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Feedback regulation is central to Delta-Notch signalling required for *Drosophila* wing vein morphogenesis

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SUMMARY

Delta and Notch are required for partitioning of vein and intervein cell fates within the provein during *Drosophila* metamorphosis. We find that partitioning of these fates is dependent on Delta-mediated signalling from 22 to 30 hours after puparium formation at 25°C. Within the provein, Delta is expressed more highly in central provein cells (presumptive vein cells) and Notch is expressed more highly in lateral provein cells (presumptive intervein cells). Accumulation of Notch in presumptive intervein cells is dependent on Delta signalling activity in presumptive vein cells and constitutive Notch receptor activity represses Delta accumulation in presumptive vein cells. When Delta protein expression is elevated ectopically in presumptive intervein cells, complementary Delta and Notch expression patterns in provein cells are reversed, and vein loss occurs because central provein cells are unable to stably adopt the vein cell fate. Our findings imply that Delta-Notch signalling exerts feedback regulation on Delta and Notch expression during metamorphic wing vein development, and that the resultant asymmetries in Delta and Notch expression underlie the proper specification of vein and intervein cell fates within the provein.

Key words: *Delta*, *Notch*, *Drosophila*, neurogenic gene, wing vein

INTRODUCTION

The *Drosophila* wing is an apt venue in which to study genetic and cellular interactions that establish coordinate axes and allocate the predominant cell fates, vein and intervein, within the adult wing blade. Wing vein morphogenesis in *Drosophila melanogaster* was originally described by Waddington (1940). Wing veins develop during an intricate metamorphic process that converts a folded, monolayer wing disc into a flat, bilayered adult wing. These processes have been described at the cellular level by Fristrom et al. (1993). The results of numerous mutant and genetic mosaic analyses imply that patterns and size in wing development are controlled by cell interactions and not by cell lineage (Diaz-Benjumea and García-Bellido, 1990; García-Bellido and de Celis, 1992; de Celis and García-Bellido, 1994b). Sturtevant and Bier (1995) have proposed a sequential model for the action of genes central to wing vein establishment and differentiation. Subsequent analyses of wing vein development have revealed that function and cross-regulation of at least three signal transduction pathways – Delta/Notch, dpp/TKV and DER – are required for differentiation of the correct number of vein cells (de Celis, 1997).

The *Delta (Dl)* locus was first identified on the basis of the thickenings of the distal termini of longitudinal wing veins (‘deltas’) in flies heterozygous for *Dl* loss-of-function and wild-type alleles (Dexter, 1914). *Dl* or *Notch* (*N)* loss-of-function genotypes result in vein thickening of varying severity (Lindsley and Zimm, 1992) and *N* gain-of-function genotypes can lead to vein shortening (Foster, 1975; Portin, 1975). These contrasting phenotypes imply that neurogenic signalling may function during two stages of wing vein development. During the first stage, neurogenic signalling would be required to define the provein anlage, i.e., a broad stripe of ‘vein-competent’ cells more numerous than those in the adult vein, that extends bilaterally beyond the adult vein boundaries. Excessive neurogenic signalling during this stage, associated with *N* gain-of-function *Abruptex* (N^{Ax}) alleles (Palka et al., 1990; Heitzler and Simpson, 1993; de Celis and García-Bellido, 1994a), would reduce the extent of the anlage and thereby lead to development of shortened wing veins. During the second stage, Delta and Notch would delineate vein and intervein cell fates within the provein anlage. Reduced neurogenic signalling during this stage, associated with reductions in *Dl* or *N* function, would lead to overcommitment to the vein cell fate within the anlage and the development of thickened veins. Analysis of vein mutants performed by Diaz-Benjumea and García-Bellido (1990) supports the premise that veins are determined during the proliferative phase of larval wing disc development and differentiate during pupal development. The fact that clones of *Dl* or *N* mutant tissue develop as vein only when adjacent to ‘normal’ vein tissue implies that there exists a ‘vein-competent’ domain within which veins normally form (García-Bellido and de Celis, 1992; de Celis and García-Bellido, 1994b).

Neurogenic signalling is central to the partitioning of cell fates within equivalence groups in many contexts during embryonic and postembryonic *Drosophila* development (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Delta functions as a signal in this process, as reflected in the cell non-autonomous behavior of Delta in somatic mosaic analyses in

the notum (Heitzler and Simpson, 1991) and wing blade (García-Bellido and de Celis, 1992). Notch functions as a receptor, as shown by its cell-autonomous function in embryos (Hoppe and Greenspan, 1986, 1990), nota (Heitzler and Simpson, 1991) and wing blades (García-Bellido and de Celis, 1992). The fact that the extracellular domain of the Delta protein is capable of interacting with the Notch protein on opposing cell surfaces implies that Delta is a ligand for the Notch receptor (Fehon et al., 1990). The similar phenotypes observed in *Dl* and *N* loss-of-function mutants imply that Delta activates the Notch receptor (Shellenbarger and Mohler, 1978; Lehmann et al., 1983; Parody and Muskavitch, 1993).

In this paper, we investigate the functions of neurogenic signalling during pupal wing vein formation. We find that neurogenic signalling is required between 22 and 30 hours after puparium formation (APF) for specification of the proper number of vein cells, i.e., for the partitioning of vein and intervein fates within the provein. Delta and Notch protein expression patterns are generally complementary during wing vein development. This observation, and the impacts of ectopic Delta expression on vein development that we observe, imply that asymmetries in Delta and Notch protein expression among adjacent cells are essential for the correct partitioning of cell fates within the provein. We show that reduction in Delta expression or ubiquitous expression of an activated Notch receptor in the pupal wing blade alters Delta and Notch protein expression, reflecting the existence of a feedback mechanism by which neurogenic signalling regulates Delta and Notch expression in developing wing veins.

