neurotic, a novel maternal neurogenic gene, encodes an *O*-fucosyltransferase that is essential for Notch-Delta interactions

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Summary

Notch signalling, which is highly conserved from nematodes to mammals, plays crucial roles in many developmental processes. In the *Drosophila* embryo, deficiency in Notch signalling results in neural hyperplasia, commonly referred to as the neurogenic phenotype. We identify a novel maternal neurogenic gene, *neurotic*, and show that it is essential for Notch signalling. *neurotic* encodes a *Drosophila* homolog of mammalian GDP-fucose protein *O*-fucosyltransferase, which adds fucose sugar to epidermal growth factor-like repeats and is known to play a crucial role in Notch signalling. *neurotic* functions in a cell-autonomous manner, and genetic epistasis tests reveal that Neurotic is required for the activity of the full-length but not an activated form of Notch. Further, we show that *neurotic* is required for Fringe activity, which encodes a fucose-specific β1,3 *N*-acetylglucosaminyltransferase, previously shown to modulate Notch receptor activity. Finally, Neurotic is essential for the physical interaction of Notch with its ligand Delta, and for the ability of Fringe to modulate this interaction in *Drosophila* cultured cells. We present an unprecedented example of an absolute requirement of a protein glycosylation event for a ligand-receptor interaction. Our results suggest that *O*-fucosylation catalysed by Neurotic is also involved in the Fringe-independent activities of Notch and may provide a novel on-off mechanism that regulates ligand-receptor interactions.

Key words: Notch signalling, Neurogenic gene, *neurotic*, *nti*, *O*-fucosyltransferase, Fucose, Fringe, Notch-Delta binding, *Drosophila*

Introduction

Communication between cells during developmental processes can occur either by direct cell-cell communication or be mediated by secreted molecules. The Notch pathway is a classical example of a signalling mechanism that relies on cell-cell contact. The Notch pathway is evolutionarily conserved in metazoans and is associated with pleiotropic functions, such as cell differentiation, proliferation, morphogenesis, and apoptosis (reviewed by Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Mumm and Kopan, 2000). *Notch* encodes a single-pass transmembrane receptor protein with 36 epidermal growth factor-like (EGF) repeats and three Notch/LIN-12 repeats in its extracellular domain, and six CDC10/Ankyrin repeats and a PEST-like sequence in its intracellular domain (Kidd et al., 1983; Wharton et al., 1985). When the Notch receptor is activated by its ligands, it is proteolytically cleaved in the transmembrane domain and the intracellular domain (Notch[ICD]) enters the nucleus (Struhl and Adachi, 1998; Mumm and Kopan, 2000). Notch[ICD] forms a ternary complex with Suppressor of Hairless and Mastermind to activate transcription of downstream genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Wu et al., 2000; Kitagawa et al., 2001). In various species, two types of Notch ligands, Delta and Serrate/Jagged, have been identified and shown to possess redundant and distinct functions (Fehon et al., 1990; Rebay et al., 1991; Gu et al., 1995; Zeng et al., 1998). Specific functions of each of these two ligands are modified by Fringe (Fng), which adds *N*-acetylglucosamine (GlcNAc) to *O*-fucose residues on the EGF repeats of Notch (Brückner et al., 2000; Moloney et al., 2000a). Fng is involved in establishing a boundary that segregates two cell groups, such as dorsal and ventral (D/V) compartments of the wing disc or leg segments of the leg disc in *Drosophila* (Irvine, 1999). This GlcNAc modification had been thought to make Notch more sensitive to Delta and less sensitive to Serrate (Fleming et al., 1997; Panin et al., 1997; Klein and Arias, 1998). More recently, an additional modification of the

GlcNAc by a galactosyltransferase has been shown to be required for the inhibition of Jagged1 (Chen et al., 2001). The *O*-fucose glycans are found at serine or threonine residues of consensus sequence CXXGGS/TC (X is any amino acids) that is between the second and third conserved cysteines of the EGF repeats (Harris and Spellman, 1993). In mammals, the *O*-fucosyltransferase that catalyses this fucosylation has been purified and cloned (Wang and Spellman, 1998; Wang et al., 2001). Recently, Okajima and Irvine (Okajima and Irvine, 2002) examined the functions of a *Drosophila O*-fucosyltransferase (OFUT1) using RNA interference (RNAi) and implicated OFUT1 in Notch signalling. More recently, Shi and Stanley (Shi and Stanley, 2003) reported a knock out of the mouse *Pofut1* gene that encodes mouse *O*-FucT-1, and demonstrated that *Pofut1*$^{-/-}$ mice have a phenotype similar to those of the embryos lacking downstream effectors of all Notch signalling pathways. However, the molecular mechanism underlying the function of *O*-fucose was not determined.

Deficiency of essential genes for Notch signalling causes overproduction of neurons in the *Drosophila* embryo: the 'neurogenic' phenotype (Campos-Ortega, 1995). Some essential components of Notch signalling are associated with the neurogenic phenotype when both the zygotic genes and maternally contributed mRNA are abolished. These genes are referred to as 'maternal neurogenic genes'. Several genes relatively recently identified as components of Notch signalling, such as Suppressor of Hairless, Kuzbanian, Presenilin and Nicastrin belong to this class of genes (Lecourtois and Schweisguth, 1995; Rooke et al., 1996; Struhl and Greenwald, 1999; Ye et al., 1999; Chung and Struhl, 2001; López-Schier and St Johnston, 2002; Hu et al., 2002). Because of the difficulty to screen for maternal phenotypes, not all maternal neurogenic genes have yet been identified. We report a novel maternal neurogenic gene, *neurotic* (*nti*; *O-fut1* – FlyBase), which encodes the *O*-fucosyltransferase. The activity of *nti* is also needed in wing margin formation, indicating that *nti* is an essential component of Notch signalling. Epistatic analysis showed that *nti* is essential for full-length Notch and Fng function but not for Notch^{ICD}. We also show that Nti is essential for the physical interaction between Notch and Delta in *Drosophila* cultured cells. Thus, our results establish Neurotic/OFUT1 as a moderator of Notch-ligand interactions, which has both Fng-dependent and Fng-independent functions.

Materials and methods

Fly stocks

We used *Canton-S* as a wild type. For misexpression analysis, *UAS-Notch* (gift from S. Artavanis-Tsakonas), *UAS-N^{act}* (Go et al., 1998) and *UAS-fng* (Panin et al., 1997) were driven by *ptc-GAL4* (*GAL4$^{559.1}$*) (Hinz et al., 1994) driver. Somatic mosaics were generated using the FLP/FRT system (Xu and Rubin, 1993) and the MARCM system (Lee and Luo, 1999). Germline mosaics were induced by the FLP-DFS technique (Chou and Perrimon, 1996).

Genetics

To generate mosaic clones, larvae were heat-shocked at 37°C for 1 hour 48-72 hours (germline mosaics, Fig. 3B,D) or 24-48 hours (others) after egg laying. Embryos that lack maternal *nti* function were laid by *y w hs-flp/+*; *FRT^{G13} ovo^{D1}/FRT^{G13} nti* female. These females

were crossed with *FRT^{G13} nti/CyO, ftz-lacZ* males. Somatic clones were made in *y w hs-flp*; *FRT^{G13} nti/FRT^{G13} Ubi-GFP* larvae, and for rescue experiment, *y w hs-flp*; *FRT^{G13} nti/FRT^{G13} Ubi-GFP*; *hs-nti/+* larvae were used.

