Notch receptors are transmembrane proteins that regulate a wide range of developmental processes. Notch is modified in its epidermal growth factor-like domains by the addition of fucose to serine or threonine residues. O-Fucosylation is mediated by protein O-fucosyltransferase 1, and down-regulation of this enzyme by RNA interference or mutation of the Ofut1 gene in Drosophila or by mutation of the Pofut1 gene in mouse prevents Notch signaling. To investigate the molecular basis for the requirement for O-linked fucose on Notch, we assayed the ability of tagged, soluble forms of the Notch extracellular domain to bind to its ligands, Delta and Serrate. Down-regulation of OFUT1 by RNA interference in Notch-secreting cells inhibits both Delta-Notch and Serrate-Notch binding, demonstrating a requirement for O-linked fucose for efficient binding of Notch to its ligands. Conversely, overexpression of OFUT1 in cultured cells increases Serrate-Notch binding but inhibits Delta-Notch binding. These effects of OFUT1 are consistent with the consequences of OFUT1 overexpression on Notch signaling in vivo. Intriguingly, they are also opposite to, and are suppressed by, expression of the glycosyltransferase Fringe, which specifically modifies O-linked fucose. Thus, Notch-ligand interactions are dependent upon both the presence and the type of O-fucose glycans.

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Notch receptors are transmembrane proteins that regulate a wide range of cell fate decisions throughout the Metazoa (for review, see Ref. 1). The broad range of processes that require normal Notch signaling is reflected in the range of human diseases that result from mutations in components of the Notch signaling pathway. These include leukemia, Alagille syndrome, CADASIL, and spondylocostal dystoses (for review, see Refs. 2 and 3). Notch receptors are activated by two types of ligands, Serrate and Delta ligands. The core components of the Notch pathway are all highly conserved, but the Drosophila genome encodes a single Notch, a single Serrate (SER), and a single Delta (DL), whereas mammalian genomes encode four Notches, three Deltas, and two Serrates (called Jagged in mammals). Interaction of ligand and receptor triggers proteolytic processing of Notch, resulting in the translocation of the intracellular domain of Notch from the plasma membrane to the nucleus (for review, see Ref. 4). In the nucleus, Notch forms a complex with other proteins to regulate the transcription of downstream target genes.

Recent studies have revealed that glycosylation is essential for normal Notch signaling. The extracellular domain of Notch includes 36 tandemly repeated epidermal growth factor-like (EGF) domains. Many of these EGF domains are subject to an unusual post-translational modification in which fucose is attached in an O-linkage to a serine or threonine residue that occurs right before the third cysteine of the EGF domain (5). O-linked fucose (O-fucose) is essential for Notch signaling as down-regulation of the enzyme that mediates O-fucosylation by RNAi in Drosophila or by mutation in mouse impairs Notch signaling (6,7). Subsequent to the action this enzyme, protein O-fucosyltransferase 1 (OFUT1 in Drosophila), O-fucose can be further elongated by the action of another glycosyltransferase, Fringe, which attaches N-acetylgalactosamine (GalNAc) in a β1,3 linkage to EGF-O-fucose (8,9). In contrast to the general positive requirement for the O-fucose monosaccharide in Notch signaling, studies of Fringe indicate that elongated forms of O-fucose glycans differentially modulate the ability of the two types of Notch ligands to activate the Notch receptor (Refs. 10–13; for review, see Refs. 15).

Genetic and cell culture studies further show that both OFUT1 and Fringe influence Notch signaling cell autonomously (7,10,13), indicating that they act in the signal receiving cell, presumably by directly glycosylating Notch, which is a substrate for their glycosyltransferase activities (5,7–9). Epistasis analyses indicate that both OFUT1 and Fringe act upstream of the activated (cleaved) form of the Notch receptor (7,10,13), which positions the requirement for O-fucose glycans upstream of the proteolytic cleavages associated with Notch activation. In principle this leaves several possibilities for how O-fucose glycans affect Notch signaling, such as an influence on intracellular trafficking, an influence on Notch-ligand binding, or an influence on Notch proteolysis. The possibility that OFUT1 affects Notch-ligand binding has not been investigated previously, and evidence both for and against the possibility that Fringe influences Notch signaling at the level of Notch-ligand binding has been presented in prior studies (9,10,16,17).

