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

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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 temperatur-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; Lecourtis 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 Di 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 veins from interveins. We find that the activities of Notch and the vein-promoting gene ve are linked: Ve is required to activate Di 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 Di 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 NotchΔ555{1} (Kidd et al., 1983), the loss-of-function alleles fad and NMlz (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 Ax18, Ax596 and Ax1672 (Kelley et al., 1987); at the Di locus, the lethal alleles DiM1, DiM2 and DiM3 (de Celis et al., 1991) and a Di LacZ line Dp{1}; at the Su(H) locus, the lethal allele Su(H)AR9 (Schweisguth and Posakony, 1994); at Hairless, the lethal allele H{1} (Bung and Posakony, 1995) and at the E(spl) locus, the deficiency E(spl)B122.2 that deletes the seven E(spl) bHLH genes (Schröms et al., 1992). As a vein-specific marker we used a Star-lacZ clone (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 ve1 (Sturtevant et al., 1993) and the viable vein allele vn1 (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 rho30, which results in ectopic ve expression (Noll et al., 1994), and in the Notch alleles fad and Ax1672. 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 K, 15 mA, 2 mm aluminium filter) 48-72 hours after egg laying. Minute clones were scored in males of the following genotypes f50a; Su(H)AR9/M(2)/Z P{F}1 {30B}; f50a; DlM1M3 (w) P{F}1 {87}, f50a; DlM1M3 (w) P{F}1 {87}, f50a; H{2}/M(3)w P{F}1 {87} and f50a; E(spl)B122/M(3)w P{F}1 {87}. Mitotic recombination proximal to the f1 insertion produces homozygous mutant cells labelled with the cell marker forked (f). Clones in Di* background were induced in flies bdl DlM1/Dp(3;3)bxdl110, Di* and bdl DiM2/Dp(3;3)bxdl110, Di*. Clones in ve vn background were induced in flies f50a; ve1 vn1 DlM1/ve1 vn1 P{F}1 {87}F and f50a; ve1 vn1 E(spl)B122/ve1 vn1 P{F}1 {87}F.

### In situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation with digoxigenin-labelled DNA probes in imaginal discs were described 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 RNA probes were used: 3 kb EcoRI Di cDNA clone (Vassin et al., 1987), 0.7 kb HindIII/Not (Noll) fragment (Deliakidis and Artavanis-Tsakonas, 1992), 3 kb BgIII/KpnI 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 Di 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 (Feihon 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 Di 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 Di, E(spl), Su(H) and H in clones. We find that lethal alleles of these genes cause vein thickening (Di, E(spl) and Su(H)) or vein loss (H) similar to that observed with Notch loss- and gain-of-function alleles respectively. Thus, Di, 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). 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...
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 DI lethal alleles analysed (Di\textsuperscript{M1}, Di\textsuperscript{M2} and Di\textsuperscript{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 Di\textsuperscript{M1} and Di\textsuperscript{M2} clones in a DI\textsuperscript{+} duplication background, to eliminate any effects of DI haploinsufficiency in neighbouring cells. In these experiments a similar phenotype of vein induction in wild-type cells was seen (Fig. 1D), indicating that DI 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 DI or Notch null alleles. Firstly the veins that differentiate in Su(H)\textsuperscript{AR9} and E(spl)\textsuperscript{b32.2} clones are narrower than those formed within comparable clones of Notch or DI 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)\textsuperscript{AR9} and E(spl)\textsuperscript{b32.2} M\textsuperscript{+} clones are comparable to those of control M\textsuperscript{+} clones (data not shown), demonstrating that these alleles do not affect cell viability.

