An *O*-fucose site in the ligand binding domain inhibits Notch activation

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Summary

Two glycosyltransferases that transfer sugars to EGF domains, OFUT1 and Fringe, regulate Notch signaling. However, sites of *O*-fucosylation on Notch that influence Notch activation have not been previously identified. Moreover, the influences of OFUT1 and Fringe on Notch activation can be positive or negative, depending on their levels of expression and on whether Delta or Serrate is signaling to Notch. Here, we describe the consequences of eliminating individual, highly conserved sites of *O*-fucose attachment to Notch. Our results indicate that glycosylation of an EGF domain proposed to be essential for ligand binding, EGF12, is crucial to the inhibition of Serrate-to-Notch signaling by Fringe. Expression of an EGF12 mutant of Notch (N-EGF12f) allows Notch activation by Serrate even in the presence of Fringe. By contrast, elimination of three other highly conserved sites of *O*-fucosylation does not have detectable effects. Binding assays with a soluble Notch extracellular domain fusion protein and ligand-expressing cells indicate that the N-EGF12f mutation can influence Notch activation by preventing Fringe from blocking Notch-Serrate binding. The N-EGF12f mutant can substitute for endogenous Notch during embryonic neurogenesis, but not at the dorsoventral boundary of the wing. Thus, inhibition of Notch-Serrate binding by *O*-fucosylation of EGF12 might be needed in certain contexts to allow efficient Notch signaling.

Key words: *fringe*, *Notch*, Fucose, Glycosylation, *O-fucosyltransferase*, *Drosophila*

Introduction

The Notch pathway is a signal transduction pathway that plays key roles in regulating cell fate decisions throughout metazoan development (Artavanis-Tsakonas et al., 1999). *Drosophila* Notch is a transmembrane receptor protein with 36 EGF repeats in its extracellular domain (Fig. 1A). The two Notch ligands in *Drosophila*, Delta and Serrate, are also transmembrane proteins with multiple EGF repeats. Interaction of Notch with ligands expressed by neighboring cells triggers proteolytic processing of Notch (reviewed by Mumm and Kopan, 2000). The intracellular domain of Notch then travels to the nucleus and, together with other proteins, regulates gene expression. In addition to activating Notch expressed on the surface of neighboring cells, Notch ligands can also inhibit Notch activation in ligand-expressing cells (Thomas et al., 1995; Doherty et al., 1996; de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997; Jacobsen et al., 1998; Sakamoto et al., 2002). This phenomenon, which has been referred to as 'autonomous inhibition' or 'cis-inactivation', is thought to reflect an ability of Notch ligands to bind to Notch expressed on the same membrane without activating it.

The activation of Notch by its ligands is regulated by glycosyltransferases that participate in the synthesis of *O*-linked fucose glycans attached to EGF domains (reviewed by Haines and Irvine, 2003). *O*-fucosylation of EGF domains is catalyzed by the enzyme O-FucT-1 (Wang and Spellman, 1998; Wang et al., 2001). Loss of O-FucT-1 activity by RNAi or mutation of the *Drosophila Ofut1* gene (Okajima and Irvine, 2002; Okajima et al., 2003; Sasamura et al., 2003), or by targeted mutation of the mouse *Pofut1* gene (Shi and Stanley, 2003), results in phenotypes that resemble those observed in the complete absence of Notch signaling.

The *O*-linked fucose monosaccharide transferred to protein by OFUT1 can be elongated by β1,3N-acetylglucosaminyltransfrases encoded by Fringe genes (Fig. 1B) (Bruckner et al., 2000; Moloney et al., 2000a). In contrast to the general positive requirement for OFUT1 in Notch signaling, glycosylation by Fringe exerts a positive influence on Delta-Notch signaling, but a negative influence on Serrate-Notch signaling (Fleming et al., 1997; Panin et al., 1997; Klein and Martinez Arias, 1998; Papayannopoulos et al., 1998; Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000a). The influence of Fringe on Notch signaling has been best studied in the *Drosophila* wing (reviewed by Irvine and Vogt, 1997). Both Fringe and Serrate are expressed by dorsal wing cells (Irvine and Wieschaus, 1994; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995), whereas Delta and Notch are initially broadly expressed (Fehon et al., 1991; Doherty et al., 1996; de Celis and Bray, 1997). Fringe facilitates Notch activation in dorsal cells by potentiating their ability to respond to Delta (Panin et al., 1997). At the same time, by inhibiting Serrate signaling, Fringe limits Serrate to signaling back across

the dorsoventral (DV) boundary to ventral cells (Fig. 1E) (Fleming et al., 1997; Panin et al., 1997; Klein and Martinez Arias, 1998). Notch activation promotes Serrate and Delta expression in the wing (de Celis and Bray, 1997; Panin et al., 1997), and although the initial expression of Notch ligands does not require Notch activation, maintenance of their expression does. Consequently, Serrate and Delta become restricted to the DV boundary, where their expression is maintained by a positive feedback loop.

Genetic and cell culture studies indicate that OFUT1 and Fringe function specifically on the receiving side of the Notch pathway, rather than on the signaling side (Panin et al., 1997; Bruckner et al., 2000; Hicks et al., 2000; Okajima and Irvine, 2002; Sasamura et al., 2003). Biochemical studies indicate that Notch is a substrate for the glycosyltransferase activities of OFUT1 and Fringe (Bruckner et al., 2000; Moloney et al., 2000a; Moloney et al., 2000b; Okajima and Irvine, 2002). These results implicate the glycosylation of Notch as the mechanism by which Fringe and OFUT1 exert their effects. Studies of proteins involved in blood clotting and fibrinolysis led to a proposed consensus site for *O*-fucosylation, $C^2XXGG(S/T)C^3$, in which S/T is the modified amino acid, and C^2 and C^3 are the second and third cysteines, respectively, of the EGF domain (Harris and Spellman, 1993). According to this consensus sequence, 11 of the 36 EGF repeats in *Drosophila* Notch could potentially be *O*-fucosylated (Moloney et al., 2000b). However, other studies have indicated that the original consensus sequence is too narrow (Wang and Spellman, 1998; Panin et al., 2002; Shao et al., 2003). Based on a broader consensus sequence, $C^2XXX(G/A/S)(T/S)C^3$, 23 of the 36 EGF repeats of *Drosophila* Notch could potentially be *O*-fucosylated (Fig. 1A). The presence of *O*-fucose is a prerequisite for glycosylation by Fringe, but additional structural constraints that influence Fringe-dependent elongation of *O*-fucose on particular EGF domains also appear to exist (Shao et al., 2003).

Confirmation that Notch is the substrate that accounts for the effects of OFUT1 and Fringe on Notch signaling requires the identification of functional sites of modification. Additionally, it remains unclear whether the different effects of OFUT1 versus Fringe, or the effects of Fringe on Delta versus Serrate, are mediated through the same or distinct sites of *O*-fucosylation. The large number of potential sites poses a challenge to the identification of biologically relevant sites. Nonetheless, prior studies of Notch suggest two possible regions as potentially important sites of *O*-fucosylation.

A series of gain-of-function alleles of *Notch*, called *Abruptex* (N^{Ax}) are caused by missense mutations that map to EGF repeats 24, 25, 27 and 29 (Kelley et al., 1987; de Celis and Bray, 2000). These mutations cause a ligand-dependent, hyperactivation of Notch (Kelley et al., 1987; Heitzler and Simpson, 1993; de Celis and Garcia-Bellido, 1994; de Celis and Bray, 2000). Formally then, a normal function of this region of Notch appears to be to negatively regulate Notch activation. Ectopic expression experiments suggest that this might occur because N^{Ax} alleles impair autonomous inhibition (de Celis and Bray, 2000). Genetic interactions between N^{Ax} alleles and *fringe* mutations have also led to the suggestion that N^{Ax} alleles might affect the sensitivity of Notch to Fringe (de Celis and Bray, 2000; Ju et al., 2000), which has itself been suggested to act through autonomous inhibition (Irvine and Vogt, 1997; Sakamoto et al., 2002). The observations that both *O*-fucose glycans and N^{Ax} alleles influence the activation of Notch by its ligands, and that the N^{Ax} region overlaps an array of potential sites of *O*-fucosylation (Fig. 1A), identify this as a region of interest.

