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# Structural requirements for Notch signalling with Delta and Serrate during the development and patterning of the wing disc of *Drosophila*

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## SUMMARY

**The Delta and Serrate proteins interact with the extracellular domain of the Notch receptor and initiate signalling through the receptor. The two ligands are very similar in structure and have been shown to be interchangeable experimentally; however, loss of function analysis indicates that they have different functions during development and analysis of their signalling during wing development indicates that the Fringe protein can discriminate between the two ligands. This raises the possibility that the signalling of Delta and Serrate through Notch requires different domains of the Notch protein. Here we have tested this possibility by examining the ability of Delta and Serrate to interact and signal with Notch molecules in which different domains had been deleted. This analysis has shown that EGF-like repeats 11 and 12, the RAM-23 and cdc10/ankyrin repeats and the region C-terminal to the cdc10/ankyrin repeats of Notch are necessary for both Delta and Serrate to signal via Notch. They also indicate, however, that Delta and Serrate utilise EGF-like repeats 24-26 of Notch for signalling, but there are significant differences in the way they utilise these repeats.**

Key words: Notch, Delta, Serrate, *Drosophila*, Signalling, Wing development

## INTRODUCTION

The *Notch* gene of *Drosophila* encodes a large protein with a single transmembrane region that has been shown to act as a receptor in various developmental events (reviewed in (Artavanis Tsakonas et al., 1995). For example, Notch is required during neurogenesis and myogenesis in the embryo, where its function has been shown to be central to the selection of neural or myogenic precursors from pools of developmentally equivalent cells (reviewed in (Baylies et al., 1998; Jan and Jan, 1993). In these processes, the product of the *Delta* gene acts as the ligand of Notch. Notch also functions during the development and patterning of the legs (Couso and Martinez Arias, 1994; de Celis et al., 1998; Sotillos et al., 1997) and the wings (reviewed in Irvine and Vogt, 1997) of Drosophila. In these instances Notch uses two ligands, the products of the *Delta* and *Serrate* genes, to direct the expression of genes that fuel growth and patterning of these structures.

Delta and Serrate proteins are structurally very similar with both proteins containing a single transmembrane domain, a small intracellular domain and large extracellular regions comprising epidermal growth factor (EGF)-like repeats, whose numbers differ between the two proteins, and an amino-terminal region, which is required for the interaction with Notch (Artavanis Tsakonas et al., 1995). Although both ligands can interact with Notch and substitute for each other in specific experimental conditions (Gu et al., 1995; Jacobsen et al., 1998; Klein and Martinez Arias, 1998), loss of function analysis indicates that they have different roles during development (Fleming et al., 1990; Thomas et al., 1991; Vässin et al., 1987; Vässin and Campos Ortega, 1987), and analysis of their signalling during wing development indicates that the Fringe protein can discriminate between the two ligands (Fleming et al., 1997; Klein and Martinez Arias, 1998; Panin et al., 1997). This raises the possibility that signalling of Delta and Serrate through Notch may be different and that the two proteins may require different regions of the Notch protein to generate a signal.

The Notch protein contains a number of conserved sequences whose functional requirements have been studied both genetically and biochemically. The extracellular domain contains 36 EGF-like repeats and three cystine-rich repeats called lin-12/Notch repeats (Kidd et al., 1986; Wharton et al., 1985). Genetic analysis of these domains has identified three regions that are functionally important: one centred around EGF-like repeats 11 and 12, a second one around EGF-like repeats 25 and 26 and a third one in the lin-12/Notch repeats (Brennan et al., 1997; De Celis et al., 1993; Hartley et al., 1987; Kelley et al., 1987; Lieber et al., 1993; Lyman and Young, 1993; Rebay et al., 1993). The first region is highlighted by loss of function mutations (Brennan et al., 1997; De Celis et

al., 1993; Hartley et al., 1987; Kelley et al., 1987; Lieber et al., 1993; Rebay et al., 1993), suggesting that this region is required for signalling. The second one is highlighted by a complex class of mutations, the *Abruptex* class, which display features of both gain and loss of function (Brennan et al., 1997, 1999; Kelley et al., 1987; Lieber et al., 1993), suggesting that this region may regulate signalling. Mutations in the lin-12/Notch repeats suggest that this domain is also required to regulate signalling through the Notch protein (Brennan et al., 1997; Lieber et al., 1993; Lyman and Young, 1993). Cell culture experiments designed to measure interactions between Notch and the Delta and Serrate proteins have extended the genetic experiments and shown that EGF-like repeats 11 and 12 are required for the direct interaction between Notch and the Delta and Serrate proteins (Lieber et al., 1992; Rebay et al., 1991), and that EGF-like repeats in the *Abruptex* region can influence this interaction (Lieber et al., 1992).

The intracellular domain of Notch contains three identifiable domains: the RAM-23 domain adjacent to the transmembrane domain, six cdc10/ankyrin repeats and a region C-terminal to the cdc10/ankyrin repeats. Genetic analysis of *Notch* alleles that mutate this region identified two major functional domains: one centred around six cdc10/ankyrin repeats that is required for signalling via Notch (Brennan et al., 1997; Lieber et al., 1993; Lyman and Young, 1993), and a second one C-terminal to this motif of unclear function, although it has been suggested that Wingless signalling can prevent Delta signalling via Notch through this region (Axelrod et al., 1996). Two hybrid and immunoprecipitation experiments have shown that these regions interact with a number of different cytoplasmic proteins. For example, it has been demonstrated that the RAM-23 domain interacts with Suppressor of Hairless (Tamura et al., 1995), the cdc10/ankyrin repeats interact with Deltex (Diederich et al., 1994) and the region C-terminal to the cdc10/ankyrin repeats interacts with Dishevelled (Axelrod et al., 1996).

Although these studies have highlighted domains of Notch that are required for its function, they have not examined which domains are specifically required for Delta and Serrate to signal, nor have they examined if there is a difference in the regions of the Notch protein required for Delta and Serrate to signal via Notch. Here we have performed an in vivo analysis of the different domains of Notch in two different functional assays for Delta and Serrate signalling. Our results demonstrate that EGF-like repeats 11 and 12, the RAM-23 and cdc10/ankyrin repeats and the region C-terminal to the cdc10/ankyrin repeats are necessary for both Delta and Serrate to signal via Notch. These studies also indicate that Delta and Serrate utilise EGF-like repeats 24-26 for signalling, but that there are differences in the way they utilise these repeats.

## MATERIALS AND METHODS

### Construction of the DNA molecules encoding the ECN and FLN proteins

All the UAS*ECN* and UAS*FLN* DNA constructs (see Fig. 1) are derivatives of two vectors, pUAST+ECN and pUAST+FLN. The generation of pUAST+ECN and the deletions of the EGF-like repeats have been described previously (Brennan et al., 1999; Jacobsen et al., 1998). The pUAST+FLN vector was obtained by cloning the region of the *Notch* cDNA encoding the intracellular domain of the Notch protein (Lieber et al., 1992) into the pUAST+ECN vector as a *Bgl*II/*Xba*1 fragment to reconstruct the complete cDNA. All the deletions within the extracellular domain were introduced into pUAST+FLN as a *Kpn*1/*Bgl*II fragment of the *Notch* cDNA, which contains EGF-like repeats 3-29, obtained from the corresponding pUAST+ECN vectors. The deletions of the RAM-23 and cdc10/ankyrin repeats and the region C-terminal to cdc10/ankyrin repeats have been produced previously in *Notch* cDNAs whose expression is regulated by a heat shock-inducible promoter (Lieber et al., 1993). These deletions were introduced into the full-length *Notch* cDNA in the pUAST vector by replacing the wild-type *Bgl*II/*Xba*1 fragment of pUAST+FLN, which encodes the intracellular domain, with the *Bgl*II/*Xba*1 fragments of the *Notch* cDNA, which contain the intracellular deletions.