### Expression of Notch, Delta and E(spl)m\textbeta 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). The expression of E(spl)bHLH proteins with DI expression reveals that, although the patterns are largely complementary with DI 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, DI and E(spl) expression occurs at the transcriptional level, DI 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 DI 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 DI (Fig. 3E,J). In contrast, the expression of Notch evolves during pupal development; it is gradually lost from intervein territories during the

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**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 DI\textsuperscript{M3} clone in the dorsal wing surface covering the position of LIV differentiates a thicker LIV vein. (C-C\textsuperscript{¢}) Dorsal DI\textsuperscript{M2} 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\textsuperscript{¢}). (D-D\textsuperscript{¢}) Two examples of bhl DI\textsuperscript{M2} clones in the dorsal LIII (D) and ventral LIV (D\textsuperscript{¢}), which induce vein differentiation in the adjacent one or two wild-type cells. DI 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\textsuperscript{¢}) which differentiates a thicker dorsal LIII (E\textsuperscript{¢}), and induces extra-vein differentiation in the ventral component of LIII (E\textsuperscript{¢}). (F) Ventral Su(H)AR9 clone covering LII, which results in the differentiation of a thicker LII. (G) DI\textsuperscript{+} clone that reduces vein differentiation in dorsal LV. (H-H\textsuperscript{¢}) Ventral E(spl)\textsuperscript{b32.2} clone that causes thickening of the ventral component of LIII (H) but does not affect vein differentiation in the opposite surface (H\textsuperscript{¢}). (I) Dorsal DI\textsuperscript{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. Black lines mark the boundaries of mutant clones.
**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)mβ 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.
Vein formation in the Drosophila wing

first 12 hAPF (Fig. 3G-H), becoming restricted in pupal wings 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; Schnepf 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 rho30 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. 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 rho30 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 rho30 pupal wings 24-28 hAPF. Ectopic Dl expression develops in places where ve is abnormally expressed (e.g. LIII/LIV, arrow).

Regulation of Notch and Delta expression in vein territories

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Fig. 5. Expression of Notch-related genes in Notch mutant pupal wings. (A-D) Expression of Notch, Dl and E(spl)mβ in fa5md and Axl272 pupal wings. The expression of (A) Notch, (B) E(spl)mβ and (C) Dl in fa5md pupal wings 24-28 hAPF follows the pattern of thicker veins that develop in these mutant backgrounds. (D) Expression of Dl in Axl272 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 NMLz 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 fa5md/NMLz the expression of Notch (red) in vein intervein boundaries is eliminated, although Notch expression between LII and LIV veins is still present. The developing veins (e.g. LII, LIII) are labelled with a specific antibody against Ventral veinless protein (green).
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 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 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. 11J), cannot rescue vein differentiation in vn mutant wings. In 24 hAPF 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

![Fig. 6. Effects of modified Notch function on ve expression and vein differentiation.](image)

(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 Ax3rd 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 for mutant 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.

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

<table>
<thead>
<tr>
<th></th>
<th>LIIId*</th>
<th>LIIv†</th>
<th>LIIIId*</th>
<th>LIIIv†</th>
<th>LIVd*</th>
<th>LIVv†</th>
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<tr>
<td>Wild type</td>
<td>1±0 (10)</td>
<td>2.7±0.5</td>
<td>3.1±0.3</td>
<td>1.3±0.3</td>
<td>3±0</td>
<td>2±0.2</td>
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<tr>
<td>l(1)N3</td>
<td>3.3±1.1 (3)</td>
<td>6.6±0.8 (9)</td>
<td>10±1.2 (5)</td>
<td>6.6±1.4 (8)</td>
<td>10±1.5 (3)</td>
<td>7±0.6 (5)</td>
</tr>
<tr>
<td>N55e11</td>
<td>2.6±0.6</td>
<td>3±0.9</td>
<td>4.2±0.5</td>
<td>4.5±0.5</td>
<td>5±1.2</td>
<td>5±2</td>
</tr>
<tr>
<td>DlM2</td>
<td>6.5±0.7</td>
<td>5±0.7</td>
<td>7±1.4</td>
<td>7.8±0.9</td>
<td>6.8±1.1</td>
<td>7±1.2</td>
</tr>
<tr>
<td>Su(H)AR9</td>
<td>5.8±0.7 (6)</td>
<td>9.4±0.9 (5)</td>
<td>9±1.4 (4)</td>
<td>9±1.3</td>
<td>9.7±0.9 (4)</td>
<td>9±1.4 (5)</td>
</tr>
<tr>
<td>E(spl)b32.2</td>
<td>6.8±0.7</td>
<td>5±1</td>
<td>7±1</td>
<td>8±1.5</td>
<td>7.2±1.2</td>
<td>8±0.7</td>
</tr>
</tbody>
</table>