Constructs and molecular cloning

Cloning of *nti* and generation of constructs were carried out according to standard protocols. *hs-nti* was constructed by ligating *nti* full-length cDNA into *Stu*I site of pCaSpeR-hs. *nti-IR* were made by ligating 1-534 and 1-745 cDNA tail-to-tail, and inserted into pUAST and pRmHa-3. *nti-myc* was constructed by inserting a Myc epitope sequence (EQKLISEEDL) after the 26th amino acid in Nti, and resulting fragment was subcloned into pRmHa-3. *nti-G3* was created using QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers (5′-GCATTCGTAAAAAGGGGAGGAGGTGTGCACGGT-TTTCC-3′ and its complementary strand), and the mutated fragment was subcloned into pRmHa-3. pRmHa-3-Dl-AP (Brückner et al., 2000) and mt-fngHA (Panin et al., 1997) were kindly provided by Stephen Cohen and Kenneth Irvine, respectively.

Immunohistochemistry

Wing imaginal discs dissected from late third instar larvae were immunostained as previously described (Matsuno et al., 2002). Immunostaining and in situ hybridisation to whole-mount embryos were performed according to standard protocols. Antibodies used for immunostaining were anti-Elav (9F8A9, dilution 1:10) (O'Neill et al., 1994), anti-Wg (4D4, 1:500) (Brook and Cohen, 1996), and anti-Notch (C17.9C6, 1:500) (Fehon et al., 1990). For observation, fluorescent microscopy (Fig. 1G,H) and confocal microscopy (BioRad Radience 2100, Fig. 1A-D, Fig. 2D, Fig. 3) were used.

Binding assay

Drosophila S2 cells were cultured in M3 medium supplemented with 10% fetal bovine serum. Binding assay was performed according to the method developed by Brückner et al. (Brückner et al., 2000) with modifications. The cells (1.67×10^6 cells/6 cm dish) were transfected with CellFECTIN (Invitrogen) with 0.56 μg of each of the expression vectors for Notch, Nti and Fng, or the empty control vector (pRmHa-3) to keep the total amount constant (1.67 μg). One day after transfection, CuSO$_4$ (final concentration, 0.7 mM) was added to the medium to induce the metallothionein promoter in pRmHa-3. The induction was carried out for 2 days. Conditioned medium was collected from the cells transfected with pRmHA-3-Delta-AP with the same method as described above. The conditioned medium containing Delta-AP supernatant was filtrated through 0.45 μm, supplemented with 0.1% NaN$_3$ and incubated with the adherent cells for 90 minutes. Cells were scraped from the dishes, washed five times with Hanks' balanced salt solution containing 0.05% BSA and 0.1% NaN$_3$, and lysed in 10 mM Tris-HCl (pH 8), 1% Triton-X100. Endogenous alkaline phosphatase (AP) activity was inactivated by heat treatment for 10 minutes at 65°C and lysates cleared by centrifugation. AP activity was determined with an AP assay kit (Wako, Japan) using p-nitrophenyl phosphate as a substrate.

Western blotting

Transfections were done as additional replicates for the binding assay. Protein was extracted from the cells with 20 mM HEPES-NaOH (pH 7.9) buffer containing 0.5% NP-40, 15% glycerol, 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM dithiothreitol, 1 mM sodium ortho-vanadate and proteinase inhibitors (Kitagawa et al., 2001). After separation by SDS-polyacrylamide electrophoresis, proteins were electrophoretically transferred onto PVDF membranes (BioRad). Primary antibodies used for blotting were anti-Notch intracellular domain (C17.9C6), anti-Myc (9E10), anti-HA (12CA5) and anti-tubulin (E7) (Chu and Klymkowsky, 1989). They were visualised with appropriate secondary antibodies conjugated with horseradish peroxidase, ECL (Amersham Pharmacia Biotech) chemiluminescent

substrate and X-ray films. In some cases, the membranes were stripped and reblotted.

Flow cytometric analysis

S2 cells were transfected with pRmHa-3-based expression vectors for GFP (gift from R. Tsuda), Notch and *nti* RNAi as described above. Cells mechanically detached from tissue culture dishes were washed in phosphate-buffered saline (PBS) once and incubated with an anti-Notch extracellular domain antibody (Rat-1, gift from S. Artavanis-Tsakonas) at 4°C for 20 minutes. After washing with PBS, the cells were incubated with a phycoerythrin-labeled anti-rat IgG antibody for 20 minutes at 4°C, washed with PBS and analysed with a FACScan (BD Biosciences).

Results

neurotic is a maternal neurogenic gene that is essential for Notch signalling

To identify novel genes involved in the Notch pathway, we screened a collection of zygotic lethal mutation with specific maternal effects (N.P., A. Lanjuin and C. Arnold, unpublished) for those associated with a neurogenic phenotype. We identified such a mutation, 4R6, in a novel gene that we named *neurotic* (*nti*). While *nti* zygotic mutant embryos have normal nervous systems (Fig. 1A,B), embryos derived from *nti* homozygous germline clones show a strong neurogenic phenotype (Fig. 1C) that is not significantly paternally rescued (Fig. 1D). Thus, the maternal effect phenotype of *nti* is similar to the neurogenic phenotypes associated with mutations that

are defective in Notch signalling. Next, we tested whether *nti* is required for other Notch-dependent developmental events, such as adult wing patterning (Greenwald, 1998; Artavanis-Tsakonas et al., 1999). In the adult wing, *nti* mutant clones result in loss of wing margin and vein thickening (Fig. 1E,F), phenotypes that are reminiscent of loss of Notch activity (de Celis and Garcia-Bellido, 1994). Notch regulates the expression of *wingless* (*wg*) at the dorsal and ventral (D/V) compartmental boundary (Fig. 1G) (Williams et al., 1993; de Celis and Garcia-Bellido, 1994; Rulifson and Blair, 1995; de Celis et al., 1996). Like *Notch* mutant clones, *nti* mutant clones across the D/V boundary are associated with loss of Wg expression (Fig. 1H). These phenotypes suggest that *nti* encodes an essential component of the Notch pathway.