MATERIALS AND METHODS

Drosophila stocks

Oregon-R (Stanford isolate) was used as a wild-type control in all analyses. *P[lArB]l(3)A326.2F3, ry^{506}/TM6C, cu Sb e Tb ca* (i.e., *Dl^{PlacZ}/TM6C*) was obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). This enhancer trap was previously shown to exhibit expression patterns in embryos and ovaries appropriate for the *Dl* locus (Wilson et al., 1989). The *Dl^{PlacZ}* insertion was remobilized to identify local transpositions that might affect *Dl* regulatory regions (S. S. Huppert and M. A. T. Muskavitch, unpublished data). *Dl^{EW}* is a transposon-induced mutation isolated during the remobilization of *Dl^{PlacZ}*. *Dl^{RF}* is a heat-sensitive allele (Parody and Muskavitch, 1993). *P[ry^{+} hsp70-Notch(intra)]* (i.e., *P[hs-N(intra)]*) was a generous gift from Gary Struhl (Columbia University; New York, NY) (Struhl et al., 1993). This strain was used to examine the impact of constitutive neurogenic signalling on wing vein development. The *1348::GAL4* driver, isolated by G. Technau (Universität Mainz; Mainz, Germany), was used to induce expression of wild-type Delta (using a *UAS::DeltaWT* responder construct; T. L. Jacobsen and M. A. T. Muskavitch, unpublished data) and a dominant-negative form of Delta (using a *UAS::DeltaD* responder construct; T. L. Jacobsen and M. A. T. Muskavitch, unpublished data). The expression pattern supported by *1348::GAL4* driver was assessed using a nuclear-targeted β-galactosidase responder construct (i.e., *UAS::βGalNUC*; T. L. Jacobsen and M. A. T. Muskavitch, unpublished data).

Phenocritical period analysis

Dl^{RF}/Dl^{PlacZ} animals were generated by crossing *Dl^{PlacZ}/TM6C* adults to *Dl^{RF}/Dl^{RF}* adults. Tubby^{+} white prepupa growing at 18°C were collected and kept in a humid chamber at 18°C, then shifted to 32° for 8 hours at various times after puparium formation (APF). *P[hs-N(intra)]* animals were grown at 25°C. White prepupae were collected and kept in a humid chamber at 25°C, then shifted to 37°C for 2 hours at various times during development. Phenotypes of adult wings were then examined.

Immunohistochemistry

Pupae were staged from white prepupa formation at 25°C. Wing discs younger than 6 hours APF were dissected in PBS (3 mM NaH_2PO_4, 7 mM Na_2HPO_4, 131 mM NaCl, pH 7.5) and fixed for 25 minutes in 4% paraformaldehyde in PBS. For wing discs aged more than 6 hours APF, staged pupae were removed from pupal cases in PBS and then incubated in 4% paraformaldehyde in PBS for no less than 12 hours at 4°C. Wing discs were then pulled from the pupal carcass and cuticles were removed.

Dissected discs were incubated with primary antibody at 1:300 (anti-β-galactosidase; Promega; Madison, WI), 1:10,000 (anti-Delta, MAb202 ascites; Parks et al., 1995), or 1:15,000 (anti-Notch, C17.9C6 purified ascites; Fehon et al., 1990) overnight at 4°C in TPBS (0.3% Triton X-100 in PBS) plus 5% normal goat serum (NGS). Discs were then incubated at room temperature for 2 to 6 hours with goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunochemicals; West Grove, PA) diluted 1:800 in TPBS plus 5% NGS. For increased sensitivity, silver was precipitated onto the peroxidase reaction product (Gallyas et al., 1982; Liposits et al., 1984). Discs were mounted in glycerol and viewed using a Zeiss Axioskop light microscope.

Immunofluorescence

Pupae were staged and wings were fixed and dissected as above. Dissected discs were then incubated with primary antibody at 1:16,000 (anti-Notch, C17.9C6 purified ascites; Fehon et al., 1990) and 1:4,000 (anti-Delta, GP581) overnight at 4°C in TPBS plus 5% NGS. Discs were next incubated overnight at 4°C with goat anti-mouse IgG conjugated to fluorescein isothiocyanate and goat anti-guinea pig IgG conjugated to Texas Red (Jackson Immunochemicals; West Grove, PA), each diluted 1:100 in TPBS plus 5% NGS. Discs were then mounted in glycerol plus 1% n-propyl gallate. The tissue was viewed with a Bio-Rad MRC600 confocal laser microscope. The guinea pig polyclonal anti-serum GP581 was prepared as described in Fehon et al. (1990). The 0.54 kb *Cla*I DNA fragment encoding Delta EGF-like repeats 4-9 (amino acids 350-529) was excised from the Dl1 cDNA (Kopczynski et al., 1988) and transferred into a β-galactosidase vector to create a fusion protein that was used to immunize guinea pigs (Pocono Rabbit Farm & Laboratory; Canadensis, PA).

Propidium iodide staining was performed as in Li and Kaufman (1996).

Whole-mount microscopy

Wings were removed from adults and mounted under coverslips in Gary's Magic Mountant (Ashburner, 1989).

RESULTS

Phenocritical periods for neurogenic signalling during pupal wing vein specification

The conditional genotype *Dl^{RF}/Dl^{PlacZ}* was employed to analyze effects of the reduction of Delta function (i.e., reduction of neurogenic signalling) on wing vein formation during larval and pupal development. Animals bearing this genotype exhibit heat-sensitive increases in the severity of *Dl* mutant wing venation phenotypes (data not shown). At the permissive temperature of 18°C, the *Dl^{RF}/Dl^{PlacZ}* genotype yields wings with mild 'deltas' where longitudinal veins intersect the margin (Fig. 1A). *Dl^{RF}/Dl^{PlacZ}* animals pulsed for 8 hours beginning at times ranging from 40 to 60 hours APF at 18°C, equivalent to 20 to 30 hours APF at 25°C, display thickened veins and marginal 'deltas' larger than those of animals raised at 18°C. The most severe impact on wing venation of this conditional *Dl* mutant

genotype results from a pulse applied from 40 to 48 hours APF (Fig. 1B). We propose that this vein thickening phenotype occurs because reduction in *Dl* function leads to reduction of neurogenic signalling within the provein. As a result, fewer cells are inhibited from adopting the vein cell fate than in wild-type animals and veins become thicker. An extreme example of the failure to inhibit cells within the provein from adopting the vein cell fate is evident in the wings of Dl^{EW}/Dl^{EW} animals (Fig. 1G).