Here, we demonstrate that the O-fucose monosaccharide is essential for the normal binding of Notch to its ligands, DL and SER. We also extend prior studies of the influence of O-fucose elongation by demonstrating that Fringe not only enhances DL-Notch binding (9) but also inhibits SER-Notch binding, and we show that OFUT1 overexpression and Fringe expression exert opposing influences on Notch-ligand binding. Our results underscore the central importance of O-fucosylation to Notch
signaling and identify a mechanism by which O-fucose glycans exert their influence.

MATERIALS AND METHODS

Expression Constructs—All proteins were expressed under control of the metallothionein promoter. N-AP and DL-AP expression constructs were from S. Cohen (9), and FNG and OFUT1 expression constructs have been described previously (7, 8, 13). We also used an OFUT1 expression construct in which V5 and His tags were added to the C terminus (pRMHa3b-Ofut1V5His). These tags have no detectable influence on its enzymatic activity. To express FcAP, the plasmid pMT/BIP/Fc-AP was constructed by amplifying DNA encoding amino acids 1–530 of human placental alkaline phosphatase, and after washing beads, the amount bound to beads was re-evaluated by Western blotting. For Western blotting, N-EGF:FLAG was detected using a mouse anti-FLAG primary antibody, a horseradish peroxidase-coupled anti-mouse secondary antibody, and Supersignal West Dura chemiluminescence substrate (Pierce) and then assayed using a Fluorochem 8800 CCD camera system (Alpha Innotech).

For Preparation of Beads—5 ml of conditioned media from cells secreting N-EGF:FLAG or control cells transfected with pMT1B1 vector was mixed with 50 ml of Eview Red anti-FLAG M2 affinity gel (Sigma) at 4 °C overnight and then washed 3 times with 5 ml of Tris-buffered saline supplemented with 5 mM CaCl2. Similar amounts of N-EGF:FLAG from normal and RNAi-treated S2 cells were loaded onto beads, the amount bound to beads was re-evaluated by Western blotting. For Western blotting, N-EGF:FLAG was detected using a mouse anti-FLAG primary antibody, a horseradish peroxidase-coupled anti-mouse secondary antibody, and Supersignal West Dura chemiluminescence substrate (Pierce) and then assayed using a Fluorochem 8800 CCD camera system (Alpha Innotech).

Cell-based Notch-Ligand Binding Assays—The binding assay was adopted from the protocol of Bruckner et al. (9) and involves mixing conditioned media containing N-AP or Fc-AP with S2 cells expressing a Notch ligand or with control S2 cells.

For Preparation of AP Fusion-conditioned Media—Cells were cultured in M3 complete medium (Sigma) in 6-cm dishes to a density of 2–3 × 10⁶/ml (~80% confluency), then transfected with expression constructs (8 μg of total DNA) using Cellfectin (Invitrogen) in Drosophila SF medium (Invitrogen) for 6 h. They were then cultured for 24 h in M3 complete medium, and then expression was induced in HyQ-CCM3 medium (Hyclone) by the addition of CuSO4 to 0.7 mM. Two days after induction, the conditioned medium was collected, centrifuged at 14,000 × g for 10 min at 4 °C to remove cell debris, and assayed for AP activity. Western blotting (not shown) confirmed that AP activity was proportional to amounts of N-AP. For the experiments in Fig. 2A, the N-AP fusion in the conditioned media was concentrated by centrifugation through Amicon Ultra centrifugal filters, 10,000 molecular weight cutoff (Millipore). The amount of AP fusion in the media was equalized among samples presented in the same panel by the addition of conditioned medium from untransfected S2 cells. When N-AP was co-transfected with OFUT1:V5His or FNG, the total amount of DNA was kept constant by adding vector DNA (pRMHa-3) as needed. Double-stranded RNA (dsRNA) was prepared as described previously (7). RNAi for Ofut1 was then achieved by adding 40 μg of dsRNA 6 h after transfection. After 1 h, 3 ml of M3 complete medium was then added, and the cells were cultured for 4 days before induction of transgene expression in HyQ-CCM3.