neurotic encodes a O-fucosyltransferase homolog

The *nti* mutation 4R6 was mapped to 50C-D by complementation tests to deficiency lines, and nucleotide sequence analysis of the mutant chromosome revealed a nonsense mutation in the annotated gene CG12366. The single mutation changes the Lysine at position 133 in the putative ORF of 402 amino acids to a premature stop codon (AAG to TAG; Fig. 2A), strongly suggesting that 4R6 causes complete loss of CG12366 function. As expected from the *nti* maternal effect, CG12366 mRNA is uniformly distributed in early embryos but decreases during later stages (Fig. 2B,C; data not shown). To confirm that the *nti* mutant phenotype is caused by a disruption in CG12366, we tested whether inducible expression of CG12366 could rescue *nti* mutant cells. As shown in Fig. 2D, Wg expression in the *nti* clones is rescued in wing imaginal discs by ubiquitous expression of CG12366 (*hs-nti*). The maternal neurogenic phenotype could also be partially rescued using *hs-nti* (data not shown). We also constructed a hairpin construct (*nti-IR*) to express in vivo a dsRNA that would inhibit CG12366 function. Expression of *nti-IR* using *ptc-Gal4* causes wing nicks reminiscent of loss of Notch activity (de Celis and Garcia-Bellido, 1994) and *nti* mutant clones, consistent with RNAi-mediated
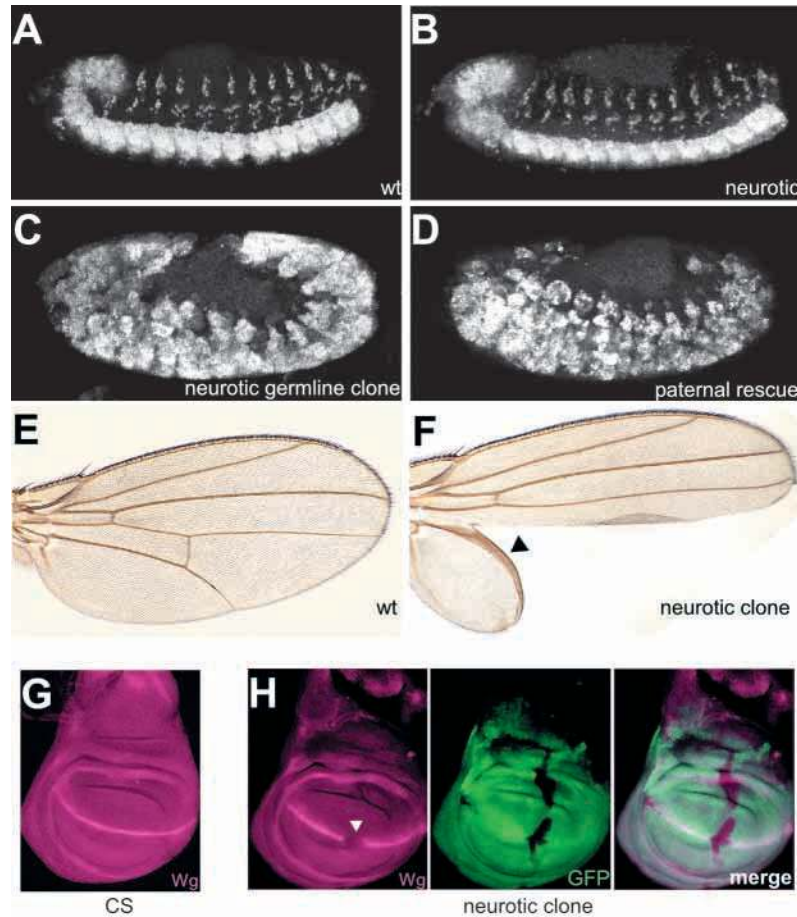
Fig. 1. *nti* mutation gives similar phenotypes to those defective in Notch signalling. (A-D) *nti* is a maternal neurogenic gene. Embryos were stained with anti-Elav to detect all differentiated neurons (Robinow and White, 1988). (A) Wild-type embryo shows normal nervous systems. (B) *nti* mutant embryos derived from heterozygous mothers also have a normal embryonic nervous system. (C) Embryos that lack both maternal and zygotic *nti* function show a severe neurohyperplasia, and their germ band fails to shorten. (D) Paternally supplied *nti* gene could hardly rescue the maternal effect neurogenic phenotype; however, germ band retraction occurred in these embryos. (E) Wild-type wing. (F) *nti* mutant clones are associated with wing nicking and vein thickening (arrowhead). Both are typical phenotypes associated with defects in Notch signalling. (G) Wg is expressed at the DV boundary in wing imaginal disc. (H) Wg expression (purple) is lost in *nti* mutant clone (arrowhead). *nti* clones are detected by lack of GFP expression (green). (A-D,G-H) Anterior is towards the left and dorsal is upwards.

A

B

C

D

nti clone; hs-nti   Wg   GFP   merge

E

UAS-nti-IR

F

G

Fng-dependent

Fng-independent
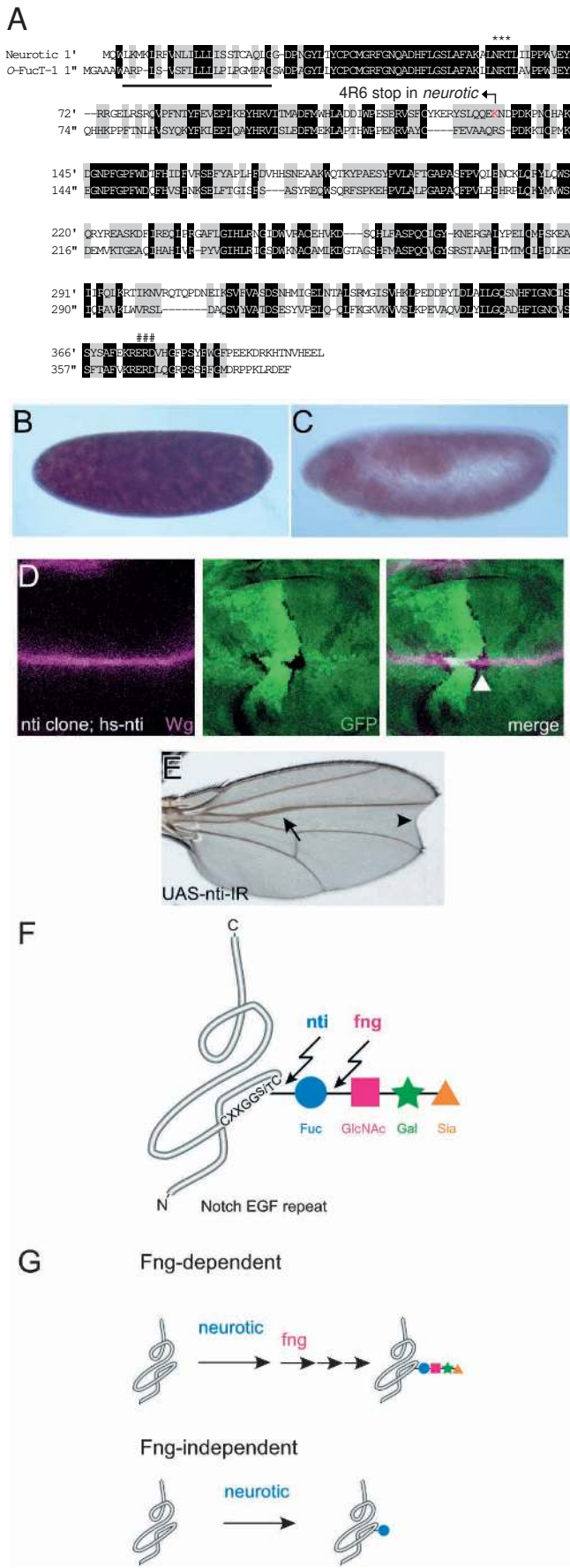
inactivation of *nti*. We used this *nti-IR* expression construct to knock down *nti* function in cultured cells (see below). These results demonstrate that CG12366 encodes *nti* function.

Database searches revealed that *nti* encodes a putative protein with significant homology to human GDP-fucose *O*-fucosyltransferase 1 (Wang et al., 2001) (*O*-FucT-1; Fig. 2A). *O*-FucT-1 is an enzyme that adds a carbohydrate, fucose, to serine or threonine residues in the consensus sequence C*XX*GGS/TC (*X* is any amino acids) between the second and third conserved cysteines of EGF repeats (Harris and Spellman, 1993; Wang et al., 2001). Notch and its ligands, Delta and Serrate, have such sequences in their extracellular EGF repeats, and all of them are *O*-fucosylated (Moloney et al., 2000a; Moloney et al., 2000b; Panin et al., 2002). Recently, Okajima and Irvine (Okajima and Irvine, 2002) have demonstrated that *Drosophila O*-FucT-1 is required for the activation of Notch by its ligands using a RNAi knockdown approach. Furthermore, Shi and Stanley (Shi and Stanley, 2003) showed that *O*-FucT-1 mutant mice have a phenotype similar to that of Presenilins or RBP-Jκ. Thus our present results confirm these previous observations and support the model that *O*-FucT-1 is an essential component of Notch signalling.