In order to analyze the impacts of constitutive neurogenic signalling during wing vein formation, we induced ubiquitous expression of a Notch variant that yields *N* gain-of-function phenotypes (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). The *P[hs-N(intra)]* transgene encodes a heat-shock-regulated Notch variant, Notch(intra), constituted solely of the Notch receptor intracellular domain (Struhl et al., 1993). When this variant is expressed during embryonic neuroblast specification, adoption of neuroblast fate is inhibited excessively and almost all ventral ectodermal cells give rise to epidermis (Struhl et al., 1993). At 25°C, Notch(intra) has no effect on vein formation and the adult wing is wild type in character (Fig. 1C). However, when Notch(intra) expression is induced by 2 hour heat pulses at 37°C initiated at times from 22 to 32 hours APF, vein loss is observed in the adult wing. The most extensive vein loss that we observe results from a heat pulse from 26 to 28 hours APF (Fig. 1D). Animals pulsed in this manner exhibit shortening of L2, L4 and L5, and some shortening of L3. We attribute the Notch(intra)-associated loss-of-vein phenotype to excessive inhibition of adoption of the vein cell fate within the provein.

The interval during which neurogenic signalling appears to be required for partitioning of fates within the provein is correlated with the second phase of cell layer apposition observed for intervein cells (Fristrom et al., 1993). During this time, the wing veins become apparent progressively. Cells on either side of the ‘intervein bands’ begin to extend basal processes and the vein cells are the only regions remaining unapposed (Fristrom et al., 1993, 1994). Neurogenic signalling apparently blocks the responses of lateral provein cells to inductive cues that specify the vein cell fate and these lateral cells then extend basal processes during the later stages of intervein morphogenesis.

Delta and Notch protein expression in larval and pupal wing discs

Delta and Notch exhibit complex and dynamic expression patterns that are correlated with the numerous embryonic and imaginal defects observed in *Dl* and *N* mutants (Fehon et al., 1991; Kooh et al., 1993). We have examined Delta and Notch protein expression patterns throughout wing vein development in order to assess relationships between protein expression patterns and the timing of neurogenic signalling.

In third larval instar wing discs, Delta is expressed in most cells within the wing pouch, although higher expression is associated with two stripes of cells along the prospective wing margin, and with regions wherein longitudinal wing veins L3, L4 and L5 will develop (Kooh et al., 1993) (Fig. 2A). Notch protein accumulates on cell surfaces and is expressed throughout the wing pouch (Fig. 2B), although expression is lower in cells in which Delta is expressed (Fehon et al., 1991; Kooh et al., 1993). At 6 hours APF at 25°C, when the wing disc has everted and the dorsal and ventral surfaces of the wing pouch have become apposed, the Delta expression pattern is similar to that observed in third instar larvae (Fig. 2C). The Notch expression pattern becomes refined such that Notch protein levels are higher in wing blade cells adjacent to Delta-expressing cells (Fig. 2D). Delta protein expression does not appear in the region within which L2 will develop until approximately 22 hours APF at 25°C (data not shown). By 30 hours APF at 25°C, the developing wing blade has the general shape of the adult wing and the vein pattern is pre-figured by Delta expression. Delta is highly expressed in all vein cells and is concentrated within subcellular vesicles at this time (Fig. 2E). This protein expression pattern is consistent with the report of de Celis (1997), indicating that *Dl*

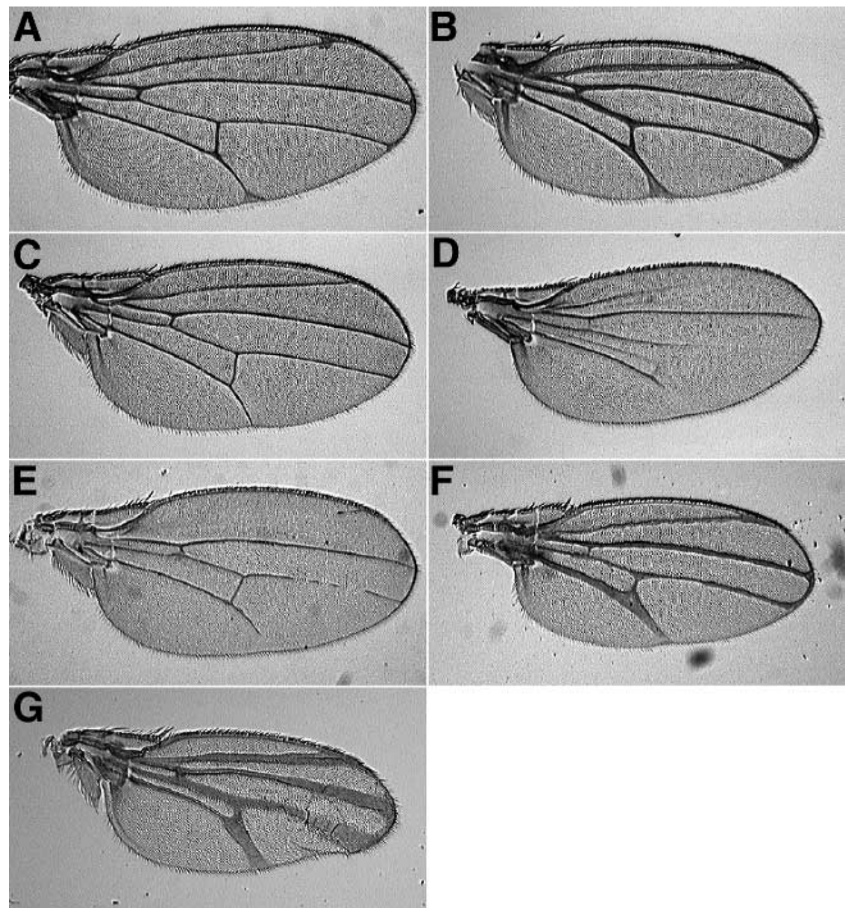

Fig. 1. Phenotypic effects associated with perturbations in neurogenic signalling during pupal wing vein development. (A,B) Adult wing phenotypes of Dl^{PlacZ}/Dl^{RF} animals reared at permissive temperature, 18°C (A), or pulsed to 32°C from 40 to 48 hours APF (B). (C,D) Phenotypes of *P[hs-N(intra)]* animals grown at 25°C (C), or grown at 25°C and heat-pulsed to 37°C from 26 to 28 hours APF (D). (E) Vein loss phenotype in a *UAS::DeltaWT/+; 1348::GAL4/+* wing from an animal grown at 25°C. (F) Vein thickening phenotype in a *1348::GAL4/+; UAS::DeltaD/+* wing from an animal grown at 25°C. (G) Extreme vein thickening phenotype of Dl^{EW}/Dl^{EW} *animals* grown at 25°C.