Studies of Notch-ligand binding suggest EGF12 as another potentially relevant site of *O*-fucosylation. In a cell aggregation assay, two EGF repeats, EGF11 and EGF12, were found to be necessary and sufficient for interaction with Delta (Rebay et al., 1991). Notably, EGF12 is one of only three EGF repeats that contains an *O*-fucose consensus site in all Notch receptors (Fig. 1A), and studies of murine Notch1 have demonstrated that it is a substrate for O-FucT-1 and Fringe (Shao et al., 2003). Moreover, both Fringe and OFUT1 can influence Notch-ligand binding (Bruckner et al., 2000; Shimizu et al., 2001; Okajima et al., 2003; Sasamura et al., 2003).

In order to begin to assess the significance of these potential sites of *O*-fucosylation on Notch, we eliminated evolutionarily conserved *O*-fucose sites in EGF12 and the N^{Ax} region. Mutation of EGF12 results in a dramatic change in Notch activation in vivo, and in its physical interactions with Serrate in vitro. Our results underscore the importance of negatively regulating Notch-ligand interactions, and suggest a novel mechanism by which such regulation might occur.

Materials and methods

Plasmid construction

Mutagenesis was conducted within pBS-N, constructed by ligating a 5.5 kb *Kpn*I-*Xho*I fragment from pUAST-N.865 into BluescriptIIKS+ (Stratagene). pUAST-N.865 was constructed by cloning a *Notch* cDNA (Wharton et al., 1985), from an *Eco*RI site at nucleotide 700 to an introduced *Xba*I site at 10500, into pUAST. PCR-based mutagenesis (e.g. to create pBS-NEGF12f) was performed as described (Panin et al., 2002), and was confirmed by sequencing. The *Kpn*I-*Xho*I fragment was cloned back into pUAST-N.865 to create constructs for transformation. pMT-N-EGF10-12, encoding a Bip signal, amino acids 408 to 529 of Notch, and V5-6×His tags, was created by amplifying DNA from pBS-N by PCR, digesting with *Eco*RI and *Xba*I, and ligating into pMT1B (N. Haines and K.D.I., unpublished). pMT-N-EGF10-12f was constructed similarly, using pBS-NEGF12f as a template. pMT-NEGF12f:AP was constructed by cloning a 2.7 kb *Kpn*I-*Bgl*II fragment from pBS-NEGF12f into pMT-N:AP (Bruckner et al., 2000).

Drosophila stocks and crosses

Multiple insertions of each *UAS-N* construct were created. Designations and chromosomes for lines analyzed are *UAS-NEGF12f[M4a]* (second chromosome), *UAS-NEGF12f[M4b]* (third), *UAS-NEGF12f[M2]* (third), *UAS-NEGF24f[a]* (first), *UAS-NEGF24f[b]* (second), *UAS-NEGF24f[c]* (third), *UAS-NEGF26f[a]* (second), *UAS-NEGF26f[b]* (third), *UAS-NEGF26f[c]* (third), *UAS-NEGF24+26f[a]* (second), *UAS-NEGF24+26f[b]* (second), *UAS-NEGF31f[a]* (second), *UAS-NEGF31f[b]* (third) and *UAS-N.865* (second). We also examined the previously described insertions *UAS-N[8]* and *UAS-N[13]* (Doherty et al., 1996). Initial analysis of these lines was conducted by crossing them to *ptc-GAL4*, and examining wing imaginal discs and adult wings. The approximate level of Notch expression generated by each line was determined by anti-Notch staining; the phenotypes described are from lines with similar levels of Notch. In most cases independent insertions gave similar results. All phenotypes described are from crosses at 25°C.

Stocks for ligand mutant clones and ectopic Notch expression were: *y w hs-Flp[122]; ptc-Gal4 UAS-lacZ; FRT82B Ubi-GFP*;

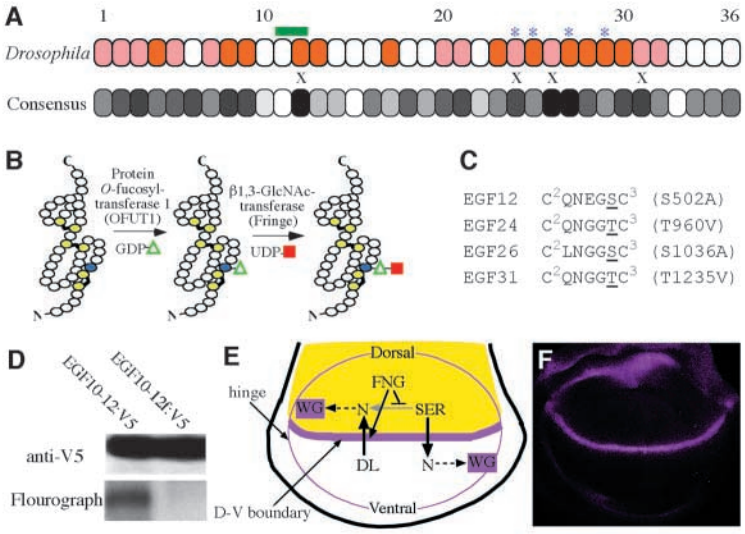


Fig. 1. *O*-fucosylation of Notch. (A) Schematic of EGF repeats in the extracellular domain of Notch. Top: *Drosophila* Notch, with EGF repeats that conform to a narrow consensus sequence for *O*-fucosylation [$C^2XXGG(S/T)C^3$] shaded pink, and those that conform only to a broader consensus sequence [$C^2XXX(A/G/S)(S/T)C^3$] shaded orange. Green bar marks EGF11-12, asterisks mark repeats to which N^{Ax} mutations map, X marks repeats mutated in this study. Bottom: Consensus *O*-fucosylation pattern, with EGF repeats shaded according to the fraction of the 15 Notch receptors analyzed that have a broad consensus *O*-fucose site in that repeat (15/15=black, 0/15=white, intermediate levels of conservation are shaded proportionately gray). (B) Schematic of an EGF domain, illustrating the first two steps in the *O*-fucosylation pathway. In CHO cells, further elongation occurs; the extent of elongation in *Drosophila* is unknown. Adapted with permission from Moloney et al. (Moloney et al., 2000a). (C) Amino acid sequences of *O*-fucose sites mutated in this study. The *O*-fucose attachment site is underlined; the mutation created is in parenthesis. (D) To characterize *O*-fucosylation of EGF12, a polypeptide including EGF10-12 and a V5 tag was expressed in S2 cells, partially purified, and assayed as an acceptor substrate for Fringe as described previously (Panin et al., 2002). Fluorography reveals that N-EGF10-12 is a substrate for Fringe, but that the N-EGF10-12f mutant is not. (E) Schematic of part of the wing disc, illustrating Notch signaling at the DV boundary. Serrate (SER) is expressed by dorsal cells and activates Notch (N) in ventral cells; it is blocked from activating Notch in dorsal cells by Fringe. Delta (DL) is expressed by dorsal and ventral cells, but activates Notch principally in dorsal cells, potentiated by Fringe. Notch activation results in the expression of downstream genes, including *wg* (magenta). (F) Portion of a wing disc, stained for WG protein.

UAS-N.865; FRT82B $Ser^{rev2-11}$ */ TM6b*; and
UAS-N.865; FRT82B Dl^{rev10} *e / TM6b*.