### *Drosophila* strains used

For every UAS*ECN* and UAS*FLN* construct injected several (at least five in each case) transformant lines were obtained and were tested for activity with various GAL4 lines. Those with the strongest effects were selected and are the ones used in these studies. For any given line the phenotypes obtained after overexpression were reproducible. In addition to the UAS*ECN* and UAS*FLN* stocks, the UAS*Dl* (Seugnet et al., 1997) and UAS*Ser* (Speicher et al., 1994) stocks were used. The GAL4 driver stocks used were *ptc*GAL4 and *ptc*GAL4*;spd*lacZ*/SM6a-TM6B*. The two stocks cause the expression of the different UAS constructs on the anterior side of the anterior/posterior compartment boundary of the wing (see Fig. 6A) (Speicher et al., 1994). *spd*lacZ allows the activity of the *spd* enhancer of the *wingless/wnt4* locus to be monitored by the expression of a β-gal reporter gene (Neumann and Cohen, 1998). The levels of ectopic expression achieved using the UAS/GAL4 system has been shown to be temperature-dependant. The overexpression experiments involving UAS*Ser* were carried out at 25°C. The levels of ectopic Serrate induced at this temperature led to a consistent effect on reporter expression that could be easily be scored, but the expression of UAS*Dl* at 25°C causes lethality and therefore we used the lower temperature of 17°C for experiments involving Delta. Overexpression of UAS*Dl* at 17°C results in flies with a consistent phenotype. Overexpression of the different Notch constructs at this temperature will, by necessity, result in lower levels of expression than at 25°C. For this reason we cannot compare the effects of Notch on Delta or Serrate although we can compare the effects of the different Notch constructs on the action of Ser or Dl alone; for a given ligand, all experiments were performed at the same temperature.

### Wing preparations

The flies used for wing preparations were collected and stored in SH solution (25% glycerol and 75% ethanol). Wings were prepared by removing them from the notum with watchmaker's forceps in a dissecting dish containing tap water and were mounted in Hoyer's medium (Ashburner, 1989). For flies that died as pharates, the wings were initially inflated by cooking the whole fly, once the pupal membrane had removed from the wings, in 10% NaOH (w/v) for 1 hour on a hot plate at 60°C in a dissecting dish. Once the wings were inflated the flies were moved into a dissecting dish containing tap water. The wings were then mounted in the same manner as described above.

### Notch expression in third instar imaginal discs

The expression of the FLN constructs was detected in discs using either the C17.9C6 monoclonal mouse antibody raised against the cdc10/ankyrin repeats (Fehon et al., 1990) or the F461.3B monoclonal mouse antibody raised against the extracellular domain of the Notch protein (gift from S. Artavanis Tsakonas). Polyclonal guinea pig anti-Coracle antibody was used to mark the cell membranes (Fehon et al., 1994). Larvae were dissected in cold PBS and immediately fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature.

After washing in 0.3% Triton X-100 in PBS and blocking in 1% BSA, 0.3% Triton in PBS for 1 hour at room temperature, specimens were incubated overnight at 4°C with the anti-Notch antibodies together with anti-Coracle diluted in PBS containing 0.3% Triton X-100 and 1% BSA. The C17.9C6 antibody was diluted 1:1000 and the F61.3B antibody was diluted 1:500. The Coracle antibody was used at a concentration of 1:2000. Specimens were then washed, blocked and incubated with biotinylated anti-guinea pig secondary antibody (Vector) diluted 1:200 and Cy5-conjugated anti-mouse secondary antibody (Jackson) diluted 1:250 in PBS containing 0.3% Triton X-100 and 1% BSA for 1 hour at room temperature. Following washing and a further 30-minute blocking period, specimens were then incubated with FITC-conjugated streptavidin (Jackson) diluted 1:250 in PBS containing 0.3% Triton X-100 and 1% BSA for 30 minutes at room temperature. They were then washed for a further 30 minutes before mounting in Vecta Shield (Vector). Specimens were examined under the confocal microscope (Leica). The gain of the microscope was kept constant to allow a qualitative comparison between relative levels of fluorescence.

### β-galactosidase activity and expression

The activity of the *spd* enhancer of the *wingless/wnt4* locus was determined by monitoring the expression of a β-gal reporter gene. The expression of β-gal was determined in two ways. The first was by X-gal staining. Selected larvae were dissected in cold PBS and fixed for 4 minutes in 2.5% glutaraldehyde in PBS. After washing in 0.3% Triton X-100 in PBS the presence of the lacZ protein was revealed using X-gal following standard procedures (Ashburner, 1989). The specimens were then mounted in 75% glycerol.

In addition we used a rabbit anti β-galactosidase antibody (Cappel) at a dilution of 1:7500 and Texas Red-conjugated anti-rabbit secondary antibody (Jackson) at a dilution of 1:250 (procedure as described above). Specimens were examined under the confocal microscope (Leica). The gain of the microscope was kept constant to allow a qualitative comparison between relative levels of fluorescence.

## RESULTS

We sought to identify the regions of the Notch protein required by Serrate and Delta to signal in vivo. We used two different approaches to do this. Firstly, we have studied the ability of Delta and Serrate to signal when coexpressed with different dominant negative Notch molecules. We reasoned that the expression of dominant negative Notch molecules with either Delta or Serrate will reduce the ability of Delta and Serrate to signal. Dominant negative Notch molecules lacking regions mediating the interaction with the ligands should not interfere with the signalling event. In these experiments, the expression of the different molecules has been driven with *ptc*GAL4, which directs their expression in a stripe along the anterior/posterior boundary of the developing wing.

Secondly, we have made use of an in vivo assay for signalling that allows us to examine if Delta and Serrate can signal with a particular Notch molecule. The definition and development of the wing margin require both Delta and Serrate