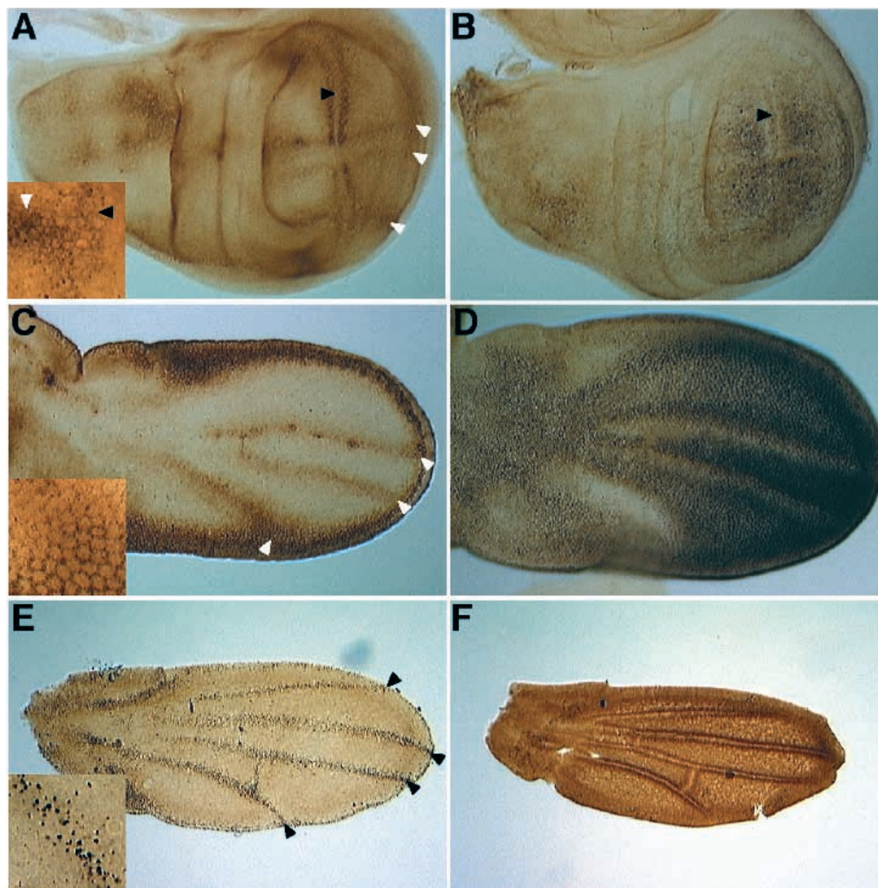

Fig. 2. Delta and Notch protein expression in wild-type wing discs. Whole-mount wing discs staged at 25°C and stained with an antibody against either Delta or Notch, as described in Materials and Methods. Discs from third instar larvae stained for Delta (A) or Notch (B). ▲, wing margin; Δ, longitudinal wing veins L3, L4 and L5. (Inset A) The intersection of the dorsal wing margin stripe (▲) and the L5 provein (Δ). Discs from 6 hour APF pupae stained for Delta (C) or Notch (D). L3, L4 and L5 marked as in panel A. (Inset C) From a region in the L5 provein. Discs from 30 hour APF pupae stained for Delta (E) or Notch (F). ▲, L2, L3, L4 and L5. Inset E, is from the region of distal L5.

transcription is limited to vein territories in the pupal wing. Notch is more highly expressed in the cells (i.e., lateral provein cells; Fig. 2F) that flank Delta-expressing cells (i.e., vein cells), and remains on the surfaces of lateral provein cells.

Simultaneous staining for Delta and Notch proteins reveals that Delta and Notch protein expression patterns are complementary, on a cell-by-cell basis, in pupal wing discs (Fig. 3). Delta is visible on the surfaces of vein cells at 24 hours APF (Fig. 3A) and accumulates in subcellular vesicles within these cells at 30 hours APF (Fig. 3B). Notch expression is higher in the intervein cells that immediately flank the Delta-expressing vein cells than in other intervein cells. These patterns become apparent by 24 hours APF (Fig. 3A) and are even more evident by 30 hours APF (Fig. 3B). This implies that Delta is expressed maximally in wing vein cells during the partitioning of fates within the provein (22 to 30 hours APF) and that Notch expression during the same period is reduced in central provein cells and elevated in lateral provein cells that will adopt the intervein fate. We have assessed the number of provein cells that express Delta and Notch by using propidium iodide to mark cell nuclei (data not shown). We find that Delta is highly expressed within proveins in stripes three cells in width in wing discs from wandering third instar larvae, prepupae at 6 hours APF, and pupae at 24 hours and 30 hours APF. Notch protein levels within proveins are lowest in regions that are three cells in width throughout wing vein development. However, the initially broad regions of cells that express high levels of Notch in the lateral proveins narrow to stripes two to three cells in width by 30 hours APF. Thus, the distributions of cells with high levels of Delta and Notch proteins appear to be complementary throughout metamorphic vein development.

Dynamic changes in the subcellular localization of Delta are also apparent during larval and pupal wing vein development. Delta expression in the wing pouch first becomes apparent

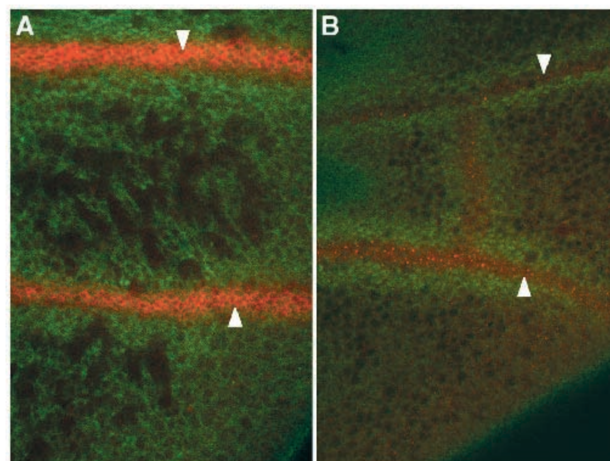

Fig. 3. Delta and Notch protein expression in pupal wing discs. Discs stained for Delta and Notch, as described in Materials and Methods. Delta signal, red; Notch signal, green. (A) 24 hour APF disc with presumptive L3 and L4 vein cells marked (Δ). (B) 30 hour APF disc with presumptive L4 and L5 vein cells marked (Δ).

