The extracellular domain of mouse Notch1 contains 36 tandem epidermal growth factor-like (EGF) repeats, many of which are modified with O-fucose. Previous work from several laboratories has indicated that O-fucosylation plays an important role in ligand-mediated Notch activation. Nonetheless, it is not clear whether all, or a subset, of the EGF repeats need to be O-fucosylated. Three O-fucose sites are invariantly conserved in all Notch homologues with 36 EGF repeats (within EGF repeats 12, 26, and 27). To investigate which O-fucose sites on Notch1 are important for ligand-mediated signaling, we mutated the three invariant O-fucose sites in mouse Notch1, along with several less highly conserved sites, and evaluated their ability to transduce Jagged1- and Delta1-mediated signaling in a cell-based assay. Our analysis revealed that mutation of any of the three invariant O-fucose sites resulted in significant changes in both Delta1 and Jagged1 mediated signaling, but mutations in less highly conserved sites had no detectable effect. Interestingly, mutation of each invariant site gave a distinct effect on Notch function. Mutation of the O-fucose site in EGF repeat 12 resulted in loss of Delta1 and Jagged1 signaling, while mutation of the O-fucose site in EGF repeat 26 resulted in hyperactivation of both Delta1 and Jagged1 signaling. Mutation of the O-fucose site in EGF repeat 27 resulted in faulty trafficking of the Notch receptor to the cell surface and a decreased S1 processing of the receptor. These results indicate that the most highly conserved O-fucose sites in Notch1 are important for both processing and ligand-mediated signaling in the context of a cell-based signaling assay.

The Notch protein is a transmembrane receptor involved in a wide variety of cell fate decisions in metazoans (1, 2). Importantly, mutations of the Notch protein and components of its signaling pathway have been implicated in an array of human diseases (e.g. CADASIL, T-cell leukemia, multiple sclerosis) (3–5). Notch becomes activated upon binding of its extracellular domain to its ligands, members of the Delta and Serrate/Jagged families, which are present on the surface of apoposed cells. The extracellular domain of Notch contains 36 tandem epidermal growth factor-like (EGF) repeats many of which contain consensus sequences for modification by O-fucose (6, 7). Some of the O-fucose moieties on EGF repeats of Notch can be further elongated by the action of Fringe, a fucose-specific β1,3-N-acetylglucosaminyltransferase (8, 9). Modification of Notch by Fringe modulates its response to ligands, inhibiting signaling from Serrate/Jagged ligands but potentiating signaling from Delta ligands (8, 10–14).

Work from several laboratories has established that protein O-fucosyltransferase-1 (O-FucT-1), which catalyzes O-fucosylation of EGF repeats, is essential for Notch function. Experiments carried out in mice and Drosophila have demonstrated that deletion or reduction in levels of O-FucT-1 results in embryonic lethality and, more importantly, that the phenotypes observed are consistent with those observed due to loss of Notch signaling (15–17). The mechanism by which O-fucose exerts its effects on Notch signaling is not entirely clear. Several studies have demonstrated that lack of O-fucose alters the interaction of Notch with its ligands (17, 18). With regard to specific O-fucose sites, a previous study revealed that Drosophila Notch bearing a mutation of a highly conserved O-fucose site within EGF repeat 12 is still able to support neurogenesis while showing defects in wing development (19). This mutant also demonstrated increases in both Delta and Serrate binding to Notch in in vitro binding assays. This effect was specific to the EGF repeat 12 mutant, as mutation of other O-fucose sites did not result in changes in Notch activity. The role of individual O-fucose sites in mammalian Notch signaling has not yet been established. The recent demonstration that O-FucT-1 is localized to the endoplasmic reticulum where it could function in quality control or possibly even as a molecular chaperone raises the possibility that specific O-fucose sites may be necessary for the processing and maturation of the Notch receptor (20, 21). Thus, evaluation of the role of O-fucose at various sites is essential to dissect the mechanism by which these sugars affect Notch function.