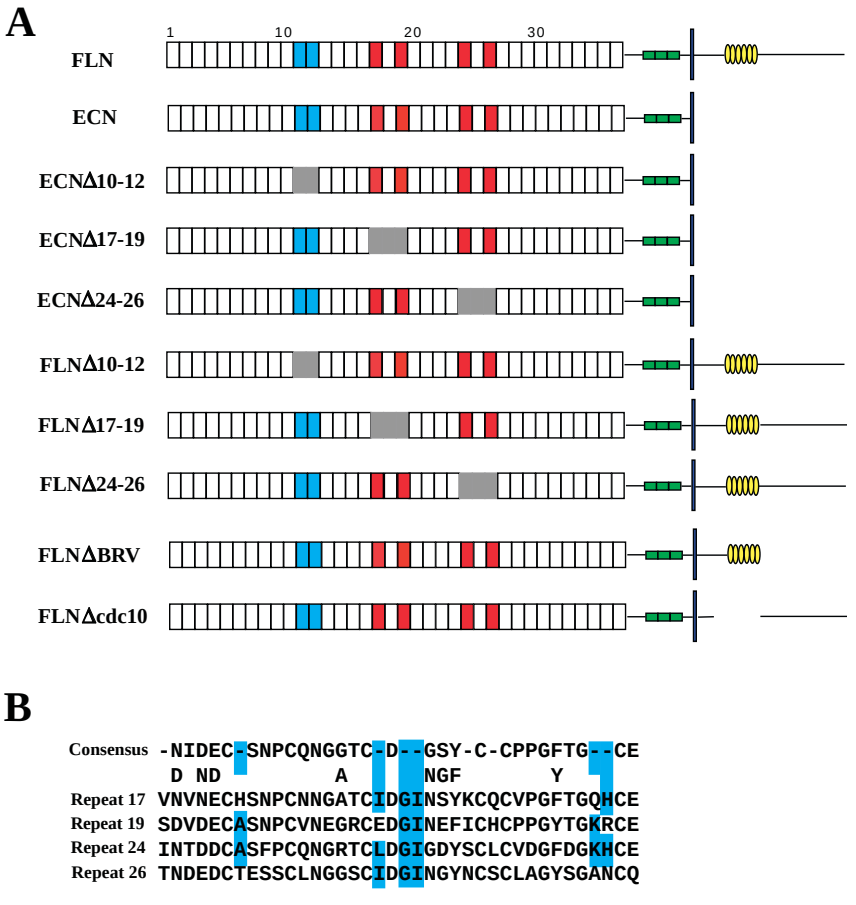


**Fig. 1.** Structural elements of Notch and deletions of EGF repeats in dominant negative (ECN, extracellular notch) and full-length Notch (FLN) molecules used in this study. (A) Schematic diagram showing the full-length Notch molecule and the Notch constructs used in this study. The extracellular domain contains 36 tandemly arranged EGF-like repeats and three Cys-rich domains in the neighbourhood of the transmembrane domain. In the intracellular domain the ovals indicate six cdc10/ankyrin repeats that have been shown to be essential for function. EGF repeats 11 and 12, which are involved in the interaction with Delta, are shaded in blue, and EGF repeats 17, 19, 24 and 26, which are structurally (see B), and possibly functionally, related are highlighted in red. Δ10-12, Δ17-19 or Δ24-26, deletion of the EGF-like repeats 10-12, 17-19 or 24-26, respectively; Δcdc10, a deletion of the RAM-23 domain and the cdc10/ankyrin repeats; ΔBRV, a deletion of the Dishevelled binding domain using *Bam*HI and *Eco*RV restriction sites within the intracellular domain. All constructs were cloned into the pUAST vector (see Materials and Methods). (B) Alignment of EGF repeats 17, 19, 24 and 26 with the consensus EGF repeat of *Drosophila* Notch (modified from Kidd et al., 1986). The hyphens indicate residues for which a consensus cannot be drawn. A consensual residue is defined when it is present at a given position at least in 10 of the 36 EGF repeats (Tateson, 1998; Kidd et al., 1986). The areas that form a $Ca^{2+}$ binding pocket are the most variable regions between EGF repeats and therefore could govern the proteins which an individual repeat can interact with. We observe sequence conservation in these regions between EGF repeats 17, 19, 24 and 26, which suggests that they might be involved in a related interaction.

signalling through Notch, and result in the activation of margin markers such as *wingless*. Disruption of Delta or Serrate signalling leads to the loss of margin marker expression and the failure of margin development, resulting in a nick in the adult wing (Couso et al., 1995; De Celis et al., 1996; Diaz Benjumea and Cohen, 1995; Doherty et al., 1996; Kim et al., 1995). On the other hand, overexpression of Delta or Serrate in the developing wing leads to the ectopic expression of margin markers (see Figs 6C and 5C, respectively; Doherty et al., 1996; Panin et al., 1997). However coexpression of Delta or Serrate with Notch leads to greater ectopic expression of the margin markers and in the case of Serrate to signalling over the dorsal surface where it is otherwise unable to signal (see Figs 6D and 5D, respectively; Klein et al., 1997; Klein and Martinez Arias, 1998). Therefore we reasoned that by combining Delta and Serrate with Notch molecules deleted for specific domains in this assay, we should be able to define structural requirements for signalling. Expressing a Notch protein lacking a region required for signalling with Delta or Serrate should not increase the signalling ability of the ligands on their own. To assay Delta and Serrate signalling, we overexpressed the different proteins under the control of *ptc*GAL4 and we used as a reporter an enhancer of the *wg/wnt4* locus (*spd*lacZ) that is expressed at the wing margin under the control of Notch signalling (Neumann and Cohen, 1996).

This work focuses on three different deletions within the EGF-like repeats found in the extracellular domain of Notch (see Fig. 1A). The first removes EGF-like repeats 10-12, which have been shown previously in cell culture experiments to be required for Notch to interact with Delta and Serrate (Lieber et al., 1992; Rebay et al., 1991); the second removes repeats 24-26, a region to which many of the *Abruptex* mutations have been mapped (Brennan et al., 1997; De Celis and Garcia Bellido, 1994; Kelley et al., 1987); and the third removes EGF-like repeats 17-19, which we have observed to be related in sequence to 24-26 (see Fig. 1B). In addition we tested a Notch molecule lacking both the 17-19 and 24-26 repeats, because we felt that the structural similarity between these repeats might conceal some functional redundancy. We have also studied the effects of two deletions in the intracellular domain (see Fig. 1A). One removes the RAM-23 domain and cdc10/ankyrin repeats, which previous experiments have indicated are required for the interaction with Su(H) and the transcription of genes dependent upon Delta and Serrate signalling (Hsieh et al., 1996; Jarriault et al., 1995; Tamura et al., 1995). The other removes the domain C-terminal to the cdc10/ankyrin repeats, which has been shown to interact with the Dishevelled protein and to be involved in the regulation of Delta signalling via Notch (Axelrod et al., 1996).

## EGF-like repeats 10-12 and 17-26 are required for interactions with ligands

We have examined whether EGF-like repeats 10-12, 17-19 and 24-26 of Notch are required for its interaction with Delta and Serrate by making use of the first assay described above. The basic dominant negative molecule used in this experiment was a Notch molecule that consists of the extracellular and transmembrane (ECN) domains only (see Fig. 1). As this molecule lacks the intracellular domain, it is expected to be unable to transduce the signal of interacting ligands and as a consequence should reduce ligand signalling when

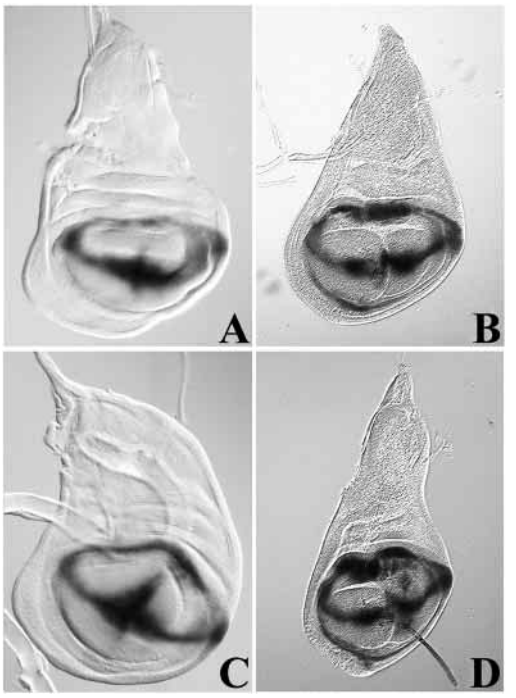


**Fig. 2.** Effects of coexpression of FLN and ECN with *ptc*GAL4 on wing development and patterning monitored by the expression of *spd*lacZ in the third larval instar. (A) Expression of *spd*lacZ in a wild-type disc (for comparison). Within the wing pouch *spd*lacZ is expressed in a ring that outlines the hinge and in a stripe along the dorsal/ventral boundary. (B) Overexpression of ECN leads to a disruption of *spd*lacZ expression at the dorsal/ventral boundary. In addition, an ectopic fold is induced in the pouch along the anterior/posterior boundary where the ECN construct is being expressed. (C) Overexpression of full-length Notch has little effect on the expression of *spd*lacZ. (D) Coexpression of full-length Notch and ECN leads to a phenotype very similar to that of expression of ECN alone (compare with 2B).

overexpressed. This appears to be the case, as expression of this molecule does not rescue Notch mutant phenotypes (our unpublished observations) and its overexpression in a wild-type background produces Notch mutant phenotypes (see Figs 2B and 3B; our unpublished observations). In principle, the dominant negative effects of ECN could be due to its forming inactive heterodimers with wild-type receptors, or to titration of ligands that are present in limiting amounts. Coexpression of FLN with ECN does not aleviate the effects caused by ECN alone (Fig. 2D), suggesting that ECN does not simply form heterodimers with the wild-type receptor and that it is likely to cause its effects principally by interacting with ligands. This possibility is further supported because deletion of particular EGF repeats in the ECN molecule alters the phenotypes produced by ECN alone (see below; our unpublished observations), which can only be explained if the effects of the ECNs are mediated by its interactions with Notch ligands.