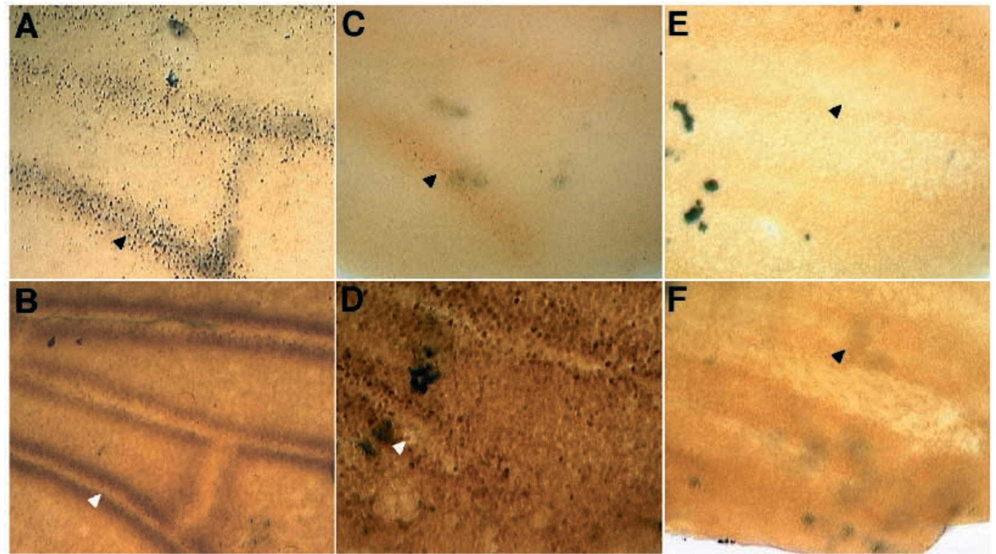

Fig. 4. Delta and Notch protein expression associated with altered neurogenic signalling in pupal wing discs. Discs were staged at 25°C and dissected at 30 hours APF. (A,C,E) Delta protein; (B,D,F) Notch protein. (A-D) Images of the region in which longitudinal veins L4 and L5 (Δ or ▲) and the posterior crossvein intersect. (E,F) Images of the distal portions of L3 (▲) and L4. (A,B) Wild-type discs; (C,D) *P[hs-N(intra)]/P[hs-N(intra)]* discs from animals grown at 25°C and heat-pulsed to 37°C from 26 to 28 hours APF, and allowed to recover 2 hours at 25°C before dissecting and staining. (E,F) *Dl^{EW}/Dl^{EW} discs* from animals grown at 25°C.

during the mid-second larval instar. Doherty et al. (1996) report that Delta accumulates in the membranes of cells at the dorsal/ventral boundary during the second larval instar, primarily in cells within the ventral compartment. Subcellular localization of Delta in the developing wing blade varies dynamically during the third larval instar and early pupal life, presumably as the result of successive cycles of cell surface targeting and endocytic down-regulation (Kooh et al., 1993; Parks et al., 1995). During the late third larval instar, we find that Delta is localized in subcellular vesicles and is also accumulating on cell surfaces (Fig. 2A inset). Next, Delta is predominantly vesicular between 2 and 3 hours APF (data not shown). Around 6 hours APF, the majority of Delta protein is again found on cell surfaces (Fig. 2C inset). Still later, by 30 hours APF, the majority of Delta protein in vein cells has been down-regulated into subcellular vesicles (Fig. 2E inset). This latter phase of Delta protein down-regulation appears to be correlated with the end of Delta-dependent signalling within the provein, and completion of the partitioning of provein cells between vein and intervein fates.

Neurogenic signalling affects levels and patterns of Delta and Notch protein expression

In order to investigate regulatory mechanisms that bear on the Delta-Notch signalling pathway during wing vein morphogenesis, we have analyzed the effects on Delta and Notch protein expression of constitutive Notch receptor activity and reduced Delta signal activity. This analysis focused on the pheno-critical period during which we find that neurogenic signalling is required for final partitioning of vein and intervein cell fates within the provein.

We employed the *P[hs-N(intra)]* genotype to investigate the impacts of constitutive neurogenic signalling on Delta protein expression. Constitutive Notch receptor activity was induced by the pupal heat pulse regime that we had found resulted in

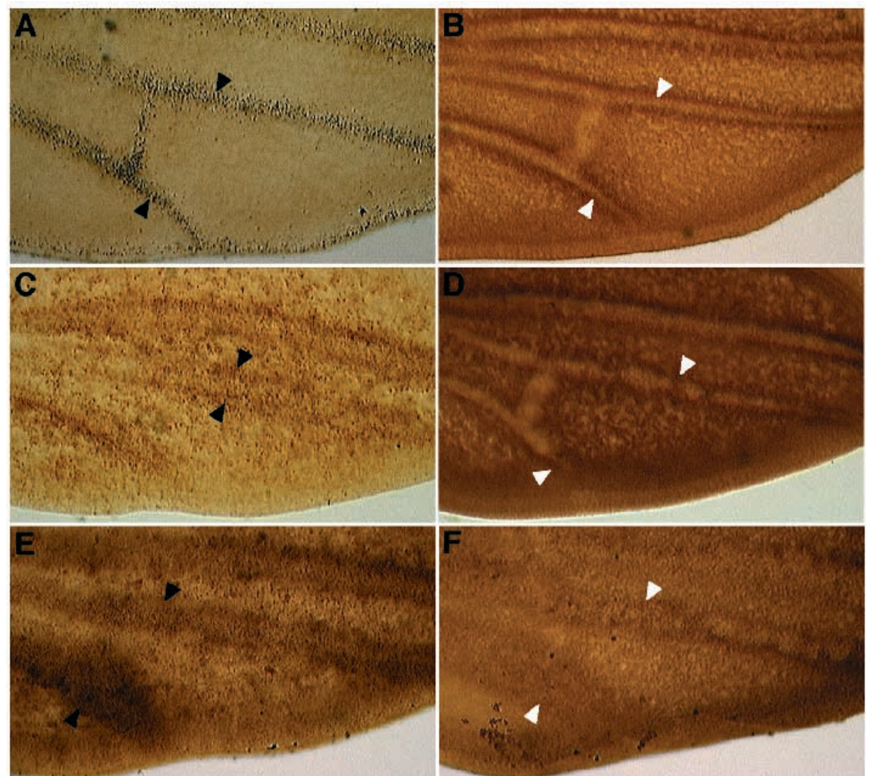

Fig. 5. Effects of ectopic Delta expression on Delta and Notch expression in 30 hours APF wing discs. Animals grown at 25°C. (A,C,E) Delta protein; (B,D,F) Notch protein. (A,B) Discs from wild-type animals with longitudinal veins L4 and L5 marked (Δ or ▲). (C,D) Discs from *UAS::DeltaWT/+; 1348::GAL4/+* animals. (C) Region of L4 marked (▲) where Delta is highest in the lateral provein cells. (D) Longitudinal veins L4 and L5 marked (Δ) where Notch accumulates throughout the provein. (E,F) Discs from *1348::GAL4/+; UAS::DeltaD/+* animals with longitudinal veins L4 and L5 marked (Δ or ▲).