Fringe (Fng), a modulator of Notch signalling, adds *N*-acetylglucosamine (GlcNAc) onto the *O*-fucose moieties of Notch and its ligands (Brückner et al., 2000; Moloney et al., 2000a). Modification of Notch by Fng, and subsequent glycosylation of Notch, modulates the binding of Delta and Serrate to Notch (Fleming et al., 1997; Panin et al., 1997; Moloney et al., 2000a; Brückner et al., 2000; Chen et al., 2001). These reports and our results suggest that *nti* enables Notch to respond to its ligands by adding *O*-linked fucose to the Notch EGF repeats. However, our finding may also be

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Fig. 2. (A) Sequence comparison of Nti and human GDP-fucose *O*-fucosyltransferase (*O*-FucT-1). The identical amino acids are reversed, conserved changes are shaded. The proteins are 42% identical and 75% similar. The position of the 4R6 mutation is shown in red (replace Lysine in Nti by stop in 4R6). The putative type II transmembrane domain is underlined. The positions of the potential *N*-glycosylation (*) site, and the E*X*D motif that is important for glycosyltransferase activity (#), are also shown. (B,C) In situ hybridisation to whole-mount embryos stained with a *nti* cDNA probe revealed that *nti* mRNA is uniformly expressed in stage 3 embryos (B), and gradually disappears by stage 11 (C). *nti* mRNA is not detectable after stage 14 (not shown). (D) Wg expression in wing imaginal disc was recovered in *nti* mutant cells with *hs-CG12366* (*hs-nti*; lack of GFP shown by green, arrowhead), indicating that *nti* is CG12366. (E) Expression of a *nti* inverted repeat RNA driven by *ptc-GAL4* generates wing phenotypes (vein thickening and wing nicking indicated by arrow and arrowhead, respectively) that resemble wings containing *nti* homozygous mutant clones (Fig. 1F), indicating that *nti-IR* is indeed blocking *nti* function. (B-D) Anterior is towards the left and dorsal is upwards. (F) A schematic diagram of the tetrasaccharide, Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc, attached to a EGF repeat of Notch. Nti adds *O*-linked fucose, and *O*-fucose residues is further elongated by Fng, β1,3-*N*-acetylglucosaminyltransferase. The *N*-acetylglucosamine (GlcNAc) is further elongated by an endogenous β1,4-galactosyltransferase and a β2,3-sialyltransferase. (G) Model for Fng-dependent and Fng-independent glycosylation of a Notch EGF repeat. (Upper) *O*-fucose residue attached by Nti is elongated to yield the tetrasaccharide in Fng-dependent manner. (Lower) Without Fng function, EGF repeats of Notch is *O*-fucosylated by Nti.

unexpected because no report has ever been associated *fng* mutations with the neurogenic phenotype (Irvine, 1999). Therefore, *nti*, like Notch, is essential for lateral inhibition during neuroblast segregation, a process for which *fng* is probably dispensable. Furthermore, the *nti* mutant phenotype in adult wings is also distinct from that of *fng* (Irvine, 1999). These results indicate that *nti* has a function that is independent of *fng*. Such a *fng*-independent function was also proposed

from the analysis of the neurogenic phenotype induced by RNAi of Nti/OFUT1 in *Drosophila* bristles (Okajima and Irvine, 2002).

Neurotic functions between full-length Notch and Notch[ICD], and is essential for Fng function

To define precisely the function of *nti* in the Notch signalling cascade, we investigated the epistatic relationship between *nti* and other genes involved in Notch signalling. We first asked whether the activity of the Notch intracellular domain (Notch[ICD]), which acts as a constitutively active Notch receptor (Struhl et al., 1993), requires *nti*. As shown in Fig. 3A, expression of Wg is absent in *nti* mutant clones. However, Notch[ICD] could induce Wg expression in *nti* mutant clones (Fig. 3B) as it can in wild-type cells (Diaz-Benjumea and Cohen, 1995). A similar experiment was carried out with an overexpressed full-length Notch receptor, which in wild type also induces ectopic Wg (Fig. 3C) (Matsuno et al., 2002). Interestingly, in contrast to Notch[ICD], the full-length Notch could not induce ectopic Wg expression in *nti* mutant clones (Fig. 3D), indicating that *nti* is required for the activity of full-length Notch but not for its intracellular domain. These interactions are consistent with the idea that Nti modifies the extracellular domain of Notch or its ligands.

Next, we investigated the epistatic relationship between *nti* and *fng*. From the structure of the *O*-linked tetrasaccharide attached to the EGF repeats of Notch and from enzymatic functions of Nti and Fng, it was speculated that Nti activity is a prerequisite for the function of Fng (Brückner et al., 2000; Moloney et al., 2000a). As previously reported, ectopic
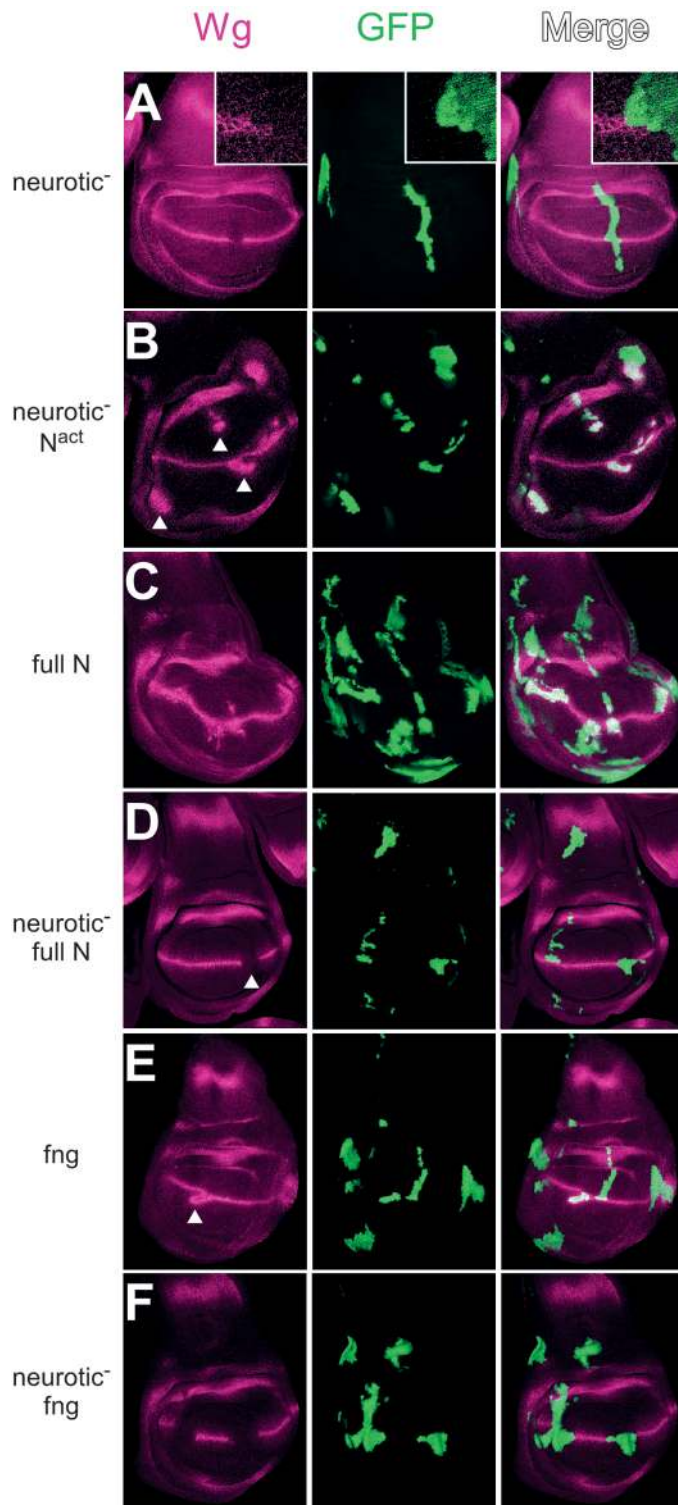
Fig. 3. Epistatic analyses between *nti*, *Notch* and *fng*. In all figures, Wg expression is shown in purple and somatic clones that lack *nti* function, and/or that overexpress specific genes, are shown in green. Anterior is towards the left and dorsal is upwards. (A) *nti* somatic clones showed cell-autonomous loss of Wg expression. Higher magnifications of the clones at the DV border are shown in the insets. *y w hs-flp/+; FRT[G13] nti/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP* larva wing imaginal disc is shown. *nti* homozygous clones are shown by GFP fluorescence (green). (B) Cell-autonomous Wg ectopic expression was detected when an activated form of Notch was expressed in *nti* mutant cells (arrowhead) as in wild-type cells (data not shown), indicating that *nti* is not needed for Notch[ICD] function. Genotype of the wing imaginal disc is *y w hs-flp/+; FRT[G13] nti/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP UAS-N[act]*. Thus, GFP indicates *nti* homozygous cells overexpressing Notch[ICD]. (C) Overexpression of *Notch* is associated with ectopic Wg expression near the DV boundary. The genotype is *y w hs-flp/+; FRT[G13]/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP UAS-Notch*. GFP shows full-length Notch overexpressing cells. (D) Full-length Notch effect was suppressed in *nti* mutant cells, indicating that *nti* is epistatic to full length Notch receptor. The genotype is *y w hs-flp/+; FRT[G13] nti/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP UAS-Notch*. GFP shows *nti* homozygous cells overexpressed for full-length Notch. (E) Ectopic expression of *fng* in the ventral compartment of the wing imaginal disc is associated with ectopic Wg expression near the margin. The genotype is *y w hs-flp/+; FRT[G13]/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP UAS-fng*. GFP shows Fng-overexpressing cells. (F) Ectopic expression of *fng* in *nti* mutant cells had no effect on Wg expression, showing that Nti is essential for Fng function. The genotype is *y w hs-flp/+; FRT[G13] nti/FRT[G13] tubP-GAL80; tubP-GAL4/UAS-GFP UAS-fng*. GFP shows *nti* homozygous cells overexpressing Fng.