*LIIId, LIIId, LIVd: Dorsal veins LII, LIII and LIV, respectively.
†LIIv, LIIv, LIVv: Ventral veins LII, LIII and LIV, 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 \(fa^{md}\)NM12 pupal wings (Fig. 5E-F), suggesting that this late expression of Notch depends on Notch signalling.

The expression of Notch, DI and \(E(spl)\)β also changes when Notch signalling is modified using other Notch alleles (\(fa^{md}\) and \(Ax^{16752}\)) in a manner that relates to the final pattern of veins in the mutant wings. In \(fa^{md}\) pupal wings, the expression of DI is detected in broader stripes of cells, and in addition Notch and \(E(spl)\)β 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 \(Ax^{16752}\) we observe that Notch, DI and \(E(spl)\)β 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 \(fa^{md}\) 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 \(E(spl)\)β. 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 \(E(spl)\)β 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-\(E(spl)\)β 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-\(E(spl)\)β; Fig. 6FJ). Vein suppression by ectopic \(E(spl)\)β expression in vein territories is rescued by the simultaneous presence of ectopic Ve (Gal4-sal UAS-\(E(spl)\)β/UAS-ve flies, Fig. 6L), suggesting that most effects of \(E(spl)\)β 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, DI or \(E(spl)\) mutant cells to differentiate as vein, and ectopic expression of \(ve\) rescues the vein loss characteristic of ectopic expression of \(E(spl)\)β, 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 DI. Thus in the third instar imaginal disc high levels of DI 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 DI in the developing veins, but the pattern of Notch expression is unchanged. The dependence of DI expression on Ve, and the regulation of \(ve\) by \(E(spl)\)β, 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 DI functions throughout pupal development (Shellenberger 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, DI, \(Su(H)\) or \(E(spl)\) and the expression pattern of \(E(spl)\)β. 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
Notch loss-of-function alleles (fam11\textsuperscript{Mflc}) 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 \(E(spl)m\beta\), 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 (fam11\textsuperscript{Mflc}) 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 \textit{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 DI in vein cells: clones of DI 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 DI 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 \textit{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 DI expression are detected in pro-vein cells which also accumulate Notch and \(E(spl)m\beta\). We do not know if DI expression here is also required for Notch activation, but the fact that \(E(spl)m\beta\) 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 DI helps to explain the vein phenotypes produced when the gene dosage of Notch and DI is altered (de la Concha et al., 1988), which indicates that the relative levels of Notch and DI expression are critical for normal Notch signalling.

\textbf{Notch and Delta operate in vein differentiation through other elements in addition to \textit{E(spl)}}

The extent of extra vein differentiation detected in mosaics differs depending on which component of the Notch pathway is mutant. Thus, DI and Notch clones consistently cause stronger phenotypes than similar \textit{Su(H)} or \(E(spl)\) clones. Although in the case of \textit{Su(H)} it is possible that these differences could be the result of residual activity, the similar phenotypes observed between a lethal \textit{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 \textit{extramacrocroetaeae} (\textit{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 \(E(spl)m\beta\), also contributes to this increase in the levels of both \textit{emc} and \(E(spl)m\beta\) is more effective in the repression of vein differentiation.

\textbf{Similarities between Notch signalling in the veins and in other tissues}

The analysis of loss-of-function alleles of \textit{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
Vein formation in the Drosophila wing


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