Stocks for generating MARCM clones (Lee et al., 2000; Struhl and Greenwald, 2001) were:

y w hs-Flp[122] tub-Gal4 UAS-GFP:nls; UAS-NEGF12f[M4a]; FRT82B tub-Gal80/TM6b;
y w hs-Flp[122] tub-Gal4 UAS-GFP:nls; UAS-N.865; FRT82B tub-Gal80/TM6b;
FRT82B $Ser^{rev2-11}$ */ TM6b*;
FRT82B Dl^{rev10} *e / TM6b*;
FRT19A N^{55e11}*/FM7; UAS-NEGF12f[M4a]*;
FRT19A N^{55e11}*/FM7; UAS-N.865*; and
FRT19A tub-Gal80 hs-Flp[122];UAS-GFP; tub-Gal4/TM6b.

Other Gal4 and UAS transgenes used, and corresponding FlyBase ID numbers (http://flybase.bio.Indiana.edu/), are *ptc-Gal4* (FBti0002124), *da-GAL4* (FBtp0001168), *Ay-GAL4* (FBti0009983), *UAS-GFP* (FBti0003040), *UAS-lacZ* (FBtp0000355), and *UAS-Fringe 22a* (Kim et al., 1995).

Cell binding assay

Cell binding assays were conducted as described previously (Bruckner et al., 2000; Okajima et al., 2003).

Antibody staining

Antibody staining was conducted as described previously (Panin et al., 1997), using as primary antibodies: mouse anti-WG (4D4, DSHB), rat anti-ELAV (DSHB), rabbit anti-Notch (E. Giniger and M. Young), mouse anti-SXL (M8, DSHB), goat anti-β-galactosidase (Biogenesis), rabbit anti-β-galactosidase (ICN), rat anti-Serrate (Papayannopoulos et al., 1998) and rabbit anti-MYC (Santa Cruz).

Sequence comparisons

The conservation of *O*-fucose sites was assessed by comparing the sequences of: Notch from *Drosophila melanogaster* (P07207), *Lucilia cuprina* (AAC36151), *Boophilus microlus* (AAN06819), *Lytechinus variegatus* (7512075), *Takifugu rubripes* (BAA20535), *Danio rerio* (18859115), *Halocynthia roretzi* (7522619), *Brachiostoma floridae* (12057020) and *Xenopus laevis* (1709335); Notch1 from *Mus musculus* (6679092), *Rattus norvegicus* (6093542) and *Homo sapiens* (27894368); and Notch2 from *Homo sapiens* (24041035), *Mus musculus* (20138876) and *Rattus norvegicus* (13242247).

Results

Drosophila Notch is *O*-fucosylated on EGF12

To begin to identify EGF domains whose *O*-fucosylation influences Notch activation, we substituted S at the *O*-fucose attachment site for A, and T for V. A or V can be found at this position in other EGF repeats of Notch or its ligands, and hence are unlikely to cause disruptions of EGF structure. We focus here on four EGF repeats of Notch: 12, 24, 26 and 31. EGF24, EGF26 and EGF31 were chosen because they lie in or near the region of Notch to which the N^{Ax} alleles map (Fig. 1A) (Kelley et al., 1987), and because they contain highly conserved *O*-fucose sites that conform to the original consensus sequence (Fig. 1C). EGF12 was chosen because it corresponds to one of two EGF repeats identified as necessary and sufficient for Notch-ligand binding in a cell aggregation assay (Rebay et al., 1991), and because it contains a potential *O*-fucose site in all cloned Notch receptors with 36 EGF repeats (Fig. 1A) (Shi and Stanley, 2003). Although this site does not conform to the original consensus for *O*-fucosylation (Fig. 1C), EGF12 of Notch1 has been shown to be glycosylated by O-FucT-1 and Fringe in CHO cells (Shao et al., 2003). *O*-fucosylation of *Drosophila* Notch EGF12 in *Drosophila* cells was confirmed by assessing the ability of a fragment of Notch isolated from S2 cells to serve as an in vitro substrate for Fringe (Fig. 1D).

A Notch overexpression assay

To assay the activity of altered Notch proteins, they were expressed under the control of the *ptc-Gal4* driver. This drives expression in a stripe of cells along the anteroposterior (AP) compartment boundary, with highest expression in cells at the

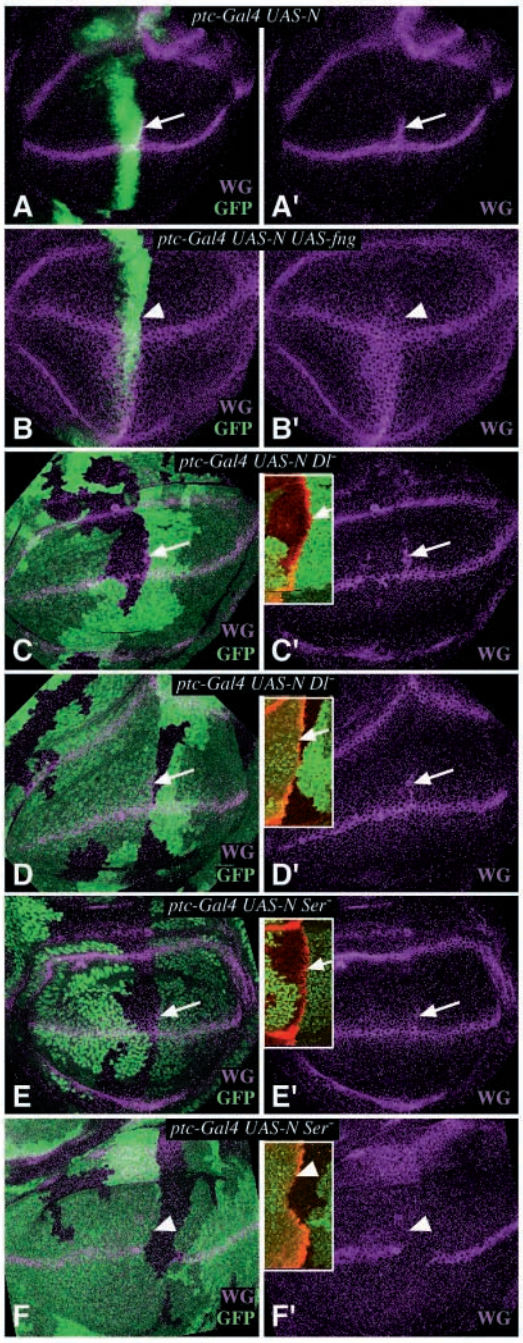


Fig. 2. An ectopic expression assay for Notch activity in the wing. Wing imaginal discs, with ventral bottom and anterior left, stained for expression of WG (magenta), GFP (green) and β-galactosidase (red). Arrows point to ectopic dorsal expression; arrowheads point to absence of expression. Panels marked with a prime show a single channel of the double stain to the left. (A,B) GFP marks the *ptc*-expression stripe. Higher or lower Notch expression can affect the length of the ectopic Notch activation stripe (i.e. how far it extends towards the proximal wing), but not the width (Notch activation is always confined to the AP boundary). (A) *ptc-Gal4 UAS-N.865 UAS-GFP*. (B) *ptc-Gal4 UAS-N.865 UAS-fng[22a]UAS-GFP*. Notch activation in ventral cells is due to potentiation of Delta signaling by Fringe (Panin et al., 1997). (C-F) GFP marks wild-type cells; insets show a portion of the *ptc* expression stripe (red). (C) *ptc-Gal4 UAS-N.865 UAS-lacZ* with anterior Dl^{rev10} clone. (D) *ptc-Gal4 UAS-N.865 UAS-lacZ* with posterior Dl^{rev10} clone. (E) *ptc-Gal4 UAS-N.865 UAS-lacZ* with anterior $Ser^{rev2\text{-}11}$ clone. (F) *ptc-Gal4 UAS-N[865]UAS-lacZ* with posterior $Ser^{rev2\text{-}11}$ clone. This clone also disrupts normal Notch activation at the DV border.

boundary, and lower expression farther away. Notch signaling was then assessed by examining the expression of Notch targets in the developing wing imaginal disc, such as Wingless (WG). WG is normally expressed along the DV border of the wing in response to Notch activation (Fig. 1E,F) (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Rulifson and Blair, 1995). WG also serves as a marker of position in developing wing discs, because it is expressed in two rings in the wing hinge that do not depend on Notch (Phillips and Whittle, 1993).