For Preparation of Cells for Binding Assays—DL-Expressing cells were a stable transfectant, and SER-expressing cells were transiently transfected (19). Control S2 cells for experiments shown were transiently transfected with vector (pRMHa-3). In separate experiments (not shown), the amount of AP activity detected was not affected by transfection of control cells. Cells were cultured to a density of ~5 × 10⁶/ml in 4 ml of HyQ CCM3 and then induced with 0.7 mM CuSO4 in HyQ CCM3 for 2 days. Cells were then washed in HyQ CCM3 and resuspended in HyQ CCM3 supplemented with 0.5% bovine serum albumin.

For the Binding Assay—0.3 ml of conditioned media and 0.3 ml of cells were mixed and incubated for 60 min at room temperature with gentle agitation. Cells were then washed 4 times at room temperature with Hanks’ balanced salt solution (Invitrogen) supplemented with 0.05% bovine serum albumin and 0.1% azide and then lysed in 10 mM Tris, pH 8.0, 1% Triton X-100 at 4 °C for 1 h. Endogenous S2 cell AP was inactivated by heat treatment for 65 °C for 10 min, and bound AP activity was detected using 6.25 mM p-nitrophenyl phosphate p-nitrophenol phosphate p-nitrophenol in 1 × diethanolamine, 5 mM MgCl2 at 37 °C for 30 min. The AP reaction was then stopped by the addition of 1 x NaOH, and AP activity was detected by the formation of a colored product using a spectrophotometer and expressed as milliabsorbance units (at 405 nm) of product formed/min. A mock assay in which S2 cell-conditioned medium was incubated with each corresponding cell type was used as the spectrophotometer blank.

For Preparation of Beads—5 ml of conditioned media from cells secreting N-EGF:FLAG or control cells transfected with pMT1B1 vector was mixed with 50 ml of Eview Red anti-FLAG M2 affinity gel (Sigma) at 4 °C overnight and then washed 3 times with 5 ml of Tris-buffered saline supplemented with 5 mM CaCl2. Similar amounts of N-EGF:FLAG from normal and RNAi-treated S2 cells were loaded onto beads, the amount bound to beads was re-evaluated by Western blotting. For Western blotting, N-EGF:FLAG was detected using a mouse anti-FLAG primary antibody, a horseradish peroxidase-coupled anti-mouse secondary antibody, and Supersignal West Dura chemiluminescence substrate (Pierce) and then assayed using a Fluorochem 8800 CCD camera system (Alpha Innotech). Amounts were then normalized by diluting N-EGF:FLAG-loaded beads with beads mock-loaded with S2 cell-conditioned medium.

For Binding Assay—5 µl of N-EGF:FLAG beads or control beads were blocked for 1 h at room temperature with 1 ml of Tris-buffered saline, supplemented with 1% bovine serum albumin, 5 mM CaCl2. Beads were then resuspended in 50 µl of conditioned media containing 3000 mAbs/min AP fusion protein, incubated for 1 h at room temperature, and then washed 5 times in 1 ml of Hanks’ balanced salt solution. AP activity was then assayed as described above.

For In Vitro Fringe Glycosylation—N-AP or N-EGF:FLAG was isolated from conditioned media using anti-AP or anti-FLAG-agarose beads (Sigma). The amount of Notch on beads was quantified by AP activity for N-AP and by Western blotting for N-EGF:FLAG, as described above.

All proteins were expressed under control of the metallothionein promoter. N-AP and DL-AP expression constructs were from S. Cohen (9), and FNG and OFUT1 expression constructs have been described previously (7, 8, 13). We also used an OFUT1 expression construct in which V5 and His tags were added to the C terminus (pRMHa3b-Ofut1V5His). These tags have no detectable influence on its enzymatic activity. To express FcAP, the plasmid pMT/BIP/Fc-AP was constructed by amplifying DNA encoding amino acids 1–530 of human placental alkaline phosphatase, and after washing beads, the amount bound to beads was re-evaluated by Western blotting. For Western blotting, N-EGF:FLAG was detected using a mouse anti-FLAG primary antibody, a horseradish peroxidase-coupled anti-mouse secondary antibody, and Supersignal West Dura chemiluminescence substrate (Pierce) and then assayed using a Fluorochem 8800 CCD camera system (Alpha Innotech). Amounts were then normalized by diluting N-EGF:FLAG-loaded beads with beads mock-loaded with S2 cell-conditioned medium as a substrate.