Notch contains multiple sites for O-fucosylation modification scattered throughout its EGF repeats. An important clue in evaluating which sites are most important for the regulation of Notch is their degree of conservation (Fig. 1) (7). Three sites, found in EGF repeats 12, 26, and 27, are invariantly conserved in all known Notch homologues with 36 EGF repeats. In this work, we have sought to determine the role these three O-fucose sites in mouse Notch1 by analyzing the effects of mutation on ligand-mediated signaling, S1 processing, and cell-surface expression. As well, we have analyzed less highly conserved O-fucose sites scattered throughout the extracellular domain. This analysis has revealed that mutation of two highly conserved O-fucose sites results in altered ligand-mediated Notch signaling and in a third site alteration of the processing of the Notch receptor.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—A full-length construct of mouse Notch1 where with C-terminal PEST domain is replaced by six tandem MYC

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**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—A full-length construct of mouse Notch1 where with C-terminal PEST domain is replaced by six tandem MYC
epitopes in pCS2+ (a kind gift of Dr. Raphael Kopan, Washington University School of Medicine) was used as a template for site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). All glycosylation sites (T) were mutated to A. All constructs were sequenced to confirm the presence of the site mutant. Revertant mutations were generated from constructs bearing site mutants to ensure that no inadvertent mutations were introduced during the mutagenesis reaction.

**Cell Lines**—COS-7 cells or COS-7 cells stably transfected with empty vector (pCDNA4) were maintained in DMEM (Invitrogen) supplemented 10% bovine calf serum and 250 μg/ml Zeocin (Invitrogen). L-cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% bovine calf serum. L-cells stably expressing rat Jagged1 and rat Delta1 (Dl-19) (generously provided by Dr. Jerry Weinmaster) were maintained in DMEM supplemented with 10% bovine calf serum.

**Co-culture Assay**—These assays were adapted from previously described co-culture assays (11). COS-7 cells (1 × 10⁸) stably transfected with empty vector (pCDNA4) were plated in a 10-cm tissue culture dish and the next day transiently transfected with 12.8 μg of wild type or mutant Notch1-pCS2+. 2.98 μg of TP-1 luciferase reporter construct (Ga981-6, a kind gift of Dr. Georg Bornkamm, Munich, Germany), and 1.49 μg of gWIZ β-galactosidase construct (Gene Therapy Systems) to normalize transfection efficiency using Lipofectamine 2000 (Invitrogen) according to manufacturer’s specifications. Four hours after transfection, Lipofectamine reagent was removed, and cells were allowed to recover in fresh DMEM for 1.5 h. L-cells (1.0 × 10⁸) or L-cells expressing Jagged1 or Delta1 were then plated in each well of a 12-well tissue culture plate. Co-culture was established by overlaying L-cells with the transfected COS-7 cells (7.5 × 10⁶). After 42.5 h of co-culture, cell lysates were prepared in reporter lysis buffer (Promega). Luciferase assays were performed as described previously (28). Each co-culture was performed in triplicate, and co-cultures resulting in significant changes were compared with co-culture of wild type Notch1 performed at least twice.

**Metabolic Labeling**—COS-7 cells were transiently transfected with pSecTag2 (Invitrogen) constructs encoding EGF repeats 11–15, 26, or 27 of mouse Notch1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The production of the EGF repeat 11–15 and 26 constructs have been described previously (6). The EGF repeat 27 construct was produced by amplifying EGF repeat 27 from full-length Notch1 template using primers: 5’-GTATAAACCTTAGTACATGAGTGTGAT and 3’-CGATCTCGAGTCACAGTTCTGGCACAG. The products were digested with HinIII and XhoI and subcloned into pSecTag2 and sequenced. Following transfection, cells were incubated with 20 μCi/ml [6-³H]fucose for 48 h. Proteins were purified from media, and O-glycans were released by alkali-induced β-elimination and analyzed by gel-filtration chromatography as described previously (8, 29).

**Determination of Percent Fucitol**—Saccharide species obtained from gel-filtration chromatography were pooled and dried in a SpeedVac evaporator (Savant). Samples were then hydrolyzed for 2 h in 2 m trifluoroacetic acid at 100 °C. The hydrolysates were dried in a SpeedVac, resuspended in water, and dried again to ensure complete removal of acid. Samples were then analyzed by high pH anion-exchange chromatography (HPAEC) on a Dionex DX300 using an MA-1 column (Dionex) as described previously (30). Samples were mixed with internal standards (1 nmol each of fucitol, fucose, and glucose) prior to injection. Fractions (0.5 min) were collected, and radioactivity corresponding to fucosyl and fucitol peaks was determined by liquid scintillation counting. The percentage of radioactivity corresponding to fucitol in each sample was determined by dividing cpm in fucitol by the total cpm in both fucitol and fucitol. These percentages were then used to calculate the amount of O-fucose in each pooled peak by multiplying the total cpm in the peak by the percent fucitol.