To test if the ECN protein can interact with Delta and Serrate in vivo, we have coexpressed ECN with the two signal proteins. Expression of either Delta or Serrate alone with *ptc*GAL4 leads to the appearance of an ectopic margin, which runs perpendicular to the extant one and alters the shape of the wing; the new margin is ventral in the case of Serrate (see Fig. 3E)

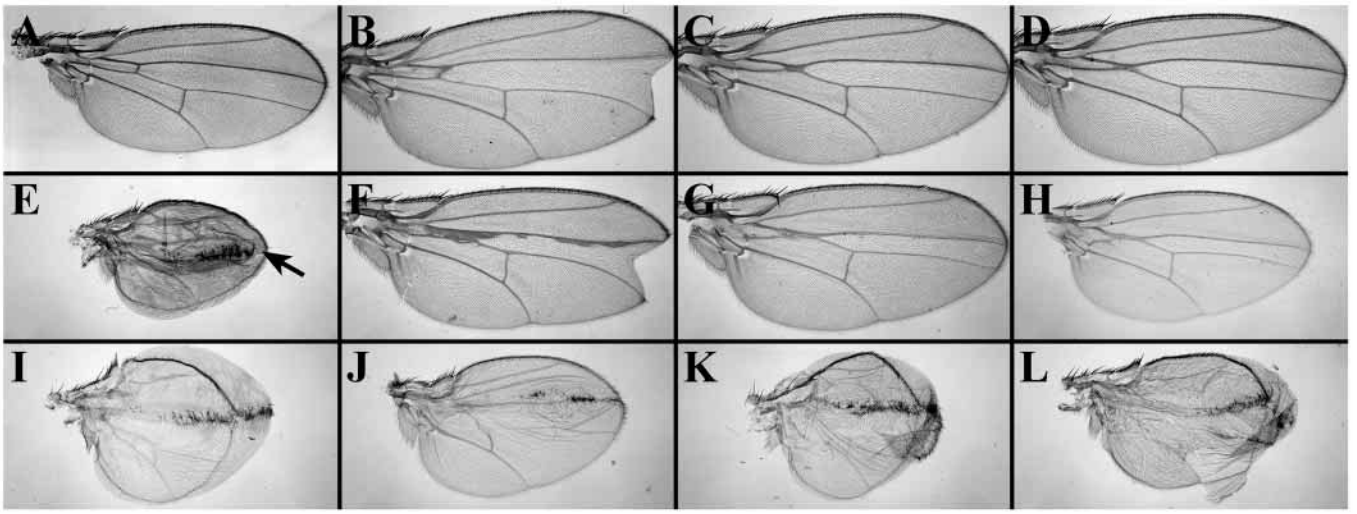


**Fig. 3.** Adult wings resulting from modulations of Notch signalling induced by expression of various extracellular Notch (ECN) constructs driven under the control of *ptc*GAL4 alone or in combination with Delta or Serrate. (A) A wild-type adult wing. (B) Expression of ECN alone leads to a nick at the wing margin between veins 3 and 4. (C) Expression of ECNΔ10-12 leads to a wing with no nick and a fusion between veins 3 and 4, as does expression of a construct harbouring a double deletion for EGF-like repeats 17-19 and 24-26 (D). (E) Ectopic expression of Serrate leads to a small wing with an ectopic margin along the anterior/posterior boundary on the ventral surface. This ectopic margin shows a nick where it meets the endogenous margin (arrow). (F) Coexpression of Serrate and ECN leads to a suppression of the phenotype induced by Serrate alone, suggesting that ECN can titrate this ligand. Coexpression of Serrate with ECNΔ10-12 (G) or ECNΔ17-19,24-26 (H) also has this effect. This suggests that both deletion constructs can still interact with Serrate. (I) Ectopic expression of Delta (Dl) leads again to a small wing pouch with an ectopic margin along the anterior/posterior boundary of both surfaces. (J) Coexpression of Dl and ECN leads to a suppression of the Dl-induced phenotype as the size of the wing shows some rescue and the ectopic margin induced by Dl is weaker and only present on the dorsal surface. (K) If EGF-like repeats 10-12 are deleted from ECN, this suppression upon coexpression with Dl is not seen, suggesting that these repeats are essential for the interaction of ECN and Dl. (L) ECNΔ17-19,24-26 expressed with Dl leads to little if any suppression of the Dl phenotype, suggesting these repeats too may be required for an interaction with Dl.

and is both, dorsal and ventral in the case of Delta (see Fig. 3I). In addition the expression of Serrate with *ptc*GAL4 produces a nick between the ectopic and normal margins that is a consequence of a dominant negative effect of Serrate on Notch (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997). Coexpression of ECN with Delta suppresses the ectopic margin caused by Delta alone, with the exception of a small patch on the dorsal side, and produces an almost wild-type shaped wing (see Fig. 3J). Similarly coexpression of ECN with Serrate eliminates the ectopic margin caused by Serrate alone (see Fig. 3F). In both cases coexpression of the ligand and ECN molecules suppresses the phenotype generated by expressing either ligand alone, suggesting that the ECN molecule can sequester both ligands and that the premise behind this experimental approach is correct.

To test if EGF-like repeats 10-12 and 17-19 and 24-26 are required for interaction with Delta and Serrate, we have coexpressed the ligands with ECN molecules that are deleted for the different EGF-like repeats (ECNΔ10-12 and ECNΔ17-19;24-26 respectively; see Figs 1, 3C,D). Delta and Serrate showed markedly different interactions with these deleted ECN molecules. The effects of ectopic expression of Serrate were suppressed by either ECNΔ10-12 (see Fig. 3G) or ECNΔ17-19;24-26 (see Fig. 3H), although the suppression by ECNΔ17-19;24-26 was not always complete (data not shown). Those of Delta were not, however, suppressed by ECNΔ10-12 (see Fig. 3K), nor very effectively by ECNΔ17-19;24-26 (see Fig. 3L).

These results indicate that Serrate can interact with both the ECNΔ10-12 and ECNΔ17-19;24-26 proteins, whilst Delta cannot interact efficiently with either of these molecules.

### EGF-like repeats 10-12 and 24-26 are required for signalling with Serrate during the development and patterning of the wing

The results presented above indicate that the interaction of Delta and Serrate with the Notch protein may utilise EGF-like repeats other than 11 and 12. To test if these repeats are required for Delta and Serrate to signal via Notch, we made use of the second assay described above.

Expression of the different full-length Notch (FLN) molecules with extracellular deletions along the anterior/posterior boundary of the wing with *ptc*GAL4 has little effect on the expression of *spd*lacZ at the margin (see Fig. 5E,G,I,K). This lack of discernible effect is not due to the absence of the mutated proteins since they are all synthesised to similar levels at the surface of the cells in the wing disc (see Fig. 4B′-H′).