expression of Fng in the ventral compartment of the wing imaginal disc induced Wg ectopically (Fig. 3E) (Panin et al., 1997). By contrast, ectopic expression of Fng in *nti* mutant clones did not induce ectopic expression of Wg, and endogeneous expression of Wg is absent in these clones (Fig. 3F). These results indicate that Nti is essential for Fng function. These findings are consistent with the RNAi experiments against Nti/OFUT1 (Okajima and Irvine, 2002).

Neurotic is essential for Delta-Notch binding

As both Notch and its ligands are *O*-fucosylated, we next analysed the effects of *nti* expression on the ligand-receptor interaction using the method of Brückner et al. (Brückner et al., 2000). S2 cultured cells were transfected with Notch and subsequently incubated with conditioned medium containing a Delta-alkaline phosphatase fusion protein (Delta-AP) prepared also by transfection to S2 cells. As S2 cells are Notch-deficient (Fehon et al., 1990), AP activity specifically bound to Notch-transfected cells will reflect the ligand binding activity of Notch. As previously reported, co-expression of Fng with Notch increases its ability to bind to Delta (Fig. 4A, compare lanes 2,3) (Brückner et al., 2000). Nti co-expression alone does not significantly alter the binding ability of Notch (Fig. 4A, lane 4). We speculated that Nti, expressed endogenously, is saturated for the Notch-Delta binding under this condition. Endogenous activity of Nti/OFUT1 in S2 cells was reported
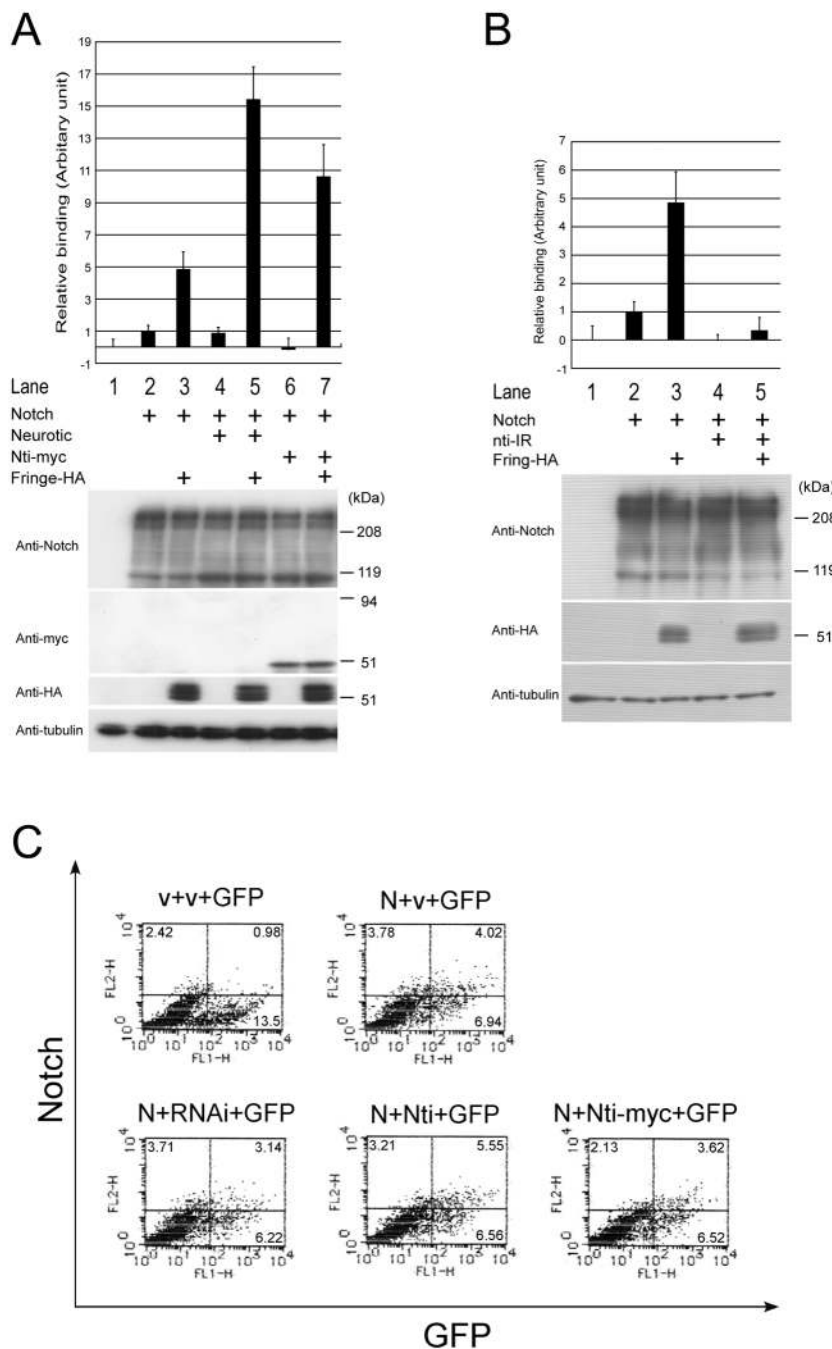
Fig. 4. Nti function is essential for Notch receptor to bind Delta ligand. (A) Co-expression of Nti and Fng with Notch synergistically increases the binding to Delta-AP. (Upper panel) Delta-AP binding to transfected S2 cells as measured by an enzymatic activity of alkaline phosphatase (Brückner et al., 2000). S2 cells were transfected with the expression vectors for the proteins indicated as + or the empty control vectors to keep the total amount of the plasmids constant. The relative binding of the ligand was expressed as an arbitrary unit that takes the normalised bound AP activity of the empty vector-transfected cells as zero, and that of the cells transfected with Notch alone as one. Means±s.d. of triplicate assays are shown. Lower panel, Western blots of S2 cell extracts from the cells prepared in parallel to those used for the binding assays presented in the upper panel. The blot was probed with the anti-Notch, anti-Myc, anti-HA and anti-tubulin antibodies. Two forms of Notch were recognised by the anti-Notch antibody (9C6) that directs a region of the intracellular domain of Notch. The upper band is the unprocessed form of Notch and the lower band is the C-terminal part of the cleaved form. (B) Co-expression of *nti-IR* (RNAi for *nti*) with Notch abolishes the binding to Delta-AP irrespective of Fng overexpression. The analysis was carried out and the results were presented as in A. (C) Expression of Notch on cell surface is not influenced by the changes in Nti expression. The cells transfected with the indicated expression vectors were analysed by flow cytometry with an antibody directed against the extracellular domain of Notch (Rat-1). The numbers denote the percentages of cells in the indicated squares.