**Cell-surface Biotinylation**—COS-7 cells (5 × 10⁵) were plated in a 35-mm tissue culture dish and transfected the next day with 4 μg of wild type or mutant Notch1-pCS2+ using Lipofectamine 2000. Cells were incubated for 24 h. Cells were washed three times with Hanks’ balanced salt solution (HBSS) (Invitrogen) and then incubated with 2.5 mM EZ-link sulfo-NHS-biotin (Pierce) or with HBSS (as a control) two times for 10 min each time. The biotinylated reagent was then removed, and cells were incubated with 100 mM Tris-HCl (pH 8.0) for 15 min to quench the biotinylation reaction. Cells were lysed in 1.0 ml of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitor mixture (Roche Applied Science). Lysates were cleared by centrifugation. ImmunoPure avidin-agarose (25 μl) (Pierce) was added to 100 μl of lysate and incubated for 16 h at 4 °C. The avidin-agarose was pelleted by brief centrifugation and the lysate removed. Avidin-agarose was washed three times with RIPA buffer and then boiled in 100 μl of SDS sample buffer. Avidin-bound biotinylated and non-biotinylated samples along with their respective lysates were then run on a 10% SDS-PAGE, transferred to nitrocellulose, and detected using a mouse anti-myc epitope antibody (generously provided by Dr. Jen-Chih Hsieh, Stony Brook University). Cadherin expression was determined by blotting samples with mouse anti-Pan cadherin

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**FIGURE 1.** Sites of O-fucosylation on mouse Notch1. The extracellular domain of mouse Notch1 contains multiple sites for O-fucosylation based on the consensus sequence CXX-(S/T)C, most of which appear to be modified (6). These sites are conserved to varying degrees when compared with Notch receptors with 36 EGF repeats (indicated by the number of asterisks from a total of 15 homologues) (adapted from Ref. 7). The O-fucose sites mutated in this study are indicated by number. The location of the ligand-binding domain (24), the negative regulatory Abruptrix region (26), and a putative region of flexibility (25) of the Notch protein are also indicated.
Conserved O-Fucose Sites Have Distinct Effects on Notch1 Function

RESULTS

Mutation of Conserved O-Fucose Sites Results in Changes in Delta1- and Jagged1-mediated Notch1 Signaling—To evaluate the effects of individual O-fucose site mutants on the ability of Notch1 to signal via Delta and Jagged ligands, a previously reported cell signaling system (11) was adapted for use in COS-7 cells. In this system, COS-7 cells were transiently transfected with a plasmid encoding mouse Notch1 along with a luciferase reporter and a β-galactosidase reporter (for normalization of transfection). The Notch1-expressing COS-7 cells were then co-cultured with L-cells or L-cells stably expressing Delta1 or Jagged1 ligand. The cells were subsequently lysed, and luciferase and β-galactosidase were assayed to determine relative-fold activation as described previously (11). Co-culture of Notch1-expressing COS-7 cells with either Delta1- or Jagged1-expressing L-cells resulted in a statistically significant increase in relative-fold activation (RFA) when compared with co-culture with L-cells expressing no ligand (Fig. 2, A and B). Interestingly, in contrast to what has been observed using the same assay system in other cells (11, 14), the activation of Notch1 in COS-7 cells by Delta1-expressing L-cells was greater than that of the Jagged1-expressing cells. This suggests the COS-7 cells may express an endogenous Fringe homologue (see below). Co-culture of control COS-7 cells (transfected with empty vector, pCS2+) and ligand-expressing L-cells resulted in a slight increase over controls, presumably due to activation of endogenous Notch in COS-7 cells. Thus, activation of exogenous mouse Notch1 by both Delta1 and Jagged1 can be assayed using the COS-7 cell system.