In contrast with the lack of effect of the expression of the different FLN molecules on their own with *ptc*GAL4, expression of Serrate leads to the expression of *spd*lacZ on the ventral side of the wing pouch in two parallel stripes along the anterior/posterior boundary (see Fig. 5C). Coexpression of Serrate and FLN induces very strong expression of the *spd*lacZ reporter in a broad stripe on both the dorsal and ventral sides of the developing pouch, indicating that Serrate signalling is occurring all along the coexpression domain (see Fig. 5D). In contrast, expression of Serrate with FLNΔ10-12, FLNΔ24-26 or FLNΔ17-19;24-26 fails to elicit Serrate signalling on the

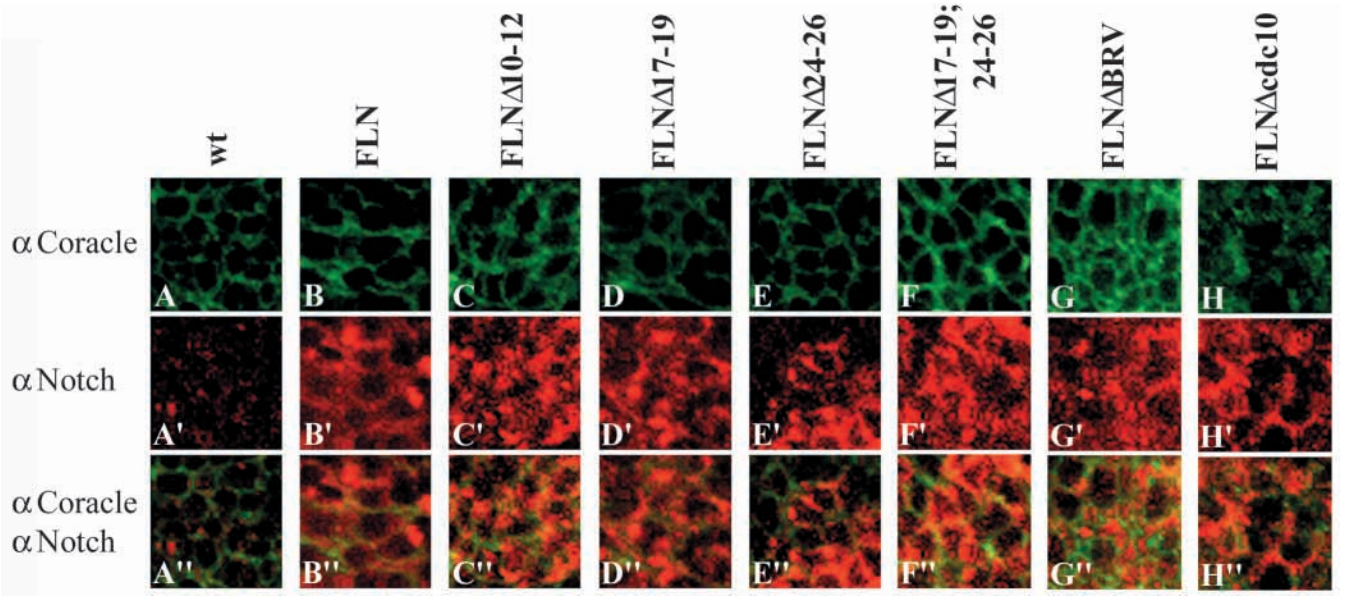


**Fig. 4.** Confocal images of the expression of the different full-length Notch (FLN) proteins under the control of *ptc*Gal4 in third larval instar wing imaginal discs visualized with anti-Notch antibodies (red). Staining with anti-Coracle antibody (green) marks the membrane of the cells (Fehon et al., 1994). In all cases we show a detail of the domain of expression over the patched stripe. (A) Endogenous Notch; (B) FLN; (C) FLNΔ10-12; (D) FLNΔ17-19 (E) FLNΔ24-26; (F) FLNΔ17-19,24-26. The settings of the confocal microscope were kept constant to allow a comparison between these panels to be made; the levels of expression of the different constructs are significantly higher than those of endogenous Notch. The antibody used recognizes the intracellular domain of Notch. In all cases it can be seen that some of the Notch protein being expressed lies in the region of the cell membrane, as shown by areas of overlap between the two channels, although it is not always coincidental with the location of Coracle. (G) FLNΔBRV; (H) FLNΔcdc10. The antibody used to detect expression of these constructs recognizes an extracellular antigen within Notch. As in A-F, the settings of the confocal microscope were kept constant to allow a comparison of the relative levels of expression of the two constructs which, as seen, are expressed at comparable levels.

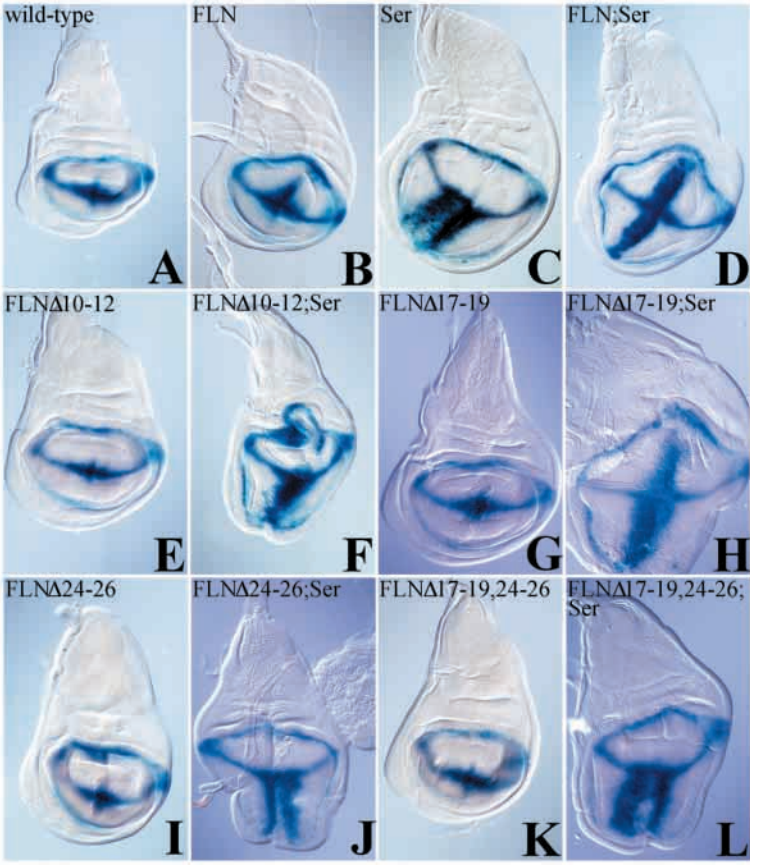


**Fig. 5.** Responses to Notch signalling using Serrate as a ligand, monitored by expression of a *spd*lacZ reporter construct in third larval instar wing imaginal discs at 25°C. All discs are shown with ventral to the bottom and posterior to the left. (A) Expression of *spd*lacZ in a wild-type disc. (B-L) Expression of the reporter in wing discs in which various UAS constructs, as indicated, have been expressed under the control of *ptc*GAL4. (B) Expression of full-length Notch (FLN) has little effect on the expression of *spd*lacZ. (C) Expression of Serrate alone leads to strong ectopic expression of the reporter in the ventral region of the disc either side of the anterior/posterior boundary. At the boundary itself there is a loss of expression due to a dominant negative effect of high levels of Serrate on Notch signalling (Klein et al., 1997). (D) Coexpression of FLN and Serrate leads to strong ectopic expression of the reporter along the anterior/posterior boundary in the dorsal and ventral regions of the disc, suggesting that a functional Notch signal is being produced. (E,G,I,K) The Notch constructs harbouring deletions in the extracellular domain, when overexpressed, have little if any affect on the reporter. (E) FLNΔ10-12, (G) FLNΔ17-19, (I) FLNΔ24-26 and (K) FLNΔ17-19,24-26. (F) A deletion of EGF-like repeats 10-12 greatly reduces the ability of the Notch molecule to signal using Serrate as a ligand. This is shown by the lack of reporter activity in the dorsal wing pouch (compare F and D). (H) A deletion of EGF-like repeats 17-19 still allows Serrate to generate a signal as shown by the dorsal activation of the reporter. (J,L) A deletion of EGF-like repeats 24-26 abolishes the ability of Serrate to signal with the ectopic Notch molecule and the expression of the reporter resembles that of when Serrate is expressed alone.