previously (Okajima and Irvine, 2002). However, co-expression of Nti with Fng potentiates Notch binding activity (Fig. 4A, lanes 5,7). Importantly, co-expression of Nti or Fng does not significantly change the expression of Notch protein in the cells (Fig. 4A, Anti-Notch). We noted that co-expression of Nti-Myc, but not wild-type Nti, with Notch inhibited the Notch-Delta binding, suggesting that Nti-Myc behaves as a dominant-negative protein (Fig. 4A, lane 6, see Discussion).

We next examined the effect of *nti* RNAi in this system. *nti-IR* reduces Notch binding activity to the level of vector-transfected cells, indicating Nti is required for Notch-Delta binding (Fig. 4B, lane 4). Knock down of Nti activity by RNAi also resulted in the disruption of Fng function and an increase in Notch-Delta binding (Fig. 4B, lane 5). This is consistent with the model that Fng attaches GlcNac to the *O*-fucose residue that is added by the Nti beforehand (Fig. 2F,G). Furthermore, these results suggest that the profound disruption of Notch signalling observed in vivo is due to the defective ligand-receptor interaction, because, during embryogenesis, only Delta is proposed to function as a ligand for Notch (Thomas et al., 1991).

To test the possibility that *nti* might affect Notch presentation on the cell surface, we examined expression of Notch in live transfected cells using flow cytometry. In this experiment, S2 cells were co-transfected with GFP and Notch expression constructs, as well as either *nti*, *nti-IR* or control constructs, and stained with an antibody raised against the Notch extracellular domain. As shown in Fig. 4C, the ratio of double positive cells for GFP and the anti-Notch (cells expressing Notch on the cell surface) was not significantly affected by either the knockdown or the overexpression of Nti (see upper-right area of each). Thus, Notch is expressed in a form accessible to the antibody and probably to the ligand irrespective of *nti* activity. These results suggest that Nti affects the physical interaction between Notch and Delta, rather than cellular distribution or transport of Notch. It has been reported that Delta and Serrate are also *O*-fucosylated (Panin et al., 2002). However, neither knockdown nor the overexpression of Nti affects the ability of Delta to bind Notch (Fig. 5). These results are consistent with the in vivo result that *nti* functions cell autonomously (Fig. 3A).

The primary structure, as well as in vivo and in vitro analyses presented here, strongly suggests that Nti encodes a protein *O*-fucosyltransferase. Nti contains a D*X*D-like motif, E*X*D (see Fig. 2A) that is conserved in the mammalian homolog and that has been shown to be important for their enzymatic activity (Wang et al., 2001; Wiggins and Munro, 1998). Fig. 6 shows that the G3 mutation that results in amino acid substitutions from ERD to GGG ('*Nti-G3-myc*') abolishes the activity of Nti when co-expressed with Fng. Nti-G3-myc also behaves as a dominant-negative protein (Fig. 6, lanes 6,7). These results suggest that glycosyltransferase activity is required for Nti function.

Discussion

### The O-fucosyltransferase Neurotic is an essential component of Notch signalling

We have identified a novel gene, *nti*, that is essential for the Notch signalling pathway, and elucidated its molecular mechanism of action. *nti* encodes a *Drosophila* homolog of an

O-fucosyltransferase that is known as *O*-FucT-1 in mammals (Wang et al., 2001). *O*-FucT-1 was identified as an enzyme responsible for adding *O*-fucose to EGF repeats (Wang and Spellman, 1998). Enzymatic modification of the *O*-fucose residues on Notch EGF has been shown to modulate binding between Notch and its ligands (Brückner et al., 2000; Moloney et al., 2000a; Chen et al., 2001). Furthermore, recently, Okajima and Irvine (Okajima and Irvine, 2002) reported that 23 out of 36 EGF repeats of Notch have potential *O*-fucosylation sites by Nti (referred to as OFUT1 in their study), and that Nti/OFUT1 has an EGF *O*-fucosyltransferase enzymatic activity in vitro. These authors also showed that Nti/OFUT1 is essential for Notch signalling by a RNAi approach. We describe the phenotypes associated with mutations in the *Drosophila O*-fucosyltransferase gene, and demonstrate that Nti is indispensable for Notch-Delta interactions. Studies on *Drosophila* Nti/OFUT1 are consistent with recent studies on the null phenotype of the *O*-fucosyltransferase gene in the mouse (Shi and Stanley, 2003).