Antibodies—Antibodies used were mouse anti-DL (CS94.9B, DSHB), rabbit anti-tubulin (Serotec), mouse anti-V5 (Invitrogen), mouse anti-WG (ID4, DSHB), rabbit anti-SER (20), mouse anti-FLAG M2 (Sigma), and guinea pig anti-OFUT1. Imaginal discs were stained as described previously (13). Guinea pig anti-OFUT1 was prepared at Pocono Rabbit Farm by immunization with a peptide sequence corresponding to the C terminus of OFUT1, amino acids 388–402, fused to keyhole limpet hemocyanin.

Drosophila Stocks and Crosses—(2SH2260 (Ofut1)50) flies were obtained from S. Hou (21). This mutation was crossed onto FRT42B/G133 by meiotic recombination, and mutant clones were generated by crossing to y w hs-flp22, FRT42B/G133 Ubi-GFP:msl flies and heat-shocking to induce flipase-mediated mitotic recombination (22).

RESULTS

A Mutation in the Drosophila Ofut1 Gene—The requirement for OFUT1 in Notch signaling has been demonstrated by RNAi in Drosophila (7) and by a targeted mutation in the murine Pofut1 gene (6). Recently, Oh et al. (21) described the results of a large scale screen for lethal transposable element insertions in Drosophila. One line, l (2)SH2260, was reported to have an insertion in the 3′ end of Ofut1 (21) and is predicted to result in replacement of the seven C-terminal amino acids of OFUT1 with four different amino acids followed by a stop codon (Fig. 1A) (21). To confirm that this insertion creates an Ofut1 mutation, we examined animals in which patches of cells were made homozygous mutants for this allele by mitotic recombination. These animals exhibit classic Notch mutant phenotypes, such as wing notching, thickened wing veins, and loss of sensory bristles on the notum (Fig. 1, B–E), consistent with the phenotypes generated by RNAi of Ofut1 (7). In developing wing imaginal discs, the expression of targets of Notch signaling, such as Wingless, is lost in cells mutant for Ofut1 (Fig. 1F). This mutation (referred to hereafter as Ofut1<sup>sh2260</sup>) thus provides
**O-Fucose Is Required for Notch-Ligand Binding**

Fig. 1. A mutation in Ofut1 results in Notch mutant phenotypes. A, predicted amino acid change in OFUT1SH. The last four amino acids (underlined) constitute a potential ER retention signal. B, wild-type wing. C, wing from an animal in which clones of cells mutant for *Ofut1* were induced. The clones are unmarked, but loss of wing tissue and thickening of wing veins (arrow) are evident. D, wild-type notum. E, notum from an animal in which clones of cells mutant for *Ofut1SH* were induced. Loss of Notch signaling is indicated by the absence of scutellar macrochaete (asterisks) and by the absence of notal microchaete (dashed outline). For comparison, corresponding regions of the wild-type notum are similarly identified. F, a wing imaginal disc with a clone of cells mutant for *Ofut1SH* (asterisk, identified by the absence of green fluorescent protein (GFP) staining, green). Mutant cells fail to express Wingless (WG, red). Panel F’ shows the WG stain only.

An independent demonstration of the requirement for OFUT1 for Notch signaling in *Drosophila* and indicates that the seven C-terminal amino acids of OFUT1 are essential for function in vivo. The last four amino acids of OFUT1 conform to a consensus signal for retention in the endoplasmic reticulum (Fig. 1A) (23, 24), and experiments are in progress to determine whether the loss of function in *Ofut1SH* is due to loss of enzymatic activity or to mislocalization.

A Cell-based Notch-Ligand Binding Assay—Genetic studies imply that O-fucose is required on Notch in order for it to be activated by its ligands (Fig. 1) (6, 7). To investigate whether this requirement reflects an influence on the binding of Notch to its ligands, we assayed the ability of an AP-tagged extracellular fragment of Notch (N:AP) to bind to ligand-expressing cells. N:AP was secreted into the culture media, and the conditioned media was used for binding studies. This assay has been employed previously to demonstrate that elongation of O-fucose by the N-acetylgalactosaminyltransferase Fringe can enhance DL-Notch binding (9). Although it is possible to assay both N:AP binding to DL-expressing cells and DL:AP binding to Notch-expressing cells, we found that changes in OFUT1 levels affected the amount of N:AP secreted from cells (not shown). Because of a concern that the amount of Notch on the cell surface might vary with different levels of O-fucosylation and the difficulty of controlling for such variations, we focused on assaying N:AP binding to ligand-expressing cells. Amounts of N:AP were determined before incubation with cells and then equalized by dilution with conditioned medium from cultured *Drosophila* S2 cells. After binding and washing, the amount of N:AP retained by cells was determined by assaying AP activity.