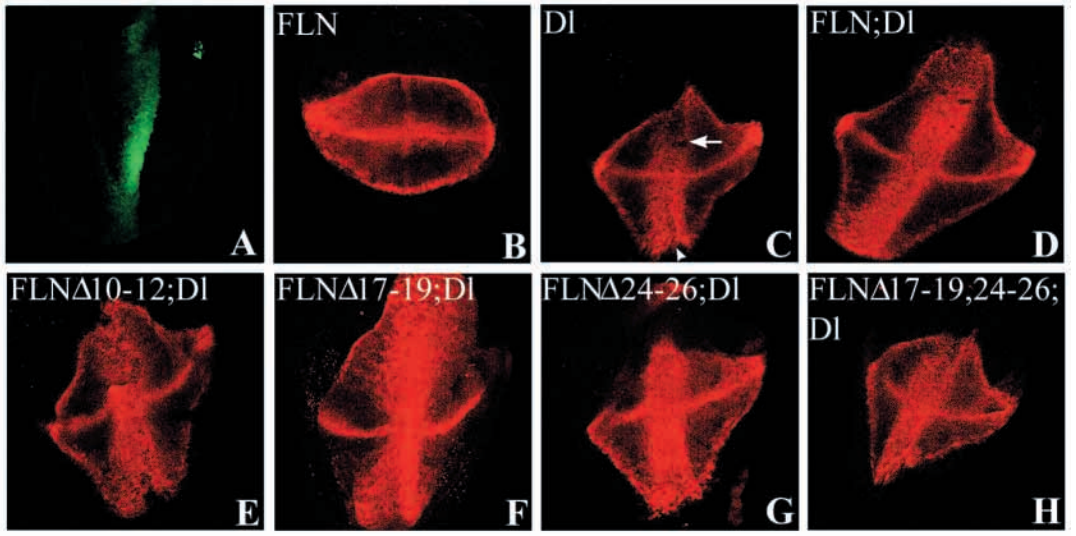


**Fig. 6.** Responses to Notch signalling using Delta as a ligand measured by the expression of a *spd*lacZ reporter construct in third instar wing discs at 17°C and monitored on a confocal microscope with an antibody against β-galactosidase. The UAS constructs indicated have been expressed under the control of *ptc*GAL4. All stainings were done simultaneously and the confocal microscope was set at a constant gain. Anterior is to the left and dorsal to the top. (A) Expression pattern driven by *ptc*GAL4 in third instar wing imaginal discs as revealed by UASGFP (see Klein and Martinez Arias, 1998). (B) Expression of FLN has little effect on the reporter at this temperature, and the same is true of all the other Notch constructs used in these experiments (not shown). (C) Expression of Delta (Dl) alone leads to strong ectopic activation of the reporter either side of the anterior/posterior boundary (arrow). The expression becomes weaker towards the middle of the domain of expression (arrowhead). This weaker expression can be explained by a dominant negative activity of high levels of Dl on endogenous Notch signalling. (D) Coexpression of FLN and Dl leads to strong ectopic expression of the reporter along the anterior/posterior boundary within the domain of patched expression. Notice that now expression is strong and uniform in the dorsal and ventral regions of the wing (compare with C). Deletion of EGF-like repeats 10-12 (E) or 17-19;24-26 (H) from Notch abolishes the ability of Dl to signal with the ectopic Notch molecule. In these instances, the expression of the reporter resembles that of when Dl is expressed alone (compare E and H with C and D; the expression of the reporter is more similar to that of C than D, and this is particularly noticeable on the dorsal side of the wing disc). Expression of a Notch molecule deleted in repeats 17-19 (F) or 24-26 (G) with Dl leads to activation of the reporter in a similar manner to that observed when FLN and Dl are expressed together (compare again with C and D). Notice that in all cases the levels of expression of the reporter at the DV boundary are similar which serves as an internal control for the differences that we observe.

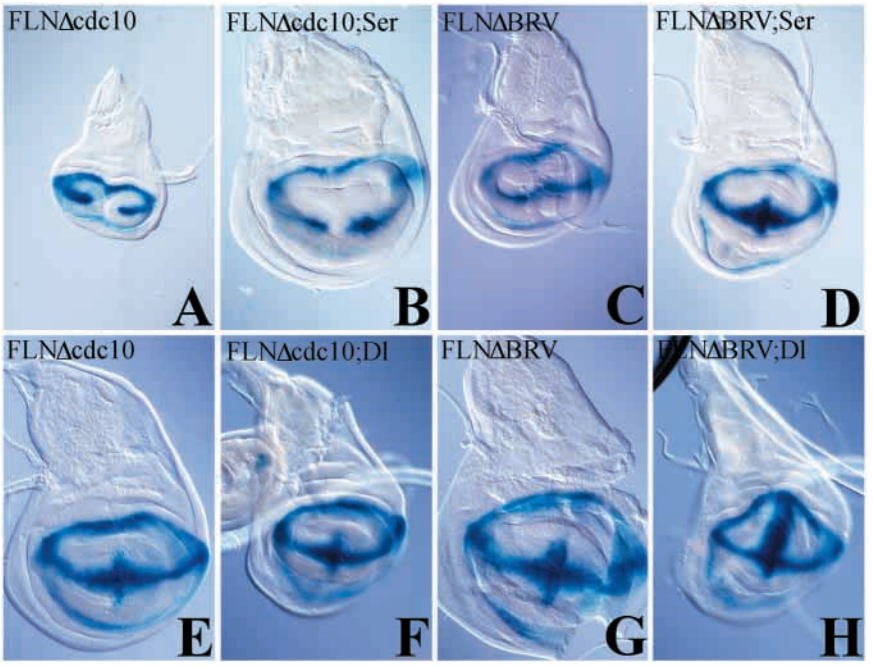


**Fig. 7.** Testing the requirement of intracellular regions of the Notch molecule for signalling by Serrate and Delta. (A-H) Expression of the *spd*lacZ reporter in third larval instar wing discs in which Notch molecules harbouring intracellular deletions have been expressed under the control of *ptc*GAL4 in the presence or absence of ligand. (A-D at 25°C; E-H at 17°C.) (A) At 25°C, ectopic expression of FLNΔcdc10 leads to a disruption of the reporter where the *ptc* stripe crosses the dorsal/ventral boundary. This result supports previous observations that this molecule acts as a dominant negative towards endogenous Notch signalling (Lieber et al., 1993). (B) When coexpressed with Serrate, the dominant negative affect of the Notch molecule is still evident and ectopic activation of the reporter by Serrate is abolished (compare with Fig. 4C). (E) At 17°C FLNΔcdc10 has little affect on the reporter; however, it is capable of abolishing the ectopic activation induced by Dl alone (F) (compare with Fig. 5C). (C) At 25°C FLNΔBRV leads to a slight disruption of the reporter at the presumptive margin. (D) This construct abolishes the ectopic signalling induced by Serrate. (G) At 17°C FLNΔBRV has little effect on the reporter; however, it is capable of abolishing ectopic activation produced by overexpression of Delta in the ventral region of the wing pouch (H).

dorsal surface, suggesting that Serrate is unable to signal with Notch molecules that lack these EGF-like repeats (see Fig. 5F,J,L). On the other hand, coexpression of Serrate with FLNΔ17-19 does lead to increased signalling; the two ventral stripes of *spd*lacZ expression are merged into a thick stripe and the reporter gene is expressed on the dorsal side (see Fig. 5H).

One possible explanation for the failure of Serrate to signal with the FLNΔ10-12, FLNΔ24-26 and FLNΔ17-19;24-26 molecules, is that although they are synthesised, these molecules are not localised to the plasma membrane. This is not so, however, since in all cases the proteins are made and placed at the cell surface (see Fig. 4). These results therefore indicate that although the Serrate protein can interact with Notch molecules that lack EGF-like repeats 10-12 or repeats 17-26 (see above and Fig. 3), it cannot produce a functional signal with these molecules. In addition, these results suggest that the lack of signalling ability of the FLNΔ17-19;24-26 molecule is mostly due to the absence of EGF-like repeats 24-26 because Serrate can signal with FLNΔ17-19.

