Fringe Modifies O-Fucose on Mouse Notch1 at Epidermal Growth Factor-like Repeats within the Ligand-binding Site and the Abruptex Region*

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Fringe plays a key role in the specification of boundaries during development by modulating the ability of Notch ligands to activate Notch receptors. Fringe is a fucose-specific β1,3-N-acetylglucosaminylltransferase that modifies O-fucose moieties on the epidermal growth factor-like (EGF) repeats of Notch. To investigate how the change in sugar structure caused by Fringe modulates Notch activity, we have analyzed the sites of O-fucose and Fringe modification on mouse Notch1. The extracellular domain of Notch1 has 36 tandem EGF repeats, many of which are predicted to be modified with O-fucose. We recently proposed a broadened consensus sequence for O-fucose, C2X3–5(S/T)C3 (where C2 and C3 represent the second and third conserved cysteines), significantly expanding the potential number of modification sites on Notch. Here we demonstrate that sites predicted using this broader consensus sequence are modified with O-fucose on mouse Notch1, and we present evidence suggesting that the consensus can be further refined to C2X1–3(S/T)C3. In particular, we demonstrate that EGF 12, a portion of the ligand-binding site, is modified with O-fucose and that this site is evolutionarily conserved. We also show that endogenous Fringe proteins in Chinese hamster ovary cells (Lunatic fringe and Radical fringe) as well as exogenous Manic fringe modify O-fucose on many but not all EGF repeats of mouse Notch1. These findings suggest that the Fringes show a preference for O-fucose on some EGF repeats relative to others. This specificity appears to be encoded within the amino acid sequence of the individual EGF repeats. Interestingly, our results reveal that Manic fringe modifies O-fucose both at the ligand-binding site (EGF 12) and in the Abruptex region. These findings provide insight into potential mechanisms by which Fringe action on Notch receptors may influence both the affinity of Notch-ligand binding and cell-autonomous inhibition of Notch signaling by ligand.

The Notch signaling pathway plays an essential role in multiple stages of development in metazoans (1). In humans, deregulation of the Notch pathway can result in a number of serious diseases, including T cell leukemia (2), cerebral arterioopathies (Cadasil) (3), and Alagille syndrome (4). Recently, defects in Notch signaling have been implicated in the pathogenesis of multiple sclerosis (5). Whereas there is only one Notch in Drosophila, four Notch homologues exist in mammalian systems. Notch becomes activated upon binding to its ligands (members of the Delta, Serrate/Jagged families) on the surfaces of adjacent cells, initiating a series of proteolytic events resulting in release of the Notch intracellular domain from the membrane (reviewed in Ref. 6). The Notch intracellular domain translocates to the nucleus, where it binds to members of the CSL (CBF1/suppressor of hairless/Lag-1 family of transcriptional regulators, activating transcription of downstream gene products.

The Notch receptor is a large, cell surface membrane glycoprotein containing multiple domains (1, 6). The extracellular domains of Notch1 and Notch2 consist largely of 36 tandem epidermal growth factor-like (EGF) repeats. Notch3 and Notch4 contain 34 and 29 EGF repeats, respectively. EGF repeats are defined by the presence of six conserved cysteine residues that form three disulfide bonds (7). Many of the EGF repeats on Notch1 contain evolutionarily conserved consensus sites for two unusual forms of glycosylation: O-fucose and O-glucose (8). The consensus sites for these modifications were determined by comparison of sites of glycosylation from the EGF repeats of several serum glycoproteins (9). Based on these analyses, O-glucose modifications were shown to occur between the first and second conserved cysteine (C1 and C2, respectively) of the EGF repeat at the sequence C2X1–5(S/T)C3. O-fucose modifications occurred between the second and third conserved cysteines (C2 and C3, respectively) at the sequence C2X3–5(S/T)C3 (9). We demonstrated that the Notch1 protein from Chinese hamster ovary cells is modified with both O-fucose and O-glucose, suggesting that these consensus sites can be used to accurately predict whether a protein will bear the modifications (8). In similar studies, we have shown that Notch ligands (Drosophila Delta and Serrate, mammalian Jagged1 and Delta1), which also contain O-fucose consensus sites, are modified with O-fucose (10). Interestingly, mutation of all of the predicted O-fucose sites on Drosophila Serrate failed to eliminate O-fucosylation, suggesting that O-fucose was modifying sites not predicted using the original consensus sequence (10). Based on these results, we proposed a broadened consensus site for O-fucosylation: C2X1–5(S/T)C3. Mutation of these sites on Drosophila Serrate eliminated O-fucosylation completely. Thus, the number of EGF repeats in Notch and other proteins predicted to bear O-fucose has increased. Nonetheless, individual O-fucosyla-

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tion sites have not been mapped on either Notch or its ligands.