S2 cells endogenously express OFUT1, but not Fringe (7, 8). N:AP isolated from otherwise wild-type S2 cells binds to DL-expressing cells (Fig. 2A) (9). Although we have not been able to saturate the binding of N:AP to DL-expressing cells, the amount bound is proportional to the amount of N:AP incubated with cells over at least a 40-fold range (Fig. 2A) and is proportional to the amount of DL-expressing cells over at least a 100-fold range (not shown). A much lower signal, which defines the background of the assay, is observed either when a control protein consisting of the Fc domain of human IgG (Fc:AP) is incubated with DL-expressing cells or when N:AP is incubated with S2 cells that do not express a Notch ligand (Fig. 2A). Importantly, we have also been able to detect SER-Notch binding with this assay (Fig. 2A), which was not detected previously (9). As for DL-expressing cells, the amount of N:AP bound to SER-expressing cells is proportional to the amount of N:AP incubated with cells (Fig. 2A).

**O-Fucosylation Is Required for Notch-Ligand Binding**—To determine whether O-fucosylation influences Notch-ligand binding, N:AP was isolated from S2 cells transfected with a N:AP expression construct and simultaneously treated with dsRNA corresponding to *Ofut1*. In S2 cells, the addition of dsRNA to the culture media is sufficient to promote mRNA degradation through RNAi (25). Western blotting confirmed that depletion of OFUT1 expression by RNAi was highly effective (Fig. 2B). When N:AP is isolated from S2 cells treated with *Ofut1* RNAi (i*Ofut1*), the binding of N:AP to DL-expressing cells is diminished to near background levels (Fig. 2C). A small amount of binding is sometimes observed, which might be due to incomplete inhibition of fucosylation by RNAi or might indicate that O-fucose potentiates binding but is not absolutely required for binding. Regardless, these results imply that O-fucose is required on Notch for normal binding to DL. Similarly, down-regulation of OFUT1 in Notch-secreting cells by RNAi also strongly inhibits SER-N:AP binding (Fig. 2C). The requirement for OFUT1 for both DL-Notch and SER-Notch binding provides an explanation for the severe Notch mutant phenotypes generated by loss of *Ofut1* function in vivo (Fig. 1) (6, 7).

**Overexpression of OFUT1 Differentially Modulates DL-Notch and SER-Notch Binding**—Intriguingly, overexpression of OFUT1 inhibits certain aspects of Notch signaling in *Drosophila* (7). To investigate whether this impairment of Notch signaling might also be effected at the level of Notch-ligand binding, we assayed the consequences of increased OFUT1 expression on the binding of N:AP to ligand-expressing cells. The ability of N:AP isolated from S2 cells that were co-transfected with an OFUT1 expression construct to bind to DL-expressing cells was substantially impaired in comparison to N:AP isolated from control cells (Fig. 3A). By contrast, co-expression of OFUT1 and N:AP actually resulted in enhanced binding of N:AP to SER-expressing cells (Fig. 3B). The distinct effects of OFUT1 overexpression on DL-Notch and SER-Notch binding provide an explanation for the partial inhibition of Notch signaling observed when OFUT1 is overexpressed in the *Drosophila* notum (7). Overexpression of OFUT1...
impairs lateral inhibition among proneural cells, which depends solely on DL but does not affect the asymmetric lineage decisions of the sensory organ precursor cells, during which Notch activation can be provided by either SER or DL (26).