### EGF-like repeats 10-12 and 24-26 are also required for signalling with Delta during the development and patterning of the wing

We also tested the ability of Delta to signal with Notch molecules bearing deletions of the different EGF-like repeats. Expression of Delta with *ptc*GAL4 results in two parallel stripes of *spd*lacZ expression, which span the dorsal and ventral regions of the developing wing pouch (see Fig. 6C). When Delta is coexpressed with FLN, the two stripes merge into a single thick one (see Fig. 6D).

Expression of Delta with FLNΔ10-12 or FLNΔ17-19;24-26 produces a pattern of *spd*lacZ expression similar to that produced by Delta alone (see Fig. 6E,H). On the other hand, coexpression of Delta with either FLNΔ17-19 or FLNΔ24-26 alters the pattern of *spd*lacZ expression, leading to a single stripe of strong expression similar to that seen when Delta and FLN are expressed together (see Fig. 6F,G). The differences in the ability of Delta to signal with the different Notch proteins is unlikely to be due to variations in the expression of the proteins, which appears to be comparable in the cases we have studied.

These results indicate that EGF-like repeats 10-12 are required for signalling of Delta, but that, in contrast with Serrate, Delta can signal with Notch molecules that lack EGF-like repeats 24-26, but only as long as repeats 17-19 are present in the molecule. This suggests that the structural similarities that we have observed between these repeats (see Fig. 1B) might reflect a functional redundancy with regard to Delta signalling.

### Domains in the intracellular region required for signalling

Mutational analysis of the intracellular domain of Notch has highlighted two regions of functional significance, six cdc10/ankyrin repeats and the associated membrane proximal RAM-23 region and a region C-terminal to this region. The cdc10/ankyrin repeats and the RAM-23 domain have been implicated in interactions with Su(H), which lead to the activation of target genes that are dependent upon Delta and Serrate signalling (Hsieh et al., 1996; Jarriault et al., 1995; Tamura et al., 1995). The region C-terminal to the cdc10/ankyrin repeats has been shown to interact with Dishevelled and is thought to be involved in negative regulation of Delta/Serrate signalling through Notch (Axelrod et al., 1996).

As before, we analysed the ability of Delta and Serrate to signal with Notch molecules lacking these domains by examining the expression of *spd*lacZ at the margin when these Notch proteins are expressed with Serrate and Delta under the control of *ptc*GAL4. Expression of a Notch molecule that lacks the RAM23 and the cdc10/ankyrin repeats (FLNΔcdc10) at 25°C blocks the expression of *spd*lacZ over the domain of expression of *ptc*GAL4 (see Fig. 7A). Coexpression of the FLNΔcdc10 protein with either Serrate or Delta stops the signal molecules from inducing the expression of *spd*lacZ (see Fig. 7B,F).

Expression of a Notch molecule deleted for the region C-terminal to the cdc10/ankyrin repeats (FLNΔBRV) also reduces expression of *spd*lacZ (see Fig. 7C) when at 25°C. Also, like FLNΔcdc10, expression of FLNΔBRV with either Delta or Serrate reduces the ability of the two signal proteins to induce the expression of *spd*lacZ (see Fig. 7D,H), although it is not able to attenuate the signalling of Serrate and Delta to the same extent as FLNΔcdc10. FLNΔcdc10 and FLNΔBRV are expressed at comparable levels in the disc and both can be found at the cell membrane (Fig. 4G,H and insets).

These results suggest that both the RAM-23 and cdc10/ankyrin repeats, and the region C-terminal to the cdc10/ankyrin repeats of Notch, are required for Delta and Serrate to signal with Notch.

## DISCUSSION

The activation of the Notch receptor requires the interaction of its extracellular domain with the ligands Delta and Serrate and of its intracellular domain with the Su(H) protein. Previous work has identified many different regions of the Notch protein that are required for its normal function (Lieber et al., 1993; Rebay et al., 1993); however, this work has not examined which domains are specifically required for Delta and Serrate to signal, or whether or not there are differences in the regions of the Notch protein required for Delta and Serrate to signal. Here we performed a functional in vivo analysis of the different domains of Notch in two different assays for Delta and Serrate signalling. Although this assay cannot determine sites involved in direct physical interactions, it can uncover sites that are required in vivo for a functional interaction between Notch and its ligands.

Our results demonstrate that EGF-like repeats 11 and 12, the RAM-23 and cdc10/ankyrin repeats and the region C-terminal to the cdc10/ankyrin repeats are necessary for both Delta and Serrate to signal via Notch. They also indicate, however, that Delta and Serrate utilise EGF-like repeats 24-26 for signalling, albeit in a differential manner.

### Dominant negative Notch receptors appear to function by sequestering ligands and not by forming non-functional heterodimers with the wild-type molecule

Truncation of the intracellular domain of a receptor leads, in general, to a molecule that exhibits dominant negative effects on the endogenous molecule. Analysis of the activity of

different cell membrane receptors has led to two principal models for the way such dominant negative receptors function (Heldin, 1995). For receptors that do not oligomerise, they are believed to function by sequestering ligands in non-functional complexes. This will reduce the number of ligand molecules that are interacting with the wild-type receptor and consequently will reduce ligand signalling. In the case of receptor molecules that need to oligomerise to form a functional receptor, dominant negative receptors disrupt signalling both by sequestering ligand and by forming non-functional receptor complexes. Both of these mechanisms reduce the number of ligand molecules that interact with wild-type receptor molecules within functional receptor complexes. The importance of these two mechanisms varies for different receptors. For receptor tyrosine kinases, which dimerise upon interaction with a ligand, it appears that dominant negative receptors disrupt signalling principally by forming non-functional receptor complexes.

The interpretation of the behaviour of certain *Notch* mutants, and of experiments with chimeric Notch proteins, suggested that the Notch protein may dimerise during signalling (De Celis and Garcia Bellido, 1994; Heitzler and Simpson, 1993; de Celis and Bray, 1997). Consequently it has been concluded that dominant negative Notch receptors disrupt signalling in a manner that is very similar to dominant negative receptor tyrosine kinase molecules, i.e. by forming non-functional heterodimers with the wild-type molecule (Heitzler and Simpson, 1993; de Celis and Bray, 1997). The observations that in our experiments the effects of ECN are not titrated by full-length Notch molecules but rather that they can titrate effectively Delta and Serrate, suggest that if the ECN molecule titrates endogenous full-length molecules, it does so through an interaction with ligand. Therefore, in the case of Notch, it appears that dominant negative molecules may work primarily by titrating ligand.

### EGF-like repeats other than repeats 10-12 are required for the interaction of Delta and Serrate with Notch

The phenotype generated by expressing Serrate with *ptc*GAL4 can be suppressed by coexpression with any of the ECN proteins that we have tested, although in the case of ECNΔ17-19;24-26 it is not always completely suppressed (unpublished observations). This suggests that none of the deleted EGF-like repeats are essential for the interaction between Notch and Serrate. At first sight these results appear to be in conflict with previous experiments, which suggested that EGF-like repeats 11 and 12 are necessary and sufficient to mediate the aggregation of Serrate and Notch expressing S2 cells. One possible explanation for this discrepancy is that there is a second site within the extracellular domain of the Notch protein, which interacts with the Serrate protein. In this situation the ECNΔ10-12 protein should still be able to interact with the Serrate protein via this second site and thus suppress the phenotype generated by overexpressing Serrate by sequestering the excess protein. The second site is likely to lie within EGF repeats 17-19; 24-26 since dominant negative molecules lacking this domain, while suppressing the effects of Serrate, are less effective at doing so than the full-length dominant negative molecule.

