between LIII and LIV veins. A positive feed-back loop regulates transcription of the Notch-related gene *lin12* during vulvar development in *C. elegans* (Wilkinson et al., 1994), indicating that it may be a general mechanism through which high levels of *Notch* expression and function can be maintained in a cell population.

The activation of Notch at vein/intervein boundaries depends on the presence of Dl in vein cells: clones of *Dl* mutant cells induce neighbouring wild-type cells to differentiate as vein so that wild-type cells appear at both sides of novel vein/intervein boundaries. As expected, expression of *Dl* persists in the vein territories during pupal development, in a domain that includes the vein and its vein/intervein boundaries (Fig. 7A). This domain is broader than that of *ve* and coincides with the expression of other genes associated with vein development, such as *ventral veinless* (de Celis et al., 1995a; and data not shown), and will be referred to as 'pro-vein' (Fig. 7B). Interestingly, the highest levels of *Dl* expression are detected in pro-vein cells which also accumulate Notch and E(spl)mβ. We do not know if Dl expression here is also required for Notch activation, but the fact that *E(spl)mβ* expression/Notch activation occurs in more that one row of cells suggests that cells in vein/intervein boundaries have simultaneously the capability to receive Notch signals and to activate Notch in neighbouring cells (Fig. 7B). The overlap in the expression of Notch and Dl helps to explain the vein phenotypes produced when the gene dosage of *Notch* and *Dl* is altered (de la Concha et al., 1988), which indicates that the relative levels of *Notch* and *Dl* expression are critical for normal Notch signalling.

Notch and Delta operate in vein differentiation through other elements in addition to E(spl)

The extent of extra vein differentiation detected in mosaics differs depending on which component of the Notch pathway is mutant. Thus, *Dl* and *Notch* clones consistently cause stronger phenotypes than similar *Su(H)* or *E(spl)* clones. Although in the case of *Su(H)* it is possible that these differences could be the result of residual activity, the similar phenotypes observed between a lethal *Su(H)* allele and a *E(spl)* deficiency suggest that additional Notch intracellular components are required in vein differentiation. One candidate to participate with *E(spl)* in the repression of vein differentiation in response to Notch activation is the gene *extramacrochaetae* (*emc*), whose expression is also increased in boundary intervein cells (de Celis et al., 1995b). It is possible that Notch, in addition to activating the expression of *E(spl)mβ*, also contributes to this increase in the levels of *emc*, and the combination of high levels of both *emc* and *E(spl)mβ* is more effective in the repression of vein differentiation.

Similarities between Notch signalling in the veins and in other tissues

The analysis of loss-of-function alleles of *Notch* and of several other members of the Notch pathway indicates that they all participate in a signalling mechanism to limit the number of cells that differentiate as vein. The requirement for Notch in vein

development shows similarities to that observed during the singling out of neural precursors: both processes appear to involve the establishment of a territory of competence and, subsequently, the restriction of cell differentiation to a subset of the competent cells. In other respects, vein differentiation reveals differences in the mode of Notch function. Thus, during neural precursor segregation, signalling operates transiently in all the cells surrounding the emerging neural precursor, leading to singling out of individual cells. In contrast, during vein formation stripes of cells acquire the same cell fate, presumably because Notch is not operative in the vein itself. Furthermore, Notch activity is required over a prolonged period of time to maintain the separation between the vein and the adjacent interveins, and its activation occurs in the cells that form the boundary of the developing vein. The basis of Notch activation here appears to be the heterogeneous distribution of the ligand and receptor of the pathway. In this respect, the establishment of vein thickness is similar to the formation of the wing margin, where the asymmetrical distribution of two Notch ligands, Dl and Serrate, result in persistent Notch activation in the dorso-ventral boundary (de Celis et al., 1996b; Doherty et al., 1996). Complementary patterns of ligand and receptor of the pathway could be a general aspect of Notch signalling in processes where Notch activity delimits cell populations that follow different fates.

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