The Influence of OFUT1 Overexpression Is Counteracted by Fringe—Intriguingly, the influence of elevated OFUT1 expression is exactly opposite to that of Fringe in the regulation of Notch signaling (Refs. 10–13; for review, see Ref. 14 and 15). Therefore, we used the N:AP binding assay to investigate the relationship between the influences of OFUT1 and Fringe on Notch-ligand binding. Expression of Fringe substantially enhances binding of N:AP to DL-expressing cells (Fig. 3A) (9). Importantly, when Fringe and OFUT1 are co-expressed, the inhibitory influence of OFUT1 on DL-N:AP binding is suppressed, and DL-N:AP binding is enhanced just as well as in the absence of exogenous OFUT1 (Fig. 3A).

The inability to detect N:AP binding to SER in a prior study precluded assessment of the influence of Fringe on Notch binding in Drosophila (9). We find that Fringe can completely inhibit binding of N:AP to SER-expressing cells (Fig. 3B). Moreover, even when Fringe and OFUT1 are co-expressed, the binding of N:AP to SER is effectively blocked by co-expression with Fringe (Fig. 3B).

Changes in Glycosylation Associated with Expression of Fringe and OFUT1—To confirm that changes in OFUT1 and Fringe expression are correlated with altered glycosylation of Notch and to provide an indication of the extent to which Notch glycosylation is altered, we employed in vitro glycosylation assays. Because glycosylation by Fringe is restricted to O-fucose on EGF domains (8, 27, 28), in vivo glycosylation by Fringe is expected to effectively cap O-fucose sites, thus precluding N:AP isolated from Fringe-expressing cells from serving as a Fringe substrate in vitro. Indeed, the N:AP isolated from Fringe-expressing S2 cells was a substantially worse substrate for Fringe (Fig. 3C). Conversely, if overexpression of OFUT1 modulates Notch-ligand binding by increasing O-fucosylation of Notch, N:AP isolated from cells overexpressing OFUT1 might be expected to be a better substrate for Fringe and a worse substrate for OFUT1. However, this was not the case, as no significant difference in in vitro glycosylation of N:AP was observed (Fig. 3C and data not shown). This raises the possibility that the modulation of Notch-ligand binding by elevated OFUT1 expression is not actually due to altered O-fucosylation. However, it is also possible that O-fucosylation of only one or a few specific EGF domains is responsible for the influence of elevated OFUT1 expression. Because there are 23 potential O-fucose sites on Notch, such a change could be too subtle to be detected in our glycosylation assays.

Notch-Ligand Binding in Vitro—We also attempted to confirm that decreased expression of OFUT1 by RNAi reduced glycosylation of N:AP. However, the decreased secretion of N:AP from iOfut1-treated cells created technical difficulties with this experiment. Therefore, we established an alternative Notch-ligand binding assay in which the EGF domains of Notch were fused to a triple FLAG epitope tag (N-EGF:FLAG). RNAi for Ofut1 reduces secretion of N:AP from cells to ~10% that...
from untreated cells, but secretion of N-EGF:FLAG is only reduced to 50% that from untreated cells (not shown). To assay Notch-ligand binding, we then bound N-EGF:FLAG to anti-FLAG-agarose beads and mixed these beads with conditioned medium from cells secreting DL:AP, SER:AP, or as a control, Fc:AP. DL:AP and SER:AP bind specifically to N-EGF:FLAG beads and not to control beads without N-EGF:FLAG (Fig 4A).

By contrast, when beads are prepared from equivalent amounts of N-EGF:FLAG (as assayed by Western blotting) isolated from cells treated with dsRNA for Ofut1, the binding of SER:AP and DL:AP is substantially decreased (Fig. 4A). This in vitro, bead-based binding assay, thus, provides an independent confirmation of the requirement for O-fucose for Notch-ligand binding. Importantly, in vitro glycosylation then confirmed that N-EGF:FLAG isolated from iOfut1-treated cells is a worse substrate for Fringe than N-EGF:FLAG from untreated S2 cells (Fig. 4B), consistent with the expectation that O-fucosylation of N-EGF:FLAG is impaired by Ofut1 RNAi (7).