Notch protein is normally expressed throughout the wing disc (Fehon et al., 1991). Nonetheless, when wild-type Notch is overexpressed, ectopic Notch activation can be induced (Fig. 2A). The pattern of Notch activation induced is instructive with regard to the mechanisms that regulate Notch. First, ectopic Notch activity is only observed near the DV boundary. This is consistent with it being due to an elevated response to Notch ligands, which are expressed preferentially in cells near the DV boundary during most of wing development (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis and Bray, 1997). Second, ectopic Notch activity is observed in cells that are along the posterior edge of the *ptc* expression stripe. This suggests that cells in which Notch is overexpressed can respond to Notch ligands expressed by neighboring cells outside the *ptc* stripe, but that their ability to signal is inhibited. This is presumably due to *cis* interactions between Notch and its ligands (Jacobsen et al., 1998). Third, although weak ectopic Notch activation can sometimes be observed in ventral cells, Notch activation is only consistently observed in dorsal cells (Fig. 2A). As Serrate is expressed exclusively by dorsal cells in the wing, this observation suggests that although both Delta-to-Notch and Serrate-to-Notch signaling can be enhanced, Serrate-to-Notch signaling is much more sensitive to Notch overexpression in the wing.

To confirm these inferences, the requirements for Notch ligands were assessed in genetic mosaics. Clones of cells that are mutant for *Delta* or *Serrate* were generated in animals in which the Notch receptor was expressed under *ptc-Gal4* control. Because the lineage restriction at the AP compartment border coincides with the sharp edge of *ptc* expression, the absence of ligand in an individual disc can be limited either to cells overexpressing Notch, or to their immediate neighbors. Absence of Serrate or Delta within the *ptc*-stripe does not prevent ectopic Notch activation (Fig. 2C,E). Conversely, absence of Serrate, but not of Delta, in cells neighboring the *ptc* stripe is sufficient to eliminate ectopic Notch activation in dorsal cells (Fig. 2D,F). These observations confirm that ectopic Notch activation in dorsal cells is due to signaling from cells outside the *ptc* stripe, and identify Serrate as the ligand that signals to activate Notch in this context.

Normally, Fringe blocks Serrate-to-Notch signaling in dorsal cells. The ability of Serrate to signal when Notch is overexpressed implies that excess Notch can partially overcome the inhibitory effect of Fringe, presumably because

at least some Notch is no longer sufficiently glycosylated. To examine this possibility, Fringe and Notch were co-expressed under *ptc-Gal4* control. Indeed, co-expression of Fringe with Notch can inhibit ectopic Notch activation in dorsal cells (Fig. 2B).

Single *O*-fucose sites in the *Abruptex* region do not influence Notch signaling

The induction of Notch activation observed when Notch is overexpressed allows the assessment of mutant forms of the Notch receptor for their ability to respond to ligand expressed by neighboring cells. At the same time, the limited ectopic activation of Notch also enables the identification of *O*-fucose sites that normally inhibit Notch activation. Expression of Notch proteins that cannot be *O*-fucosylated in EGF24, EGF26 or EGF31, as well as of an EGF24/EGF26 double mutant, all result in an ectopic activation of Notch that is similar to that generated by wild-type Notch (Fig. 3A-D). This induction of ectopic Notch activation implies that they are all functional Notch receptors that can respond to Notch ligands. At the same time, the absence of additional Notch activation implies that they remain subject to the same regulatory influences that limit normal Notch activation.

Mutation of the *O*-fucose site in EGF12 results in elevated Notch activity in dorsal cells

In contrast to the lack of significant effect of mutation of *O*-fucose sites in the *Abruptex* region, mutation of the *O*-fucose site in the ligand binding domain has a dramatic effect. When Notch with a S to A mutation at the *O*-fucosylation site in EGF12 (N-EGF12f) is expressed under *ptc-Gal4* control, Notch activation is induced in dorsal cells throughout the *ptc* expression stripe (Fig. 3E). This enhanced Notch activation is visible not only at the level of WG expression, but also through other downstream targets of Notch signaling, including the ligands Serrate and Delta, and results in an overgrowth of the dorsal wing that is visible at adult stages (not shown).

The pattern of Notch activation again suggests explanations as to its basis. First, the observation that Notch is activated throughout the *ptc*-stripe rather than just at its edge implies that cells overexpressing N-EGF12f can participate in signaling with neighboring cells. Second, the observation that a substantial increase in Notch activity is observed in dorsal cells but not in ventral cells implies that the ability of Serrate to signal to Notch is specifically increased, and/or the ability of Fringe to inhibit Serrate-to-Notch signaling is impaired. Third, the observation that Notch is activated throughout the wing pouch, rather than only near the DV border, is consistent with the observation that the ectopic Notch activation is sufficient to maintain the expression of Notch ligands.

To examine these inferences, we again examined the influences of Fringe and Notch ligands. Strikingly, co-expression of Fringe with N-EGF12f does not result in any noticeable decrease in Notch activation in dorsal cells (Fig. 3F). This suggests that mutation of the *O*-fucose site in EGF12, which is a substrate for Fringe (Fig. 1D) (Shao et al., 2003), renders Notch insensitive to the inhibitory effect of Fringe on Serrate-to-Notch signaling.

To confirm this, we assayed Notch activation in cells that simultaneously expressed N-EGF12f and were mutant for *Serrate* or *Delta*. We accomplished this using an adaptation of the MARCM method (Lee et al., 2000), in which the recombination event that creates mutant cells simultaneously results in the loss of a transcriptional repressor, Gal80, and hence allows Gal4-driven expression from UAS transgenes. *UAS-Notch* was expressed under the control of a *tubulin-Gal4* (*tub-Gal4*) driver in these experiments. Although *tub-Gal4* drives expression at lower levels than *ptc-Gal4*, the results of ectopic Notch expression are qualitatively similar. When wild-type Notch is overexpressed, Notch activation is only observed along the edge of Notch expression, and only in cells near the DV boundary (Fig. 4B). By contrast, when N-EGF12f is overexpressed, Notch activation is no longer restricted to the edge of Notch expression, and can occur anywhere in the dorsal wing (Fig. 4A).