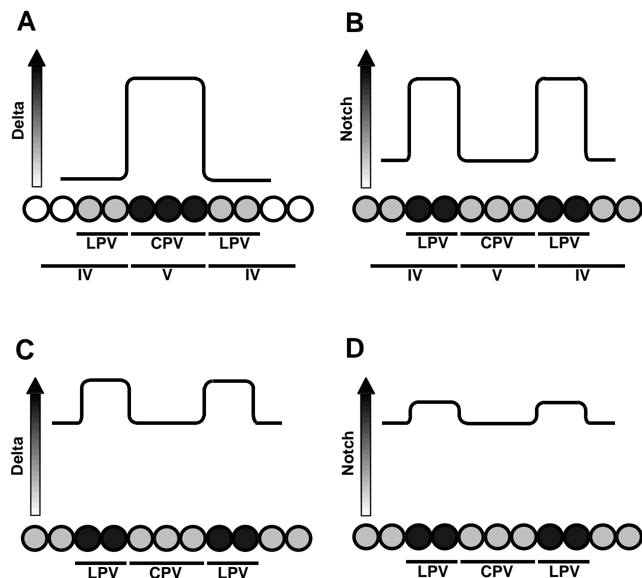

Fig. 6. A model for Delta-mediated signalling in the provein. (A,B) Wild-type situation; (C,D) the situation in which Delta is driven ectopically in intervein (IV) cells by *1348::GAL4*. (A,C) Delta protein expression; (B,D) Notch protein expression. (A) Delta protein levels are maximal in the central provein cells (CPV); very low levels are detected in the lateral provein cells (LPV). (B) Notch expression is complementary to that of Delta; maximal protein levels are observed in the LPV and the lowest levels are detected in the CPV. (C) When Delta is expressed under the control of *1348::GAL4*, Delta accumulates to higher levels in the LPV (presumptive intervein cells) than in the CPV (presumptive vein cells, V). (D) This leads to a higher level of Notch expression in the CPV compared to that in wild-type animals (B). Elevated Notch expression in CPV cells leads to reception of a neurogenic signal by those cells. This signal inhibits them from stably adopting the vein cell fate. High levels of Notch are also observed in the intervein, where *1348::GAL4* mediates ectopic Delta expression. Note: shadings of circles (i.e., cells) represent levels of protein expression relative to those in adjacent cells. More darkly shaded cells express higher levels of a given protein than more lightly shaded cells.

the most extensive vein loss in *P[hs-N(intra)]* animals: a heat pulse at 37°C from 26 to 28 hours APF (Fig. 1D). Notch receptor activation during this interval leads to drastic reductions in Delta expression (Fig. 4C) in comparison to wild-type Delta expression levels (Fig. 4A). We also observe expression and nuclear localization of the Notch intracellular domain fragment, encoded by the P[hs-N(intra)] transposon, throughout the wing blade (Fig. 4D), although it is unclear whether this nuclear localization is relevant to the *N* gain-of-function phenotype that we observe in the wing. These observations imply that Delta expression is negatively regulated by Notch receptor activity within the pupal wing pouch.

The effects of reductions in Delta signalling activity were examined in animals homozygous for the Dl^{EW} allele. This allele appears to be a regulatory mutation that disrupts *Dl* function during wing and eye development, but supports normal *Dl* function in other imaginal contexts (data not shown). Adults homozygous for the Dl^{EW} allele exhibit severe thickening of all veins (Fig. 1G), which appears to arise because all cells within the provein adopt the vein cell fate. Delta protein expression is severely reduced in Dl^{EW} mutant wing blades at 30 hours APF (Fig. 4E) compared to wild-type wing blades (Fig. 4A). Notch expression in lateral proveins fails to increase above levels observed in intervein regions in Dl^{EW} wing blades (Fig. 4F compared to Fig. 4B), implying that wild-type levels of Delta signalling are required for elevated levels of Notch expression in lateral provein cells in the pupal wing blade.

Asymmetries in Delta-Notch signalling are required for vein cell specification

To evaluate the functional significance of complementary patterns of Delta and Notch protein expression during wing vein development, we employed the GAL4-UAS driver-responder system (Brand and Perrimon, 1993) to generate ectopic expression of wild-type and mutated Delta proteins in the wing blade, using the *1348::GAL4* driver. When *UAS::DeltaWT/+; 1348::GAL4/+* animals are raised at 25°C, vein loss is observed in adult wings (Fig. 1E). We observe variable reductions of veins L2, L4 and L5. These data support the hypothesis that increased levels of Delta-mediated signalling preclude adoption of the vein cell fate. Expression of a dominant-negative form of Delta (DeltaD; T. R. Parody and M. A. T. Muskavitch, unpublished data) under control of the *1348::GAL4* driver causes vein thickening (*1348::GAL4/+; UAS::DeltaD/+*; Fig. 1F). All veins seem to be affected by the ectopic expression of this dominant-negative Delta variant. We infer that reduced levels of Delta signalling activity lead to an increase in the number of provein cells that adopt the vein cell fate, at the expense of intervein cells.

Given the dramatic effects of *1348::GAL4*-mediated Delta expression on wing vein morphogenesis, we characterized the *1348::GAL4* driver-mediated expression pattern (data not shown) in a wild-type genetic background using a responder construct that encodes a nuclear-targeted form of β-galactosidase (i.e., *UAS::βGalNUC*; see Materials and Methods). Initial patchy *1348::GAL4*-mediated expression is evident throughout the wing blade circa 12 hours APF. Expression begins to be limited to intervein areas circa 18 hours APF and is restricted to interveins by 30 hours APF (see also de Celis, 1997). Initial intervein-restricted expression evident circa 18 hours APF appears highest in regions corresponding to 'intervein bands' (Fristrom et al., 1994), and expression becomes more evenly distributed among intervein cells as pupal development proceeds.