To examine which of the O-fucose sites on mouse Notch1 are important for mediating Delta1 and/or Jagged1 activation, glycosylation site mutants were prepared (T to A). Haines and Irvine (7) showed that the O-fucose sites found in the extracellular domain of Notch are conserved to varying degrees (7) (Fig. 1). Based on their analysis, we mutated the sites that are conserved in all Notch homologues (EGF repeats 12, 26, and 27). In addition, O-fucose sites with lesser degrees of conservation in various regions of the extracellular domain were similarly mutated (EGF repeats 9, 16, 20, 24, and 30, see Fig. 1). Notch1 constructs bearing each of these mutations were then used in the COS-7 cell co-culture assay, in parallel with a co-culture assay of the wild type Notch1 construct, as described above. Analysis of the relative activation in this assay revealed that mutation of the O-fucose sites in EGF repeats 12 and 27 resulted in a nearly complete loss in Jagged1 (Fig. 3A) and a 2-fold increase in Delta1 (Fig. 3B)-mediated Notch1 signaling when compared with wild type. In contrast, mutation in the O-fucose site of EGF 26 resulted in a 4-fold increase in Jagged1-mediated signaling (Fig. 3A) and a 2-fold increase in Delta1-mediated signaling (Fig. 3B). The O-fucose site mutations in EGF repeats 9, 16, 20, 24, and 30 did not result in any significant changes with either Jagged1 or Delta1. Thus, mutation of the most highly conserved O-fucose sites resulted in significant changes in both Delta1- or Jagged1-mediated Notch1 activation, while single mutants at other sites had no apparent effect. To confirm that the observed effects on Notch1 signaling were due to mutation of O-fucose sites (and not to inadvertent mutations introduced by the PCR-mediated mutagenesis), revertant mutations, which restored the threonine residue at the site of O-fucosylation, were generated in Notch constructs bearing mutations in EGF repeats 12, 26, and 27. These revertant mutations effectively restored Delta- and Jagged-mediated signaling to wild type levels (Fig. 3C).

O-Fucose on EGF Repeats 26 and 27 Is Elongated by Endogenous Fringe—The fact that Delta1 activates Notch1 to a greater extent than Jagged1 in the COS-7 cell system suggests that COS-7 cells contain endogenous Fringe. In most contexts, Fringe stimulates activation of Notch1 from Delta1 while inhibiting activation from Jagged1 (11, 14). To examine whether any of the Fringes are modifying O-fucose on EGF repeats 12, 26, or 27, we examined the structure of the O-glycans on these EGF repeats. Although methods for analyzing O-fucose structures at individual glycosylation sites in the context of the native protein have not yet been developed, we have previously shown that individual EGF repeats encode determinants required for Fringe modification (6). Thus
analysis of the state of elongation of \( O \)-fucose on smaller fragments of the Notch1 extracellular domain (or individual EGF repeats) can be used to approximate Fringe modification at specific sites in the context of the entire Notch molecule. To investigate whether endogenous Fringe modifies \( O \)-fucose on EGF repeats 12, 26, or 27, plasmids encoding these EGF repeats were transfected into COS-7 cells. The cells were
metabolically radiolabeled with \[^{3}H\]fucose, the proteins were purified, the O-fucose glycans were released from the protein by alkali-induced \(\beta\)-elimination, and the released saccharides were analyzed by gel-filtration chromatography (Fig. 4). In the case of both EGF repeats 26 and 27, significant elongation of the monosaccharide to di-, tri-, and tetrasaccharide species is observed (Fig. 4, B–D). This indicates that COS-7 cells contain an endogenous Fringe activity. Immunoblot analysis shows the presence of Lunatic fringe in extracts of COS-7 cells, indicating the presence of at least one endogenous Fringe (data not shown). This is consistent with the higher level of Delta1 than Jagged1 activation seen in Fig. 2 and suggests that Fringe modification at EGF repeats 26 and 27 may contribute to this effect. Importantly, this also raises the possibility that the effects observed in the co-culture assay with regard to the mutations at EGF repeats 26 and 27 (Fig. 3) may in part be attributable to Fringe modulation of Notch signaling. In contrast EGF repeat 12 is modified almost exclusively by O-fucose monosaccharide (Fig. 4, A and D), suggesting that this site is a poorer substrate for Fringe than EGF repeats 26 and 27. Similar results were obtained in analysis of the glycosylation of EGF repeat 12 in CHO (Chinese hamster ovary) cells, which also express endogenous Lunatic fringe (6). These results suggest that the effects of the mutation at EGF repeat 12 in the co-culture assay (Fig. 3) are mediated largely by loss of the O-fucosyl monosaccharide.