Absence of Notch ligands in cells with normal Notch expression leads to slight increases in Notch activation (Micchelli et al., 1997), reflecting the relief of autonomous inhibition, but this effect is much milder than that associated with Notch overexpression (Fig. 4G; compare with 4B). The absence of Serrate or Delta from clones of cells expressing wild-type Notch under *tub-Gal4* control had no influence on the pattern of Notch activation (Fig. 4D,F), which was consistent with the results for *ptc*-Gal4 driven Notch expression (Fig. 2C,E). By contrast, when clones of cells are generated that express N-EGF12f but are also mutant for

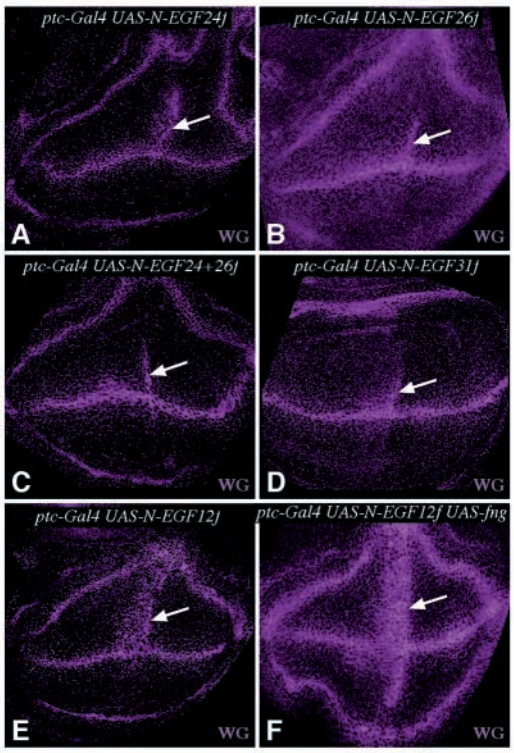


Fig. 3. Influence of *O*-fucose site mutants on Notch activity. WG expression (magenta) in wing discs expressing a Notch receptor with *O*-fucose attachment sites mutated. Arrows point to ectopic expression. (A) *ptc-Gal4 UAS-N-EGF24f[c]*. (B) *ptc-Gal4 UAS-N-EGF26f[b]*. (C) *ptc-Gal4 UAS-N-EGF24+26f[a]*. (D) *ptc-Gal4 UAS-N-EGF31f[b]*. (E) *ptc-Gal4 UAS-N-EGF12f[M4a]*. (F) *ptc-Gal4 UAS-N-EGF12f[M4a]UAS-fng[22a]*. Fringe also results in Notch activation in ventral cells.

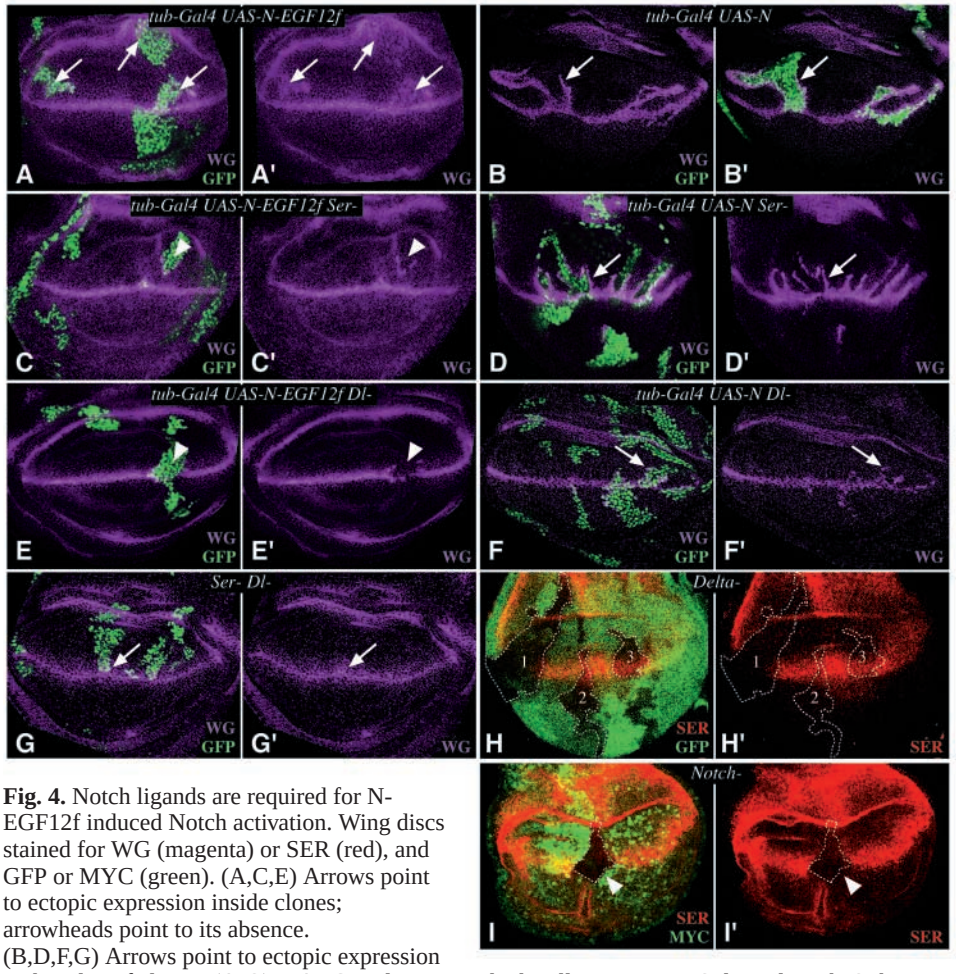


Fig. 4. Notch ligands are required for N-EGF12f induced Notch activation. Wing discs stained for WG (magenta) or SER (red), and GFP or MYC (green). (A,C,E) Arrows point to ectopic expression inside clones; arrowheads point to its absence. (B,D,F,G) Arrows point to ectopic expression at the edge of clones. (A-G) MARCM clones, in which cells expressing Gal4 under *tub-Gal4* control are identified by GFP expression. In C-G the clones are also mutant for Notch ligands. (A) *tub-Gal4 UAS-GFP UAS-N-EGF12f[M4a]*. (B) *tub-Gal4 UAS-GFP UAS-N.865*. (C) *tub-Gal4 UAS-GFP UAS-N-EGF12f[M4a] Ser$^{rev2-11}$*. (D) *tub-Gal4 UAS-GFP UAS-N.865 Ser$^{rev2-11}$*. (E) *tub-Gal4 UAS-GFP UAS-N-EGF12f[M4a] Dl^{rev10}*. (F) *tub-Gal4 UAS-GFP UAS-N.865 Dl^{rev10}*. (G) *tub-Gal4 UAS-GFP Ser$^{rev2–11}$ Dl^{rev10}*. (H) SER expression in *Dl* mutant clones (outlined by white dashes). Faint staining in some regions is from the peripodial epithelium. In a large clone that spans the DV boundary (1), SER expression is lost non-autonomously. In a narrow spanning clone (2), SER is not significantly affected. In a dorsal clone (3), SER expression is reduced but not absent. (I) SER expression is lost autonomously from *Notch* clones.

Serrate, the enhanced activation of Notch is lost, and the Notch activation pattern instead resembles that generated by wild-type Notch (Fig. 4C; compare with 4A,B). Thus, the enhanced Notch activation generated by N-EGF12f is Serrate-dependent, consistent with the inference that it results from an inability of Fringe to block Serrate-Notch signaling.

Unexpectedly however, we found that Delta is also required for the enhanced Notch activation of N-EGF12f expression (Fig. 4E). As the expression of Notch ligands in the wing is maintained by a feedback loop, we reasoned that both ligands might be required in N-EGF12f- expressing cells to generate the levels of Notch activation required to maintain their own expression. Although for simplicity Delta is generally portrayed as a ventral to dorsal signal in the wing (Fig. 1E), Delta is actually expressed to some degree on both sides of the compartment border (Doherty et al., 1996; de Celis and Bray, 1997). Prior analysis of the genetic requirements for *Delta* suggested that *Delta* expression in dorsal and ventral cells might be partially redundant, as clones of cells that are mutant for *Delta* and span the compartment boundary can result in substantial loss of wing tissue, but *Delta* mutant clones that are exclusively dorsal or ventral have milder effects (Doherty et al., 1996; de Celis and Bray, 1997). To investigate the possibility that dorsally-expressed Delta could contribute to the maintenance of Serrate expression, we examined Serrate expression in *Delta* mutant clones. Ventral or dorsal *Delta* mutant clones cause only slight decreases in Serrate expression, but large *Delta* mutant clones that span the DV boundary can eliminate Serrate expression (Fig. 4H). The loss of expression is non-autonomous within Delta mutant clones, and cell autonomous within Notch mutant clones (Fig. 4H,I), consistent with the inference that Serrate is a direct target of Notch signaling. Thus, we suggest that Delta is best thought of not as a signal from ventral cells, but rather a signal to dorsal cells, which can originate on either side of the compartment border.