We also examined the impacts of ectopic *1348::GAL4*-mediated Delta signalling on Delta and Notch expression during pupal vein development. Elevated intervein expression of Delta in *UAS::DeltaWT/+; 1348::GAL4/+* wing blades (Fig. 5C) leads to higher levels of Notch expression in intervein cells 30 hours APF (Fig. 5D) in comparison to wild-type wing blades (Fig. 5B). This suggests that Delta-mediated signalling increases expression of the Notch receptor in pupal wing blade cells. We also find that this ectopic Delta signalling leads to elevated Delta expression in two stripes of cells – the lateral provein cells – (Fig. 5C, note the arrowheads) that flank the region within which maximal Delta expression occurs in wild-type wing blades at 30 hours APF (Fig. 5A). This, in turn, causes Notch expression within presumptive vein cells to increase (Fig. 5D, note the arrowheads where Notch accumulates to uniformly high levels across the provein region), compared to that observed in wild-type wing blades (Fig. 5B). It therefore appears that Delta expression in intervein cells leads

to the reconfiguration of Delta and Notch expression patterns within and around the provein such that neurogenic signalling can lead to inhibition of adoption of the vein cell fate throughout the provein. Ectopic expression of a dominant-negative form of Delta in *1348::GAL4/+; UAS::DeltaD/+* wing blades (Fig. 5E) leads to reduced Notch expression within the lateral provein cells, such that Notch accumulates at uniformly low levels across the provein (Fig. 5F). Therefore, reduced neurogenic signal reception in lateral provein cells results in a failure to elevate Notch expression in lateral provein cells and a failure to inhibit adoption of the vein cell fate within the lateral provein.

DISCUSSION

The roles of neurogenic signalling in wing vein development

Our phenocritical period analysis implies that Delta-mediated neurogenic signalling is required for the final resolution of cell fates within the provein between 22 and 30 hours APF at 25°C. This correlates with a temperature-sensitive period for wing vein development defined by Shellenbarger and Mohler (1978) using the $l(1)N^{ts1}$ mutation. Rebay et al. (1993) also observed wing vein thickening during this time, after inducing expression of a membrane-associated Notch variant that lacks the extracellular domain. This vein-thickening phenotype is similar to the so-called 'Confluens' phenotype, which results from hyperploidy for the wild-type *N* gene (Welshons, 1965) and probably reflects titration of factors limiting for Delta-Notch signalling (Muskavitch, 1994). In contrast, expression of the constitutively active Notch(intra) variant produces the opposite phenotype, wing vein loss, as would be expected if constitutive Notch signalling inhibits adoption of the vein cell fate throughout the provein. We also note that a comparable, but earlier, induction of wing vein loss was observed by Lyman and Yedvobnick (1995) following expression of a different constitutively active Notch intracellular domain variant (Lieber et al., 1993) from 16 to 24 hours APF. Sturtevant and Bier (1995) also found that reductions in *Dl* or *N* function during the early pupal stage led to broadening of expression domains for *rhomboid*, a marker for wing veins, in the wing blade. These results, in aggregate, indicate that Delta signalling and Notch receptor activity function during the same interval during pupal wing vein development and imply that Delta acts as the signal for the Notch receptor during the neurogenic signalling process required to limit the number of vein cells that are specified among the 'vein-competent' cell population within the provein. These phenocritical periods correlate with the developmental interval during which wing veins are progressively emerging, as cells determined to become intervein cells become apposed by extending basal processes (Fristrom et al., 1993). Thus, Delta-Notch-mediated neurogenic signalling functions to partition cells within the provein between vein and intervein cell fates, presumably by inhibiting the lateral provein cells from adopting the vein cell fate until the proper number of intervein cells have been specified.

A feedback mechanism regulates levels of Delta and Notch protein expression in cells involved in neurogenic signalling

Heitzler and Simpson (1991) proposed that Delta and Notch expression could be subject to feedback regulation by Delta-Notch signalling, leading to complementary patterns of Delta and Notch expression, based on somatic mosaic analysis of Delta and Notch functions during microchaeta development. Seydoux and Greenwald (1989) had previously suggested that expression of lin-12, a Notch homolog, could be subject to signalling-dependent feedback regulation, based on somatic mosaic analysis in *C. elegans*. Wilkinson et al. (1994) subsequently found that lin-12 receptor activity represses activation of transcriptional regulatory sequences of *lag-2*, which encodes an apparent ligand for the lin-12 receptor (Tax et al., 1994; Henderson et al., 1994), implying that signalling activity exerts negative feedback regulation on ligand expression for the lag-2/lin-12 ligand/receptor pair.

We present three lines of evidence supporting the hypothesis that Delta-Notch signalling exerts feedback control on Delta and Notch expression in wing vein morphogenesis. First, Delta and Notch protein expression patterns are generally complementary within the developing wing blade. In general, cells that express higher levels of Delta and lower levels of Notch are flanked by cells that express higher levels of Notch and lower levels of Delta. This complementarity becomes more pronounced as pupal development proceeds and proveins are partitioned into vein and intervein cell types. We observe similar regionally complementary patterns of Delta and Notch expression in the pupal notum during sensory organ precursor specification (Parks et al., 1997). Second, elevated Notch expression is dependent on Delta activity in two cell populations in the pupal wing. The Dl^{EW} mutation, which drastically reduces Delta expression in presumptive vein cells, leads to the absence of elevated Notch expression in lateral provein cells. In addition, when a dominant-negative form of Delta is expressed in lateral provein cells, Delta-mediated signalling is hindered, leading to the absence of elevated Notch expression in lateral provein cells. Elevated Notch expression in lateral provein cells is therefore dependent on Delta activity in presumptive vein cells. Elevation of Delta expression in intervein cells leads to elevated Notch expression in these cells, relative to the levels of Notch expression observed in intervein cells in wild-type pupal wings. These observations imply that Delta-mediated signalling from presumptive vein cells positively regulates Notch expression in lateral provein cells and that Delta-mediated signalling in intervein cells can positively regulate Notch expression in these cells. Third, constitutive activation of the Notch receptor, mediated by uniform expression of a gain-of-function Notch(intra) variant (Struhl et al., 1993), represses Delta expression in the pupal wing blade. Analogous repression of Delta expression is observed after ectopic Notch activation in the pupal notum (Parks et al., 1997) and retina (A. L. Parks and M. A. T. Muskavitch, unpublished data). These findings imply that Delta-Notch signalling activates Notch expression and represses Delta expression in lateral provein cells during pupal wing vein development, and indicate that Delta-Notch signalling exerts feedback regulation on Delta and Notch expression within the provein anlage. Our data suggest that feedback regulation of ligand and receptor expression may be a general property of Notch-mediated signalling mechanisms in *Drosophila*.