**Mutation of O-Fucose Site in EGF Repeat 27 Results in a Reduction of Cell-surface Expression and S1 Cleavage of Notch1**—The variations in Delta1- and Jagged1-mediated Notch1 signaling observed in the co-culture assays may be attributable to changes in the cell-surface expression of the mutated Notch1 proteins. To assess this possibility, COS-7 cells were transfected with either wild type Notch1 or Notch1 bearing O-fucose site mutants in EGF repeats 12, 26, or 27. Cell-surface biotinylation was then carried out, followed by streptavidin precipitation (Fig. 5A). Controls with a cell-surface protein (cadherin) and a cytoplasmic protein (\(\beta\)-actin) were performed with each sample to demonstrate the efficacy of the biotinylation procedure. A ratio of cell-surface Notch1 to cell-surface cadherin was performed to normalize for any differences in sample loading. This analysis showed that Notch1 bearing O-fucose site mutations in either EGF repeats 12 or 26 were expressed on the cell surface similar to wild type. In contrast, there was decreased cell-surface expression of Notch bearing an O-fucose mutation in EGF repeat 27 compared with wild type. Thus, mutation of the O-fucose site in EGF repeat 27 results in reduced cell-surface expression of the Notch protein.

Notch is cleaved by a furin-like convertase in the Golgi, which is required for efficient translocation to the cell surface (22). Loss of multiple O-fucose sites in mouse Notch3 results in defective S1 cleavage (23). To investigate the role of O-fucose site mutations on Notch1 processing, COS-7 cells were transfected with wild type Notch1 or Notch1 bearing mutations at EGF repeats 12, 26, or 27. The cells were lysed and examined by immunoblot analysis. S1-processing of Notch1 bearing a mutation in EGF repeat 12 was similar to wild type, while that with a mutation in EGF repeat 26 showed a slight decrease in efficiency (Fig. 5B). However, the mutation in EGF repeat 27 caused a significant reduction in S1-cleaved Notch1. Thus, mutation of the O-fucose site at EGF repeat 27 results in decreased S1-mediated processing and subsequent cell-surface expression of the Notch protein. These results provide an explanation for the reduction in Notch1 signaling caused by the mutation in EGF repeat 27.

**DISCUSSION**

In this work we have sought to determine the role of highly conserved O-fucose sites on mouse Notch1. Mutation of any of the three invariant sites across species, located in EGF repeats 12, 26, and 27, resulted in changes in both Delta1- and Jagged1-mediated Notch1 signaling. In contrast, there was no detectable effect of mutation of less conserved O-fucose sites. Interestingly, the mutations in each of the highly conserved sites resulted in a distinct effect on Notch1 function. This suggests that the effect of the mutation at EGF repeat 12 is due to...
Conserved O-Fucose Sites Have Distinct Effects on Notch1 Function

mainly to the loss of the monosaccharide form of O-fucose, while the mutations at EGF repeats 26 and 27 may be due to the loss of elongated forms.

The mutation in EGF repeat 12 resulted in loss of activation by both Delta1 and Jagged1 but no change in S1 processing or cell-surface expression. The fact that it proceeds through endoplasmic reticulum quality control checkpoints and is properly processed suggests that the relative importance of the O-fucose modification on EGF repeat 26 may in fact function as a site for O-fucose glycosylation site at EGF 12. Presence or absence of O-fucose on EGF repeat 12 appears to modulate Notch-ligand interactions, but it does not appear to be essential in all contexts (e.g. Delta-mediated activation of Notch during neurogenesis in Drosophila). The specific context in which Notch exists, with respect to ligands, Fringe, and other modulators of Notch activity, appear to affect the relative importance of the O-fucose at this site. The fact that mutation of the O-fucose site on EGF repeat 12 has such a profound effect on Notch activation in COS-7 cells suggests that these cells do not have mechanisms to compensate for this loss.