N-EGF12f is a functional Notch receptor, but does not rescue DV boundary formation

The experiments described above demonstrate that the ectopic activation induced by N-EGF12f is ligand-dependent. However, these experiments were all carried out in cells that retain endogenous Notch. To exclude the possibility that Notch activation induced by expression of N-EGF12f is mediated through endogenous Notch, we used the MARCM method to create clones of cells that simultaneously expressed N-EGF12f and were mutant for a null allele of *Notch*, *N^{55e11}*. Such clones in the dorsal part of the wing continued to display elevated Notch activity (14/15 dorsal clones; Fig. 5B). Thus, N-EGF12f is a functional Notch receptor, which can transduce ligand-dependent signals, resulting in the expression of downstream target genes.

However, MARCM clones expressing N-EGF12f but mutant for endogenous *Notch* do not fully rescue normal Notch activation at the DV boundary. Although Notch is activated within dorsal cells of such clones, they also often (11/13 clones spanning the boundary) show a gap in the normal stripe of Notch activation along the DV boundary (Fig. 5C). Although differences in the perdurability of Gal80 versus Notch might result in a lag between when endogenous Notch is lost and when N-EGF12f is expressed in the MARCM method, control

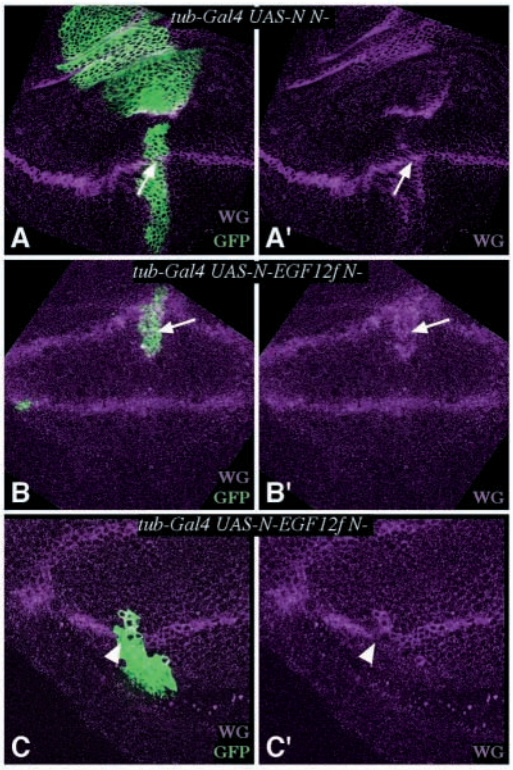


Fig. 5. N-EGF12f is a functional receptor, but cannot rescue DV boundary formation. Wing discs stained for expression of WG (magenta), with MARCM clones expressing a Notch receptor under *tub-Gal4* control and mutant for the endogenous *Notch* gene identified by GFP (green). (A) N^{55e11}; *tub-Gal4 UAS-GFP UAS-N.865*. Notch activation is continuous through a clone that spans the DV border (arrow). (B) N^{55e11}; *tub-Gal4 UAS-GFP UAS-N-EGF12f[M4a]*. Notch is activated ectopically within a dorsal clone (arrow). (C) N^{55e11}; *tub-Gal4 UAS-GFP UAS-N-EGF12f[M4a]*. Notch activation is disrupted at the DV boundary (arrowhead).

MARCM clones mutant for N^{55e11} but expressing wild-type Notch under *tub-Gal4* control always (15/15 clones) fully rescue DV boundary formation (Fig. 5A).

To explore the activity of N-EGF12f further, we tested its ability to rescue Notch function during embryonic neurogenesis. Notch plays a key role in limiting the number of cells that will adopt the neural fate (reviewed by Baker, 2000). In the absence of zygotic Notch function, early development proceeds normally because of the maternal contribution of Notch, but by stage 11 a neurogenic phenotype is observed, in which ectodermal cells on the ventral side of the embryo become neural instead of epidermal. The neurogenic phenotype of *Notch* mutants can be rescued by the expression of Notch under the control of a heterologous promoter (Seugnet et al., 1997). ELAV, a nuclear protein expressed specifically in neurons (Robinow and White, 1991), can be used to visualize neural fate. *Notch* mutant embryos display a massive increase in ELAV staining (Fig. 6A), but this increase is suppressed by the expression of Notch under *da-Gal4* control (Table 1) (Seugnet et al., 1997). Expression of N-EGF12f in *Notch* mutant embryos under *da-Gal4* control similarly rescues the neurogenic phenotype of *Notch* mutant embryos (Table 1, Fig. 6B). This observation further supports

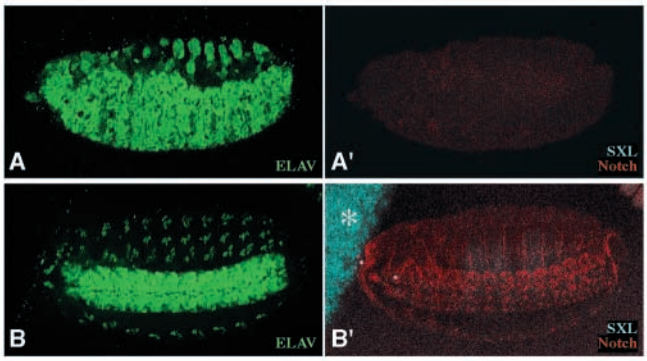


Fig. 6. N-EGF12f can rescue neurogenesis in *Notch* embryos. Embryos from a cross of N^{55e11}*/FM7; UAS-NEGF12f[M4a]/+* to *da-Gal4*, stained for expression of ELAV (green), Notch (red) and SXL (cyan). Absence of SXL identifies embryos as male. Anti-Notch staining allowed effective identification of embryos with Notch overexpression, but did not allow effective discrimination between zygotically wild-type and *Notch* mutant embryos. Staining with the markers thus allowed division of male embryos into four classes, as indicated in Table 1. (A) Class II embryo. (B) Class III embryo. A portion of a female embryo is also visible (asterisk).

the conclusion that N-EGF12f is a functional Notch receptor. Moreover, because Delta is the only ligand that functions during embryonic neurogenesis, this rescue experiment indicates that N-EGF12f can respond to Delta signaling.

A cell binding assay reveals that N-EGF12f has increased affinity for ligands

Although N-EGF12f can rescue the neurogenic phenotype of *Notch* mutant embryos, it behaves abnormally during wing development, displaying ectopic activation of Notch in dorsal cells, but an impaired ability to participate in signaling at the DV boundary. To explore the molecular basis for this, we assayed the influence of N-EGF12f on Notch-ligand binding. The extracellular domain of Notch, when fused to alkaline phosphatase and secreted from cells (N:AP), can bind specifically to Delta-expressing cultured cells but does not bind significantly to control cells (Fig. 7A) (Bruckner et al., 2000; Okajima et al., 2003). The EGF12 mutation, when introduced into N:AP (N-EGF12f:AP), actually results in a slight enhancement of binding to Delta-expressing cells (Fig. 7A). Co-expression of N:AP with Fringe significantly enhances its binding to Delta-expressing cells (Fig. 7A) (Bruckner et al., 2000). Fringe can still enhance the binding of N-EGF12f:AP to Delta-expressing cells, albeit to a slightly lesser degree than for wild-type N:AP (Fig. 7A). These binding studies indicate that *O*-fucosylation of EGF12 is not required for the ability of Notch to bind Delta. Moreover, although EGF12 is a substrate for Fringe, modification of EGF12 by Fringe is not required for the potentiation of Delta-Notch binding by Fringe.