**DISCUSSION**

O-Fucose Is Required for Notch-Ligand Binding—The studies presented here indicate that O-fucosylation is required for the physical binding of Notch to its ligands DL and SER. These

\[ \text{2 } \mu\text{g of OFUT1:V5His or FNG expression construct DNA, and vector DNA (pRMHa-3) as needed, to a total of 8 } \mu\text{g of DNA transfected. C, in vitro Fringe glycosylation assays conducted with aliquots of the same proteins employed for the binding assays. The amount of product formed is indicated by the amount of GlcNAc transferred divided by the amount of N:AP.} \]
Notably, OFUT1 is required for efficient binding of Notch to both SER and DL. This is consistent with the severe Notch phenotypes observed in vivo when Ofut1 is impaired by mutation or RNAi (Fig. 1) (6, 7). By contrast, elongation of O-fucose by the GlcNAc transferase Fringe exerts opposing influences on the ability of Notch to bind to SER and DL. Evidence that Fringe influences Notch-ligand binding has been reported previously (9, 16), but other studies have yielded conflicting results (10, 17). In our hands, Fringe has clear and reproducible effects on both DL-Notch and SER-Notch binding. Importantly, these effects of Fringe on Notch-ligand binding recapitulate its effects on signaling by these two ligands in Drosophila. The ability of both the O-fucose monosaccharide and elongated forms of O-fucose to influence Notch-ligand binding, the influence of O-fucosylation on binding by both ligands, and the consistent correlations between the effects of O-fucosylation on binding in vitro and its effects on signaling in vivo all argue that O-fucose glycans act at the ligand binding step of Notch signaling. Beyond their importance to understanding regulation of Notch signaling, these observations, thus, provide a striking example of glycosylation as a mechanism for modulating protein-protein interactions.

With the determination that O-fucosylation affects Notch-ligand binding, attention must now be turned to elucidating the mechanistic basis for this effect. OFUT1 and Fringe always act in Notch-expressing cells to influence Notch signaling and Notch-ligand binding, which implicates Notch itself as the relevant substrate. However, the actual sites of glycosylation on Notch that mediate the effects of these glycosyltransferases remain to be identified. It is also not yet clear whether the importance of O-fucosylation reflects a role for lectin-like recognition of Notch by its ligands or other co-factors or whether instead O-fucose glycans influence Notch-ligand binding indirectly by altering the conformation or oligomerization of Notch.

**Differential Modulation of Notch-Ligand Interactions**—By contrast to the positive requirement for OFUT1 demonstrated by RNAi, overexpression of OFUT1 enhances SER-Notch binding but inhibits DL-Notch binding. It is intriguing that elevated OFUT1 expression provides a mechanism for differentially modulating the activity of different Notch ligands to interact with the Notch receptor. Previously, the only factor known that could discriminate between the ability of Delta to activate Notch and that of Serrate to activate Notch was Fringe. Indeed, elevated OFUT1 expression might be a mechanism for increasing the sensitivity of cells to the presence or absence of Fringe. In vivo, Fringe only affects a subset of Notch signaling events, and it remains unclear why certain processes are sensitive to Fringe, whereas others are insensitive (for review, see Refs. 15 and 29). Because OFUT1 acts oppositely to Fringe, yet its effects can be blocked by Fringe, the relative impact of Fringe on DL-Notch or SER-Notch interactions is expected to be greater in tissues where OFUT1 is expressed at higher levels. Indeed, even though expression of Fringe alone has no obvious effect on the patterning of natal bristles, it has a strong effect when OFUT1 is also overexpressed. Overexpression of OFUT1 inhibits DL-Notch signaling, resulting in the formation of excess sensory bristles, but this effect is partially inhibited by co-expression with Fringe (7).

In addition to increasing the sensitivity of Notch signaling events to the presence or absence of Fringe, elevated OFUT1 expression presents a potential mechanism for modulating Notch signaling independently of Fringe. Although the in vivo relevance of Notch-ligand modulation by increased expression of OFUT1 at endogenous levels of expression remains uncertain, we note that certain Unidentified ligands of the lymph gland, express much higher levels of Ofut1 than surrounding cells (7). Intriguingly then, in most Drosophila tissues DL is the sole or major Notch ligand. However, in the larval lymph gland, a role for Notch signaling in regulating cell fate decisions during hematopoiesis has recently been described, and SER, rather than DL, is the ligand that regulates Notch in this tissue (30, 31). These observations provide some support for the possibility that transcriptional regulation of Ofut1 might provide a mechanism for Notch pathway regulation (7) and suggest developmental contexts in which this issue may be investigated further.

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Modulation of Notch-Ligand Binding by Protein O-Fucosyltransferase 1 and Fringe
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