The mutation in EGF repeat 26 results in an increase in Notch activation by both ligands: a 2-fold increase in Delta1-mediated signaling and a 4-fold increase in Jagged1 signaling. The hyperactivation caused by this mutation does not correlate with alterations in processing or cell-surface expression, but it is reminiscent of the Abruptex mutations. These are gain-of-function point mutations in Drosophila Notch, which cluster in EGF repeats 24–29 (Fig. 1). The Abruptex region of Notch is thought to act as a negative regulatory domain possibly mediated by this mutation does not correlate with alterations in processing or cell-surface expression, but it is reminiscent of the Abruptex mutations. It is possible that mutation of the O-fucose site in EGF repeat 26 in some manner mimics the effect of Abruptex mutations. Additionally, it has been observed that Abruptex mutations are refractory to Fringe in Drosophila, suggesting that the Abruptex mutations may induce a change in Notch similar to that induced by Fringe. Fringe modifies O-fucose residues in the Abruptex region (6). Taken together, these observations suggest that the O-fucose modification on EGF repeat 26 may in fact function as a site for Fringe to negatively regulate Notch activation. Loss of O-fucose at this site, and as consequence, loss of the ability of Fringe to modify EGF

FIGURE 5. O-Fucose site mutation in EGF repeat 27 causes a reduction in cell-surface expression and S1-processing of Notch1. A, to evaluate cell-surface expression, COS-7 cells were transiently transfected with wild type Notch1 or Notch1 bearing an O-fucose site mutation in EGF repeats 12, 26, or 27. Cells were subjected to the cell-surface biotinylation procedure described under “Experimental Procedures.” Immunoblot analysis of cell lysate and avidin-bound material was performed using an anti-myc epitope antibody (to detect transfected Notch1). As a positive control, lysate and avidin-bound material were immunoblotted with an anti-cadherin antibody and as negative control with an anti-β-actin antibody. Densitometry analysis of immunoblot indicates that there is no difference in the ratio of Notch to cadherin between wild type Notch and Notch with O-fucose site mutations in EGF repeat 12 (1.04 versus 1.05 ± 0.01, respectively) and EGF repeat 26 (0.40 ± 0.01 versus 0.35 ± 0.01 respectively). However, there is an ~2-fold difference in the ratio of wild type Notch to cadherin versus Notch bearing an EGF repeat 27 site mutant and cadherin (0.72 ± 0.06 versus 0.37 ± 0.01, respectively). Thus, there is a reduction in cell-surface expression of Notch bearing an EGF repeat 12–O-fucose site mutant. B, to analyze S1 cleavage of wild type Notch and Notch bearing O-fucose site mutants, COS-7 cells were transfected with wild type Notch or a Notch construct bearing an O-fucose site mutant in EGF repeats 12, 26, or 27. Cells were lysed and lysate was resolved by SDS-PAGE on an 8% gel and analyzed by immunoblot with an anti-myc epitope antibody. The migration positions of S1 cleaved fragment and full-length uncleaved Notch are indicated.
Conserved O-Fucose Sites Have Distinct Effects on Notch1 Function

No Fringe: 
Moderate Fringe: 
High Fringe: 

FIGURE 6. Proposed effects of progressive O-fucose elongation at EGF repeats 12, 26, and 27 on the Notch extracellular domain. The 36 tandem EGF repeats of the Notch extracellular domain are represented by ovals, and those implicated in ligand binding (L) (24) and as a flexible region (F) (25) are indicated by lines. Fucose is indicated by a triangle, GlcNAc by a square, galactose by a circle, and sialic acid by a diamond (8). With moderate levels of Fringe, elongation occurs at EGF repeats 26 and 27, followed by elongation at EGF repeat 12 with high levels of Fringe. Modification at EGF repeats 26 and 27 is proposed to alter the flexibility at this region. The conformations shown (at "No Fringe" and "Moderate Fringe") are only one example of many possible structures.

repeat 26, may prevent negative regulation of Notch signaling by Fringe, thus resulting in a hyperactivatable form of the receptor. Hambleton et al. (25) have proposed that the majority of the EGF repeats in the Notch extracellular domain are fairly rigid, due to the presence of calcium-binding motifs between EGF repeats. Interestingly, EGF repeats 26, 28 and 29 (overlapping the Aropuptex region) do not contain calcium-binding motifs and are thus predicted to be more flexible (see Fig. 1 (25). It is conceivable that the conformation of Notch may be altered by the extent of elongation on O-fucose moieties at this specific location (Fig. 6). The fact that the O-fucose glycans on both EGF repeats 26 and 27 are elongated supports this idea. Thus, we propose that the change in Notch activation caused by mutation at EGF repeat 26 may be caused by a change in the overall conformation of the extracellular domain.