N:AP can also bind to Serrate-expressing cells (Okajima et al., 2003) (Fig. 7B). Interestingly, N-EGF12f:AP binding to Serrate-expressing cells is elevated to about four times that exhibited by wild-type N:AP (Fig. 7B). Thus EGF12 is an inhibitory site for Serrate-N binding.

We also used the N:AP binding assay to investigate the influence of Fringe on Notch-Serrate binding. When N:AP is isolated from Fringe-expressing cells, its ability to bind to

Table 1. Rescue of *Notch* mutant embryos by *UAS-N* transgenes

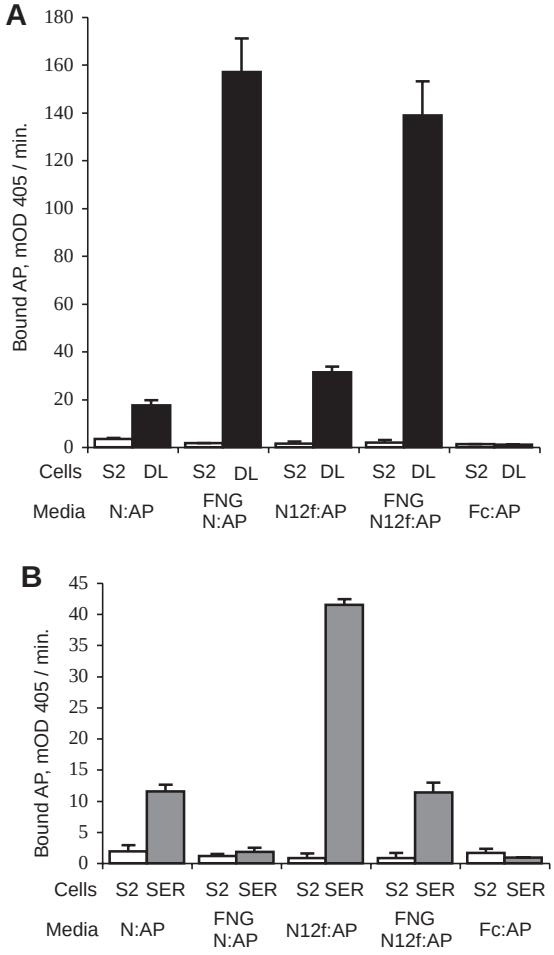
Phenotypic class	Genotype: If no rescue	Genotype: If rescue	Distribution of male embryos *UAS-N.865* cross (*n*=71): Expected if no rescue	Expected if rescue	Observed	Distribution of male embryos *UAS-N-EGF12f* cross (*n*=78): Expected if no rescue	Expected if rescue	Observed
I (Normal ELAV, no excess N)	*FM7; +; da-Gal4*	*FM7; +; da-Gal4*	17-18	17-18	2	19-20	19-20	2
II (Neurogenic ELAV, no excess N)	N^{55e11}*; +; da-Gal4*	N^{55e11}*; +; da-Gal4*	17-18	17-18	33	19-20	19-20	36
III (Normal ELAV, excess N)	*FM7; UAS-N; da-Gal4*	*FM7; UAS-N; da-Gal4 and* N^{55e11} *UAS-N; da-Gal4*	17-18	35-36	36	19-20	39	40
IV (Neurogenic ELAV, excess N)	N^{55e11} *UAS-N; da-Gal4*	N/A	17-18	0	0	19-20	0	0

Embryos from a cross of N^{55e11}*/FM7; UAS-N/+* to *da-Gal4* were stained and analyzed as described in the legend to Fig. 6. Assuming normal segregation, one half of the male embryos are expected to be zygotically mutant for *Notch*, but in practice the segregation of FM7 was skewed such that there were fewer FM7 males than *Notch* males (compare class I with class II). Although we could not unambiguously determine the genotypes of individual class III embryos, rescue of *Notch* by the *UAS-N* transgenes could be inferred from the absence of class IV.

n, number of stage 12 and older embryos scored.

N/A, not applicable.

Serrate-expressing cells is effectively abolished, as the level of detectable binding is indistinguishable from binding to control cells (Fig. 7B) (Okajima et al., 2003). Importantly, when Fringe is co-expressed with N-EGF12f:AP, binding to Serrate-expressing cells is only partially blocked, and the level of N-EGF12f:AP binding to Serrate in the presence of Fringe is comparable to that of N:AP in the absence of Fringe (Fig. 7B).



Discussion

EGF12 is a biologically relevant site of *O*-fucosylation

O-fucose is attached to a S or T. Consequently, when that amino acid is changed to one that lacks a terminal hydroxyl group, *O*-fucosylation of the EGF domain cannot occur. Consistent with this, the S to A mutation eliminates the ability of a Notch fragment including EGF12 to serve as a substrate for Fringe (Fig. 1D). For several reasons, we attribute the observed differences between N-EGF12f and wild-type Notch to this absence of glycosylation, rather than to the amino acid change per se. Substitution of a S with an A is a conservative change, and the two amino acids differ only by an oxygen atom. A is found at this location in other EGF repeats (e.g. EGF36 of *Drosophila* Notch, and EGF7 and EGF19 of mammalian Notch1), and hence is unlikely to disrupt the EGF structure. Indeed, this same mutation in EGF26 does not result in a detectable phenotype (Fig. 3B). A distinct amino acid change in EGF12, the E491V mutation in N^{M1}, results in a strong loss-of-function phenotype (de Celis et al., 1993), as would be predicted for a gross structural change in the ligand-binding domain.

By contrast, the phenotype of N-EGF12f is consistent with that which would be expected of a Notch receptor that had lost a functional site of glycosylation by Fringe. Expression of N-EGF12f results in an ectopic activation of Notch in dorsal wing cells that is insensitive to Fringe, yet dependent upon endogenous ligand expression. Binding studies further show that Serrate is able to bind to this mutant form of Notch even in the presence of Fringe, which contrasts with the lack of

Fig. 7. Mutation of the *O*-fucose site in EGF12 enhances Notch-ligand binding. Histograms show the results of binding assays conducted with conditioned media, including 4800 mOD/minute AP activity of the indicated fusion proteins (Media), mixed with (A) Stably transfected DL-expressing cells, (B) transiently transfected SER-expressing cells, or, as a control, transfected S2 cells. Fc:AP is a control protein, consisting of the Fc domain of human IgG fused to AP. Values represent an average of three experiments, error bars indicate one s.d.

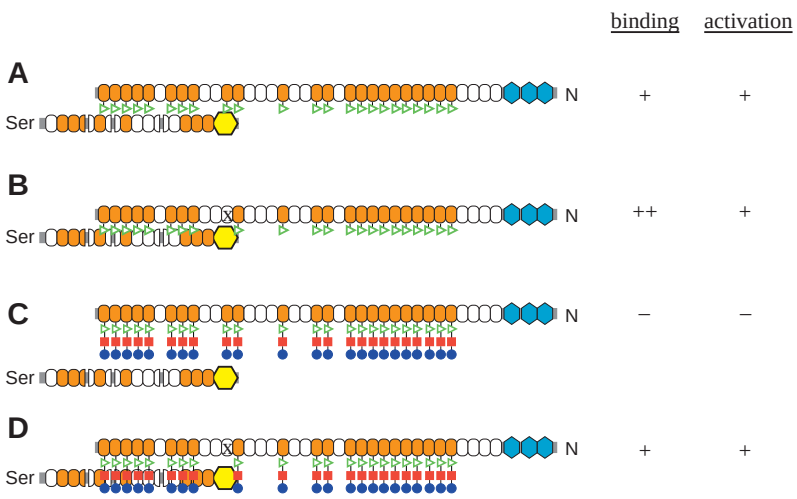


Fig. 8. Influence of *O*-fucosylation of EGF12 on Notch-Serrate interactions. Schematic of the extracellular domains of Notch (N) and Serrate (SER), with EGF domains that include consensus *O*-fucose sites (orange), LN repeats (blue) and the DSL motif (yellow). (A) In the absence of Fringe, SER binds to and activates Notch. The structure of the SER-N complex is unknown, but the DSL motif of ligands and EGF11-12 of Notch are crucial, and might interact (Fleming, 1998). (B) When EGF12 can not be *O*-fucosylated, SER-N binding is enhanced. (C) When *O*-fucose is extended by Fringe, SER can not bind or activate Notch. The *O*-fucose glycan is shown extended to the trisaccharide, which appears to be crucial for inhibition of Jagged1-Notch1 signaling in CHO cells (Chen et al., 2001). (D) When the *O*-fucose site in EGF12 is mutant, SER can still bind despite the presence of elongated *O*-fucose glycans at other sites, and consequently can still activate Notch. Green triangles, fucose; red squares, N-acetylglucosamine; blue circles, galactose.