Our findings regarding Delta and Notch expression in presumptive vein cells raise the intriguing possibility that these cells, which exhibit elevated Delta expression and reduced Notch expression, are refractory to this Delta-Notch-mediated feedback regulation. The fact that presumptive vein cells are capable of

responding to Notch receptor activity is demonstrated by the repression of Delta expression in these cells that we observe following ectopic induction of constitutive Notch receptor activity in pupal wings. However, levels of Delta in a wild-type genetic background may be sufficient to eliminate, in a cell-autonomous manner, the ability of these cells to receive a normally regulated Delta-Notch signal. Such cell-autonomous inhibition of neurogenic signal reception has been described by Micchelli et al. (1997). Cells that express high levels of Delta or Serrate along the developing wing margin do not exhibit the elevated expression of cut and wingless normally induced by the receipt of neurogenic signals, even though expression of margin-like levels of cut and wingless are induced in cells that lack Delta and Serrate and are adjacent to highly expressing cells. Within the provein, analogous inhibition of neurogenic signal reception in presumptive vein cells would lead to the observed persistence of elevated levels of Delta expression and a failure to induce increased Notch expression in central provein cells.

Asymmetry in Delta and Notch protein expression is required for wing vein development

Asymmetries in Delta and Notch expression – and in the generation and reception of neurogenic signals – appear to underlie the partitioning of vein and intervein cell fates within the pupal provein. The juxtaposition of cells that express elevated levels of Delta or Notch, respectively, establishes a boundary condition that arises as a result of feedback regulation by neurogenic signalling of Delta and Notch expression in lateral provein cells. Presumptive vein (i.e., central provein) cells, on one side of this boundary, express elevated levels of Delta and send the neurogenic signal. Presumptive intervein (i.e., lateral provein) cells, on the other side, express elevated levels of Notch and receive this signal, which inhibits adoption of the vein cell fate by lateral provein cells.

Disruption of the wild-type pattern of asymmetric Delta and Notch expression within the provein, by means of ectopic elevation of Delta expression in lateral provein cells using the GAL4-UAS system, can lead to the failure of presumptive vein cells to stably adopt the vein cell fate. Elevation of Delta expression in the lateral provein disrupts the Delta-Notch boundary condition that exists in wild-type proveins (Fig. 6). As a result of this disruption, lateral provein (i.e., presumptive intervein) cells express higher levels of Delta than central provein (i.e., presumptive vein) cells, and central provein cells exhibit elevated levels of Notch expression compared to their counterparts in wild-type proveins. This implies that Delta expression in lateral provein cells can induce elevated Notch expression in central provein cells when levels of Delta expression in presumptive intervein cells exceed levels in presumptive vein cells. This newly induced ability of central provein cells to receive a neurogenic signal can apparently lead to inhibition of adoption of the vein cell fate within the proveins for L2, L4 and L5. Adult veins are therefore shortened or gapped because central provein, as well as lateral provein, cells are now inhibited from adopting the vein cell fate.

Qualitative characteristics of Delta signalling during vein development

The fact that Delta expression in central provein cells affects specification of lateral provein cells in wild-type wing discs provides additional evidence for the nonautonomy of Delta function in vivo (Heitzler and Simpson, 1991; Parks et al., 1995). We also find that expression of Delta in intervein (i.e., lateral provein) cells, in *1348::GAL4/+;UAS::DeltaWT/+* animals, inhibits adoption of the vein cell fate by central provein cells. This outcome must also reflect nonautonomous Delta function within the provein because expression of constitutively active Notch receptor [i.e., Notch(intra)] under control of the *1348::GAL4* driver does not inhibit the development of vein cells (S. S. Huppert, T. L. Jacobsen, and M. A. T. Muskavitch, unpublished data). If the silencing of vein cell fate in *1348::GAL4/+;UAS::DeltaWT/+* animals were the result of cell-autonomous neurogenic signalling, then expression of the constitutively active Notch receptor under control of the same driver would also inhibit adoption of the vein cell fate.

Serrate, another Notch ligand (Rebay et al., 1991), can substitute for Delta in some developmental contexts (Gu et al., 1995; T. L. Jacobsen and M. A. T. Muskavitch, unpublished data). However, we find that expression of Serrate under control of the *1348::GAL4* driver exerts no discernible effects on Delta and Notch expression patterns or on the specification of vein cell fates (data not shown). This implies that Serrate cannot function as an effective ligand for Notch in the signalling process that underlies partitioning of vein and intervein cell fates within the provein during metamorphosis. In this developmental context, then, Serrate cannot substitute for Delta as an activator of neurogenic signalling.

Our observation that veins develop in the virtual absence of pupal Delta expression in the Dl^{EW} genetic background implies that Delta function, per se, is not required for direct specification of the vein cell fate or vein cell differentiation in metamorphic wing discs. Within the pupal provein, Delta function is required solely to prevent adoption of the vein cell fate by lateral provein cells. This is consistent with the observation that morphologically and functionally normal anchor cells develop even when lag-2 activity is severely reduced by mutation in *C. elegans* (Lambie and Kimble, 1991). In these two instances, neurogenic signaling activity is required to modulate the adoption of cell fates, but is not required for the differentiation of particular cell types after they have stably adopted their respective fates.

Finally, our data reveal that there are qualitative differences within the provein among the relationships between signalling activity and feedback regulation of receptor expression for the three signal transduction pathways known to influence wing vein development during metamorphosis. Delta/Notch signalling activity leads to up-regulation of receptor expression in lateral provein cells. In contrast, dpp/TKV (de Celis, 1997) and DER (Sturtevant et al., 1994)-dependent signalling are believed to lead to the down-regulation of receptor expression in central provein cells. This implies that ligand-dependent signalling, for TKV and DER pathways, within the provein may be self-limiting and required for only brief periods during wing vein development. In contrast, Delta-dependent signalling within the provein may be required for an extended period of time during metamorphosis for the correct partitioning of vein and intervein cell fates.

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