In contrast to the effects of Serrate, those of Delta are suppressed by ECN (see Fig. 3) but not by either of the deleted ECN molecules. The inability of an ECN molecule lacking EGF-like repeats 10-12 to suppress the effects of Delta overexpression confirms that repeats 10-12 are necessary for the interaction between Delta and Notch. However, the lack of effect of ECNΔ17-19;24-26 is surprising.

There are a number of reasons why the ECNΔ17-19;24-26 protein may not suppress the phenotype generated by expressing Delta effectively. For example, it may be that in our experiments the ECN protein is present in much greater quantity than the ECNΔ17-19;24-26 due to the different levels expression that are obtained from different UAS inserts (Brand and Perrimon, 1993). A second possibility is that the EGF-like repeats deleted in the ECNΔ17-19;24-26 protein are required for the interaction of the ECN and Delta proteins. In both cases this would mean that more of the Delta protein is sequestered in the experiment with the ECN molecule than the ECNΔ17-19;24-26 molecule, which would lead to a greater reduction in the signalling of Delta and hence suppression of the phenotype when Delta is coexpressed with the ECN protein. The first possibility is unlikely because both proteins have effects on their own in the wild type and can suppress the effects of Serrate expression, suggesting that they are expressed at comparable levels. In addition we observe that increasing the doses of the different dominant negative molecules does not alter their effects on the activity of Delta (our unpublished observations). Taking this into consideration, we believe that the most likely reason for the apparent difference in the ability of the ECN and ECNΔ17-19;24-26 proteins to suppress the phenotype generated by overexpressing the Delta protein is that the deletion of EGF-like repeats 17-19 and 24-26 reduces the ability of the Delta and ECN protein to interact in vivo.

The analysis that we have carried out indicates that EGF-like repeats 10-12 are essential for the interaction of Delta with Notch. However they also indicate that even when EGF-like repeats 10-12 are present, removal of repeats 17-19 and 24-26 reduces the interaction between Delta and Notch in vivo. Previous experiments have suggested that these EGF-like repeats may be important for the interaction of the Notch and Delta proteins in tissue culture, because the aggregation of Notch and Delta expressing S2 cells is reduced by deletion of these EGF-like repeats from the Notch protein (Rebay et al., 1991) and by the $Ax^{E2}$ point mutation in EGF-like repeat 29 (Lieber et al., 1992).

### EGF-like repeats 24-26 are required for Serrate and Delta to signal via Notch

The above analysis with the different ECN proteins has highlighted that a number of different EGF-like repeats are involved in the interaction of Delta and Serrate with Notch in vivo. To test if these EGF-like repeats are also required for Delta and Serrate to signal via the Notch protein, we expressed FLN proteins lacking these repeats with Delta and Serrate in an assay for Delta and Serrate signalling (see Results and Figs 5 and 6).

Expression of Delta or Serrate with FLN and FLNΔ17-19 led to the ectopic induction of *spd*lacZ expression, whilst coexpression of Delta and Serrate with FLNΔ10-12 did not alter the effects of Delta and Serrate on their own, suggesting that removal of these repeats inhibited the signalling event. A requirement for EGF-like repeats 10-12 in Notch signalling is

expected and correlates well with our findings with the ECN molecules (see above) and with previous suggestions of the requirement for these repeats (De Celis et al., 1993; Lieber et al., 1992; Rebay et al., 1991).

On the other hand, expression of Delta and Serrate with molecules that lack EGF-like repeats 24-26 produced different results. Delta can signal through a molecule that lacks these repeats, but Serrate cannot. This requirement for EGF-like repeats 24-26 is surprising and indicates that the influence of these repeats on the interactions between Notch and its ligands in cell culture (Lieber et al., 1992; Rebay et al., 1991) and in vivo (see above) might reflect a requirement for these repeats in signalling. Interestingly, there are differences in the way Delta and Serrate require these repeats. In the case of Serrate, the requirement for signalling is absolute, whereas in the case of Delta it is conditional on the presence of EGF-like repeats 17-19, which we have shown are structurally related to 24-26. These differences are made more clear when comparing the interactions of Delta and Serrate with the ECNs. Delta cannot signal with FLN molecules that lack repeats 10-12 or 17-19 and 24-26, nor interact with ECN molecules that lack these repeats. On the other hand, although Serrate cannot signal with FLN molecules lacking these repeats, it can interact with the corresponding ECN molecules. This suggests that whereas in the case of Delta a direct interaction with Notch amounts to signalling, this might not be sufficient for Serrate (see also Klein et al., 1997). In the case of Serrate, further modifications of Notch or interactions with other proteins might be required to trigger a signal.

Many of the *Abruptex* mutations of *Notch* map to these EGF-like repeats but these mutations, in different assays, do not display phenotypes to suggest that Delta or Serrate signalling is disrupted (Brennan et al., 1997; De Celis and Garcia Bellido, 1994; Kelley et al., 1987). In fact some of these *Abruptex* mutations appear to behave as gain of function mutations because during wing development these alleles show ectopic expression of genes, whose expression is regulated by Delta and Serrate signalling, like *wingless* and *cut* (De Celis et al., 1996). In contrast with the deletions used in this study, these mutations are single amino acid changes.

The different requirements of Delta and Serrate that we have uncovered might be relevant in vivo. Interestingly, ectopic gene expression in *Abruptex* mutants, which map around EGF-like repeats 24-26, is restricted to the dorsal region of the disc (see Fig. 7 in De Celis et al., 1996). This feature suggests that rather than the Notch proteins encoded by these alleles being constitutive active receptors, the effects of the *Abruptex* mutations on wing development could be due to increased signalling of a patterned ligand. Such a ligand dependence of some *Abruptex* mutants has been proposed previously during sensory bristle development (Heitzler and Simpson, 1993). The dorsal bias of the ectopic signalling suggests that the ligand responsible for the *Abruptex* phenotype in the wing could be Serrate. Serrate is expressed over the dorsal side of the developing wing, but it is prevented from signalling by the activity of the *fringe* gene product away from the wing margin (Fleming et al., 1997; Klein and Martinez Arias, 1998; Panin et al., 1997). It may be that the Abruptex phenotype has an important component of the alteration of the interactions between Serrate and EGF repeats in the 24-26 region.

### Intracellular requirements for Serrate and Delta signalling

We also tested the ability of Serrate and Delta to signal with Notch molecules that lack regions of the intracellular domain. Our results show that both the RAM-23 and cdc10/ankyrin repeats and the region C-terminal to the cdc10/ankyrin repeats are required for Serrate and Delta signalling (see Fig. 7). The requirement of the RAM-23 and cdc10/ankyrin repeats correlate well with previous experiments, as this region has previously been shown to be required for the intracellular domain to interact with Suppressor of Hairless (Fortini and Artavanis Tsakonas, 1994; Tamura et al., 1995) and is required for the intracellular domain of Notch to act as a transcriptional activator (Hsieh et al., 1996; Jarriault et al., 1995).

The requirement of the region C-terminal to the cdc10/ankyrin repeats was unexpected, however. This region has been previously shown to interact with the Dishevelled protein, a component of the Wingless signalling pathway (Axelrod et al., 1996). It has been proposed that this interaction allows Wingless signalling to negatively regulate Delta signalling through Notch. Therefore deletion of this region would be expected to increase the ability of Delta and Serrate to signal via Notch as the negative regulation by Wingless signalling cannot occur. In contrast, our results indicate that this region of Notch is essential for Serrate and Delta to signal via Notch. This difference is similar to that discussed above for the effect the *Abruptex* mutations and our deletion of EGF-like repeats 24-26 have on Serrate signalling and may reflect a similar regulatory role and requirement for signalling of the region C-terminal to the cdc10/ankyrin repeats in both Serrate and Delta signalling.

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