detectable Serrate binding to wild-type Notch expressed in the presence of Fringe. Based on these observations, we conclude that EGF12 is an essential site for inhibition of Serrate-to-Notch signaling by the Fringe glycosyltransferase (Fig. 8).

The effects of OFUT1 and Fringe involve multiple *O*-fucose sites

Although the *O*-fucose site in EGF12 is essential for Fringe inhibition of Serrate signaling in the wing, Fringe still reduces N-EGF12f:AP-Serrate binding. The decrease in binding is not sufficient to prevent N-EGF12f activation, but there must nonetheless be multiple sites that can contribute to the inhibition of Serrate signaling by Fringe. There must also be distinct sites that mediate the potentiation of Delta-Notch signaling by Fringe, because N-EGF12f:AP-Delta binding is potentiated almost as effectively as N:AP-Delta binding. Importantly then, the effects of Fringe on Delta versus Serrate signaling appear to be mediated, at least to some extent, through distinct sites of *O*-fucosylation.

The importance of additional *O*-fucose sites is further underscored by the distinct consequences of removal of *O*-fucose only at EGF12 by the S to A mutation, compared with removal of *O*-fucose at all sites by *Ofut1* mutation or RNAi. Using a cell aggregation assay, Rebay et al. found that EGF11 and EGF12 of Notch have a key role in ligand binding (Rebay et al., 1991). Deletion of EGF11 and EGF12 prevents aggregation between Notch-expressing cells and Delta-expressing cells, and a construct including only EGF11 and EGF12 of Notch is able to confer Delta-binding activity upon cells, albeit with decreased efficiency compared with full-length Notch. Although a role for other EGF repeats in ligand binding has been suggested based on the consequences of expressing fragments of Notch in the wing imaginal disc (Lawrence et al., 2000), and by cell aggregation experiments with mutant Notch proteins (Lieber et al., 1992), EGF11 and EGF12 have generally been considered to be the key EGF domains for ligand binding. However, because RNAi of *Ofut1* in S2 cells indicates that *O*-fucose is required on Notch for binding to its ligands (Okajima et al., 2003; Sasamura et al., 2003), yet *O*-fucosylation of EGF12 is not required for ligand binding, other *O*-fucosylated EGF domains must also be required for Notch-ligand interactions. Thus, multiple sites are subject to *O*-fucosylation, but with different phenotypic consequences.

Among the 15 Notch receptors with 36 EGF repeats in sequence databases, an average of 20 of the 36 EGF repeats contain potential sites for *O*-fucosylation. However, only three EGF repeats contain *O*-fucose sites in all of these 15 Notch receptors: EGF12, EGF26 and EGF27 (Fig. 1A). Thirteen other EGF domains contain sites that are somewhat conserved (i.e. an *O*-fucose site is found in that repeat in 11 or more of the 15 Notch protein sequences), including EGF24 (13/15 Notch receptors) and EGF31 (14/15 Notch receptors). These conserved sites for *O*-fucosylation cluster in an N-terminal region, and in a more C-terminal region centered around the N^{Ax} mutations (Fig. 1A). This general pattern of conservation – most Notch receptors have many sites, but only a few sites are absolutely conserved – suggests that at least some aspects of OFUT1 and Fringe regulation might be achieved through glycosylation of regions of Notch, rather than through glycosylation of specific EGF repeats. The lack of effect of mutation of individual, highly conserved EGF repeats in the N^{Ax} region is consistent with this suggestion, and experiments to analyze the consequences of mutation of arrays of *O*-fucose sites are in progress.

Relationship between Notch-ligand binding affinity and signaling

Notch ligands activate Notch receptors expressed by neighboring cells, but inhibit Notch receptors expressed by the same cell. Elevated expression of the Notch extracellular domain can also inhibit the ability of ligands to signal to neighboring cells (Jacobsen et al., 1998). Thus, one apparent consequence of the transmembrane nature of Notch ligands is that Notch activation depends not simply on the ability of ligand to bind receptor, but also on a competition between intracellular and intercellular interactions. Previously, most attention has focused on the impact of different levels of expression on this competition. But the balance in this competition can also be shifted by adjusting the affinity between Notch and its ligands. Indeed, even though most studies have focused on the ability of Fringe to inhibit the

response of a cell to Serrate, the ability of cells to send a Serrate signal appears to be enhanced by co-expression with Fringe (Panin et al., 1997; Klein and Martinez Arias, 1998), which is consistent with the idea that decreasing intracellular Serrate-Notch interactions increases the amount of Serrate available to signal to neighboring cells.

Cell-based binding assays indicate that the *O*-fucose site in EGF12 is not just important for Fringe-dependent inhibition: even the presence of the *O*-fucose monosaccharide at this site inhibits Serrate binding. The presence of an inhibitory site of *O*-fucosylation in EGF12 was unexpected given the general positive requirement for *O*-fucose in Notch signaling. However, we suggest that it can be rationalized in terms of a competition between intracellular and intercellular Notch-ligand interactions. The competition model implies that it is important, at least in certain contexts, for Notch not to bind too strongly to its ligands. One such context is probably the DV boundary of the *Drosophila* wing, because Notch ligands are expressed on both sides of the compartment boundary, and Notch is activated on both sides of the compartment boundary. Thus, we suggest that N-EGF12f is unable to rescue normal Notch activation at the DV boundary because its increased affinity for ligands enhances intracellular binding to a degree that interferes with the ability of a cell to send and receive Notch signals. Notably, EGF12 is apparently essential for both intercellular and intracellular Notch-ligand interactions (Rebay et al., 1991; Jacobsen et al., 1998; Lawrence et al., 2000).

The highly conserved presence of an *O*-fucose site in EGF12 suggests that inhibition of ligand binding by the *O*-fucosylation of EGF12 might be of widespread importance. However, if *O*-fucosylation of EGF12 was constitutive, it would simply counteract the positive influence of *O*-fucosylation at other sites. If, by contrast, *O*-fucosylation of EGF12 was regulated, then differential *O*-fucosylation of EGF12 could occur, and could serve as a mechanism of Notch regulation. Intriguingly then, EGF12 is distinguished from other potential *O*-fucose sites by the presence of an acidic amino acid (E or D) at the –2 position relative to the *O*-fucose attachment site (Fig. 1C). None of the other EGF repeats in Notch contain an acidic amino acid at this position, yet 13/15 Notch receptor proteins contain an acidic amino acid at this position in EGF12. We do not yet know what fraction of Notch receptors in a cell are modified at any of the potential sites of *O*-fucosylation, but the presence of this conserved sequence difference suggests that EGF12 might be *O*-fucosylated under different conditions, or with a different efficiency, than other EGF domains, and hence that differential fucosylation of this site might serve as a regulatory mechanism.

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