Notch signalling is involved in two major classes of cell-cell signalling: lateral inhibition and inductive signalling (Artavanis-Tsakonas et al., 1999). Neuroblast segregation in early embryogenesis is a well known example of lateral inhibition, and failure of the Notch signalling pathway during this process leads to an hypertrophy of neuroblasts, known as the neurogenic phenotype (Campos-Ortega, 1995). However, induction of several genes, such as *wg*, along the dorsal/ventral compartmental boundary of the wing disc depends on inductive Notch signalling. In both classes of Notch signalling events, the *Notch* and *nti* mutant phenotypes are identical, indicating
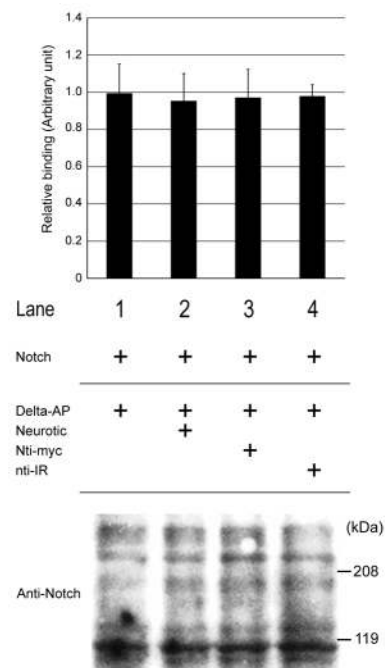
Fig. 5. Neither overexpression Nti nor co-expression of *nti-IR* with Delta-AP affects the ability to bind to Notch. Conditioned media were prepared from S2 cells transfected with Delta-AP and either the empty vector, Nti, Nti-myc or *nti-IR* in 1:1 ratio as indicated. Binding to the S2 cells co-transfected with Notch and Fng are measured.
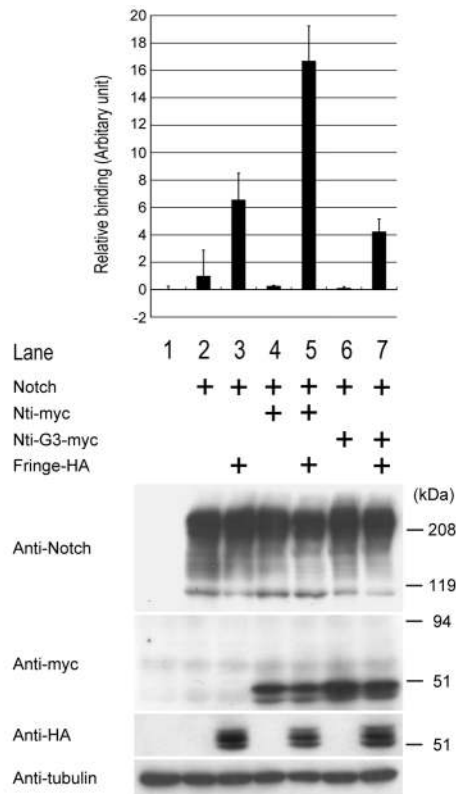
Fig. 6. The signature motif for glycosyltransferases is necessary for the Nti activity. Notch-Delta binding was assayed with Nti-G3-myc that was created from Nti-myc by inducing amino acid changes ERD (shown by # in Fig. 2A) to GGG. ERD is thought to be important for glycosyltransferase activity. The analysis was done and the results were presented as in Fig. 4A. Compared to the binding observed in Nti-myc, Notch and Fng co-expressing cells, Nti-G3-myc co-transfection with Notch and Fng does not enhance Notch-Delta binding. Instead Nti-G3-myc acts as dominant-negative form of Nti, because the Notch-Delta binding is lower than observed in Notch and Fng co-transfected cells.

that Nti is essential for Notch signalling. Our finding differs with the recent report using *nti* RNAi (Okajima and Irvine, 2002), as apparent embryonic neurogenic phenotypes were not noted. This discrepancy is probably due to the partial knockdown effect generated by RNAi.

There are a considerable number of genes, including the ligands of the Notch receptor, that encode putative proteins that contain EGF motifs with consensus sequences for *O*-fucosylation in the *Drosophila* genome (Haltiwanger, 2002). However, mutant phenotypes of *nti* are strikingly similar to those of the Notch receptor. In fact, in addition to the wing phenotype shown above, *nti* somatic clones exhibit mutant phenotypes that are characteristic of Notch loss-of-function mutations. These include defects on the notum bristles, rough eye and the leg segment fusion (data not shown). These phenotypes were also observed in the knockdown study of *nti* (Okajima and Irvine, 2002). These results indicate that the primary target of *O*-fucosylation of Nti is Notch, while there may be unrevealed signalling pathways that *nti* function is required. Roos et al. (Roos et al., 2002) have reported that there is another putative *O*-fucosyltransferase in *Drosophila* and

mammals. Thus, it is tempting to speculate that each *O*-fucosyltransferase has distinct target(s) that are involved in specific cell signalling pathways.

Neurotic has both Fng-dependent and Fng-independent function in Notch signalling

As *O*-fucose on Notch has been shown to act as a molecular scaffold for GlcNAc that is elongated by Fng, one would expect that the phenotypes of *O*-fucosyltransferase mutant might be the same as those of *fng*. Unexpectedly, however, we found that the *nti* and *fng* mutant phenotypes are quite different. Strikingly, the embryonic neurogenic phenotype that is evident in *nti* mutant, and is an indication of its essential role in Notch signalling, has never been reported in *fng* mutants (Irvine, 1999). Furthermore, it is thought that Fng does not have a significant role in lateral inhibition, while it is involved in generation of the cell boundary between cells expressing Fng and cells not expressing Fng (Irvine, 1999). Additionally, our in vitro binding assay revealed that Nti is essential for binding between Notch and Delta. Based on the previous findings and our present results, we propose that *O*-glycosylation of Notch EGF repeats has two distinct roles for binding to Delta (Fig. 7). First, *O*-fucosylation catalysed by Nti is an absolute requirement for binding between Notch and the ligand, and this binding is sufficient to accomplish lateral inhibition. For this function, no additional glycosylation to *O*-fucose residue is required. This idea is also supported by the observation that in the tissues and organisms that do not express *fng*, Delta is competent to activate the Notch receptor (Panin et al., 1997). In this respect, it is worth noting that in the *C. elegans* genome there is a highly conserved *nti*, while a *fng* homolog is not found (Wang et al., 2001). Second, addition of GlcNAc to the *O*-fucose residue by Fng enhances the interactions between Notch and Delta, modulating the receptor-ligand interactions (Fleming et al., 1997; Panin et al., 1997; Klein and Arias, 1998). In fact, it is known that the expression of *fng* has high degree of regional specificity, and the boundary of the cells expressing and not expressing Fng often defines the border of distinct tissue structures (Irvine and Wieschaus, 1994; Dominguez and de Celis, 1998; Cho and Choi, 1998). Thus, the region-specific expression of *fng* allows modulation of Notch signalling, resulting in generation of complex structure of organs. As expected from the second function of Nti, its function is essential for Fng-dependent modulation of Notch signalling as well as Fng-independent function. In the wing disc, *nti* is epistatic to *fng*, and *fng* requires *nti* to induce Wg at the dorsal and ventral compartment boundary. Additionally, in in vitro binding assay, Fng depends on Nti to enhance the binding between Notch and Delta. These lines of evidence indicate that Nti is involved in Fng-dependent modulation of Notch signalling, which is consistent with an *O*-glycan structure of the Notch EGF repeats (Brückner et al., 2000; Moloney et al., 2000a). This Fng-dependent function of Nti is not contradictory to our first model.

Given that *nti* has novel functions, what is the significance of Nti in Notch signalling at the level of cells and the organism? We propose a model for *nti* function during development. This model involves a novel receptor on-off mechanism. In early embryo, *nti* mRNA was detected uniformly (Fig. 2B). However, Okajima and Irvine (Okajima and Irvine, 2002) have reported that expression of *nti/OFUT1* was spatially regulated