Mutation of the O-fucose in EGF repeat 27 results in a significant reduction in both Delta1- and Jagged1-mediated Notch1 activation that is most likely due to a reduction in S1 processing. It is possible that this highly conserved O-fucose site serves as a "regulatory checkpoint" for further processing of the Notch protein. Since EGF repeat 27 lies in the putative flexible region of Notch (Fig. 6), alteration of its glycosylation state could alter the overall conformation of Notch, and this could affect the ability of the furin-like convertase to process Notch. Indeed, this would not be the first example of glycosylation altering the processing of a protein. Processing of the low density lipoprotein (LDL) receptor-related protein 1 (LRP1) is regulated by differential glycosylation (27). It is conceivable that the conformation of Notch may be altered by the extent of elongation on O-fucose moieties at this specific location (Fig. 6). The fact that the O-fucose glycans on both EGF repeats 26 and 27 are elongated supports this idea. Thus, we propose that the change in Notch activation caused by mutation at EGF repeat 26 may be caused by a change in the overall conformation of the extracellular domain.

Mutation of the O-fucose in EGF repeat 27 results in a significant reduction in both Delta1- and Jagged1-mediated Notch1 activation that is most likely due to a reduction in S1 processing. It is possible that this highly conserved O-fucose site serves as a "regulatory checkpoint" for further processing of the Notch protein. Since EGF repeat 27 lies in the putative flexible region of Notch (Fig. 6), alteration of its glycosylation state could alter the overall conformation of Notch, and this could affect the ability of the furin-like convertase to process Notch. Indeed, this would not be the first example of glycosylation altering the processing of a protein. Processing of the low density lipoprotein (LDL) receptor-related protein 1 (LRP1) is regulated by differential glycosylation (27). Alternatively, it is possible that loss of the O-fucose site in EGF repeat 27 causes retention of the Notch protein in the endoplasmic reticulum. Mutation of O-fucose sites has been observed to result in a decrease in the secretion of Drosophila Notch fragments or S1 processing of Notch3, suggesting that they are trapped in the endoplasmic reticulum (20, 23).

The finding that mutation of highly conserved O-fucose sites affects Notch processing and signaling supports the idea that O-fucosylation of individual sites is important for Notch function. Surprisingly, there appears to be heterogeneity in the roles of these highly conserved O-fucose sites. This may in part be explained by the fact some of these sites may also be important for Fringe regulation of Notch signaling. Indeed, our data suggests that the effects observed from the O-fucose on EGF repeat 26 might be most readily attributable to an alteration in the Fringe effect. In contrast, the effects of the O-fucose on EGF repeat 12 appear to be mediated mainly by the monosaccharide form of O-fucose. Interestingly, the O-fucose on EGF repeat 12 is mainly in the monosaccharide form in both CHO (6) and COS-7 (Fig. 4) cells, even though both cells express endogenous Lunatic fringe. In contrast, O-fucose on EGF repeat 26 is significantly elongated in both cell types. Overexpression of Lunatic or Manic fringe in CHO cells results in significant increases in O-fucose elongation at EGF repeat 12 (6) and loss of Jagged1-dependent activation (8, 13). Thus, cells expressing low levels of Fringe (e.g. Cos-7 or CHO) preferentially modify EGF repeats 26 and 27 and have an intermediate effect on Delta1 activation and Jagged1 inhibition (see "Moderate Fringe", Fig. 6). Increased expression of Fringe causes O-fucose elongation at EGF repeat 12 and further inhibition of Jagged1-dependent activation (see "High Fringe", Fig. 6). This suggests that EGF repeat 12 may play a key role in the ability of Fringe to fully inhibit Serrate/Jagged-dependent signaling. This is consistent with the results reported by Lei et al. (19) using the EGF repeat 12 mutant in Drosophila wing discs. Determination of the Fringe effect at specific sites and determination of the glycosylation effect on the overall conformation of the Notch extracellular domain are needed for further clarification of the roles of these O-fucosylation sites. Identification of specific amino acids within an EGF repeat necessary for recognition by Fringe will allow us to generate mutations that allow O-fucosylation but prevent Fringe elongation at a particular EGF repeat. This will allow us to differentiate between the effects of O-fucose monosaccharide and the elongated tetrasaccharide at a particular site (e.g. EGF repeat 26).

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Conserved O-Fucose Sites Have Distinct Effects on Notch1 Function

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