during the late stages of embryogenesis and the imaginal discs. Therefore, *O*-fucosylation of Notch EGF repeats is probably regulated temporally and spatially, and may provide a novel mechanism to regulate Notch signalling. For example, in cells
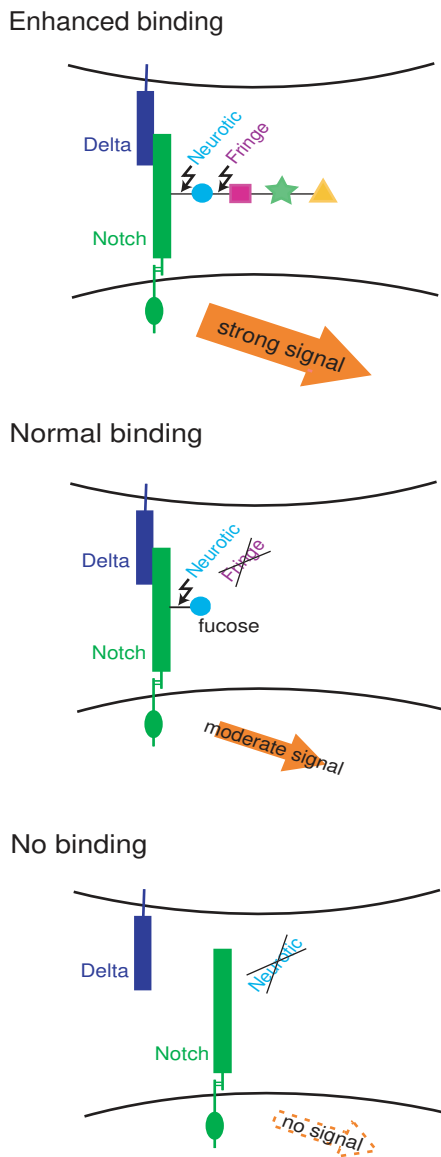
Fig. 7. Schematic drawing of Nti molecular function. *O*-fucose-containing glycans attached to the specific EGF repeat of Notch is the tetrasaccharide: Sia-α2,3-Gal-β1,4-GlcNac-β1,3-Fuc-α1-*O*-Ser/Thr. First, *O*-fucose is attached to a serine or threonine within a consensus sequence of Notch EGF repeat by Nti. *O*-fucosylation is essential for binding between Notch and Delta. Thus, if there is a lack of *O*-fucosylation, activation of Notch does not occur (lower panel). In the cells that do not express Fng, *O*-fucose on Notch does not elongate further (middle panel). However, Notch modified by *O*-fucose is competent to bind to the Delta and Serrate. Next, *N*-acetylglucosamine is added to the *O*-fucose residue by Fng, then *O*-fucose glycans are elongated by β1,4-galactosyltransferase and α2,3-sialyltransferase (upper panel). Notch modified by these *O*-linked glycans has an enhanced activity to bind to Delta. Thus, in the cells expressing Fng and the other two endogenous glycosyltransferases, Notch become more potent to receive signal than neighboring cells that do not express Fng.

expressing *nti* at higher level than their neighbours, Notch may become more potent to receive a signal. Removal of *O*-fucose from the Notch EGF repeats may be part of this regulation.

In this work, we failed to demonstrate an effect of *nti* on Notch-Serrate binding, because, as reported before, Notch-Serrate-AP binding could not be detected by this binding assay (Brückner et al., 2000). However, phenotypes observed in the somatic clones of *nti* generated in the wing disc strongly suggests that Nti affects the Notch-Serrate binding (Fig. 1H). For example, in the *nti* clones near the dorsal and ventral boundary from the ventral side, induction of Wg is impaired, indicating that *nti* clones fail to receive the Serrate signal. Thus, we propose that the requirement of *nti* for Notch-ligand binding is not restricted to Delta. However, the neurogenic phenotype of *nti* could be wholly explained by the effect on Notch-Delta binding, as Delta is the only the ligand of Notch involved in neuroblast segregation during early embryogenesis (Thomas et al., 1991).

In our Notch-Delta binding assay, co-expression of Nti and Fng with Notch increased the Notch-Delta binding over that of Fng with Notch, whereas co-expression of a wild-type Nti with Notch did not increase this binding. We speculate that the amount of Nti endogenously expressed in S2 cells, as evidenced by the effectiveness of *nti-IR* shown in Fig. 4B, saturates the ability of fucose alone to promote the ligand binding. Indeed, it was shown that S2 cells endogenously expressed Nti/OFUT1 (Okajima and Irvine, 2002). However it does not imply that addition of *O*-fucose to Notch by Nti is also saturated under these conditions. Namely, the increase of *O*-fucosyltransferase activity, which does not lead to elevation of Delta binding per se, could increase the Notch-Delta binding after elongation of the *O*-fucose residue by Fng. These results suggest that linear range by which fucose-GlcNAc moiety can elevate the ligand binding is wider than that by which fucose alone can. Multiple sites that are possibly *O*-fucosylated were found in the EGF repeats of Notch (Okajima and Irvine, 2002). Thus, it is possible that some of these potential *O*-fucosylation sites may influence the Delta binding only after modification by Fng. We also noticed that Nti-myc probably has a dominant-negative function (Fig. 4A, lane 6). This dominant-negative activity of Nti-myc is apparently restored by co-expression with Fng, which makes the ligand binding well over that by Fng expression alone (Fig. 4A, lanes 3,7). This is in contrast with the fact that the decreased Notch-Delta binding associated with RNAi of *nti*, which is supposed to merely reduce the endogenous Nti protein, hardly restored by co-expression with Fng (Fig. 4B). Therefore, we speculate that the Myc-tag does not simply disrupt the enzymatic activity of Nti-myc. Some kind of competition among Nti-myc, endogenous Nti, Fng and Notch may be involved in this dominant-negative effect. In support of this model, physical interaction between Notch and Fng has been reported (Ju et al., 2000). This dominant-negative effect may also account for the reduction of the Notch-Delta binding associated with Nti-G3-myc. We speculate that Nti-myc and Nti-G3-myc, both of which carry the same Myc-tag, have similar dominant negative effects, which do not depend on the enzymatic activity of *O*-fucosyltransferase and which are restored by co-expressing Fng. The slight reduction of the Notch-Delta binding associated with Nti-G3-myc and Fng over that of Fng alone may also be caused by this dominant-negative effect of Nti-G3-myc.

Role of O-fucosylation in cell signalling

The importance of glycoconjugates in cell adhesion and cell-cell communication has been believed for many years, since nearly all cells were found to be covered with numerous carbohydrate-rich molecules (Roseman, 2001). More recently, gene disruption of a number of glycosyltransferases resulted in embryonic lethality in mice, underscoring their importance in development of multicellular organisms. However, most of the reports are only descriptive of the phenotypes and the molecular mechanisms are still elusive (Furukawa et al., 2001; Dennis et al., 1999). Here, we have presented one of the very few examples of molecular explanation for such mechanisms of action. Furthermore, it is an unprecedented example of an absolute requirement of a protein glycosylation event for a ligand-receptor interaction. The simplest interpretation of these phenomena would be that association of Delta to its receptor involves lectin-like protein-carbohydrate interaction. This hypothesis is consistent with the result that O-fucosylation induces little conformational change in EGF repeat of factor VII (Kao et al., 1999).

The first protein shown to be O-fucosylated was urinary type plasminogen activator (uPA) (Kentzer et al., 1990), followed by tissue-type plasminogen activators and several clotting factors. It has been shown that O-fucosylation of uPA is essential for activation of the uPA receptor (Rabbani et al., 1992) that has diverse functions including plasminogen activation (Dear and Medcalf, 1998), although uPA receptor-deficient mice survive to adulthood with no overt phenotypic abnormalities and are fertile (Bugge et al., 1995). Another O-fucosylated protein is Cripto, a member of the EGF-CFC family. Cripto is thought to act as an essential co-factor for Nodal signalling (Minchiotti et al., 2002). Amino acid substitution of threonine to alanine, which prevents O-fucosylation reduced the signalling activity of Nodal, suggesting that O-fucosylation is important for Cripto function (Schiffer et al., 2001; Yan et al., 2002). Until now, more than ten proteins have been found to carry the consensus sequence of O-fucosylation site (Haltiwanger, 2002). In this paper, we have shown that O-fucosylation is mandatory for Notch signalling. Although the O-fucosyltransferase presented here has remarkable functional specificity to the Notch receptor, there seems to be plurality of such enzymes (Roos et al., 2002). Therefore, the regulation of protein activity or function by O-fucosylation could be a general mechanism for many signal transduction systems.

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