Recent studies have demonstrated that O-fucosylation plays an essential role in Notch function. Reduction of O-fucosyltransferase expression in Drosophila using RNAi (11) or in mice by gene ablation\(^a\) causes Notch-like phenotypes, suggesting that O-fucosylation is essential for Notch function. In addition, we and others have shown that Notch activation is modulated by extension of O-fucose on Notch with the fucose-specific β1,3,4-N-acetylglucosaminyltransferase Fringe (for recent reviews, see Refs. 12 and 13). Fringe was first identified in Drosophila and shown to inhibit Notch’s ability to respond to Serrate but to potentiate its ability to respond to Delta (14). Three Drosophila Fringe homologues have been identified in mammals: Lunatic fringe (Lfng), Manic fringe (Mfng), and Radical fringe (Rfng) (15). The β1,3,4-N-acetylglucosaminyltransferase activity of Fringe proteins is essential for their biological activity in Drosophila (16-18) and in cell-based Notch signaling assays (19), and the O-fucose residues are required for Fringe to modulate Notch activity (16, 19). Thus, Fringe mediates its effects on Notch signaling by the addition of GlcNAc to O-fucose moieties. Nonetheless, neither the specific role of O-fucose in Notch function nor the mechanism of how a change in sugar structure alters Notch function is known.

To better understand the mechanism of how the O-fucose structures modulate Notch function, we have begun to map sites of O-fucose and Fringe modification on mouse Notch1. Here we used fragments of the mNotch1 extracellular domain to identify sites of O-fucose modification and subsequent elongation by Mfng. We show that the broadened O-fucose consensus sequence (C\(^{\beta2}\)X\(_{\beta5}\)S\(_{\beta7}\)T/C\(_{\beta4}\)) (10) can be used to precisely predict sites of O-fucosylation, and we observed that Fringe modifies O-fucose on EGF repeats that have important biological roles, including ligand binding and cell-autonomous inhibition by ligand. These findings suggest specific mechanisms for how the change in O-fucose glycan structure can modulate Notch function.

### Experimental Procedures

**Materials—** N-Acetylenuraminidase I (α2,3-specific sialidase) was obtained from Glyko, Inc., and β-galactosidase (Diplococcal sialidase) was purchased from Roche Molecular Biochemicals. [\(^6\)\(^{3}\)H]Fucose was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). A plasmid expressing full-length mouse Notch1 with a C-terminal Myc tag (pcDNA-Notch1/myc) was generously provided by Dr. Jefferey Nye (Northwestern University). Drosophila Fringe bearing a N-terminal His\(_6\) tag was generously provided by Dr. Kenneth Irvine (Rutgers University). The Chinese hamster ovary (CHO) Lc1 cell line (20) and Lc1 cells stably transfected with either mouse Fringe or control vectors (16) were developed in and generously provided by the laboratory of Dr. Pamela Stanley (Albert Einstein College of Medicine). All CHO cells were grown as described previously (8). All other reagents were of the highest quality available.

| Name | Sequence |
|------|----------|
| EGF 1–5 | 5'-GTAAAAGCTTCCGGCGCTAGCCCTCCAGC-3' |
| EGF 6–10 | 5'-CGATCTCTAGAGTTCCGAGGACATAGG-3' |
| EGF 11–15 | 5'-CGATAGTCTCAGGGGTCTGACATGACCT-3' |
| EGF 16–18 | 5'-CGATAGTCTCAGGGGTCTGACATGACCT-3' |
| EGF 19–23 | 5'-GTAAAGCTTCCGGCGCTAGCCCTCCAGC-3' |
| EGF 24–28 | 5'-CGATCTCTAGAGTTCCGAGGACATAGG-3' |
| EGF 29–36 | 5'-CGATAGTCTCAGGGGTCTGACATGACCT-3' |
| EGF 24 | 5'-CGATCTCTAGAGTTCCGAGGACATAGG-3' |
| EGF 26 | 5'-GTAAAAGCTTCCGGCGCTAGCCCTCCAGC-3' |
| EGF T12A | 5'-GGTCCCAGGCAAGCGGCGTCATTCTGACATG-3' |
| EGF T20A | 5'-GGTCCCAGGCAAGCGGCGTCATTCTGACATG-3' |
| EGF S23A | 5'-GGTCCCAGGCAAGCGGCGTCATTCTGACATG-3' |
| EGF T26A | 5'-GGTCCCAGGCAAGCGGCGTCATTCTGACATG-3' |

\(^a\) S. Shi and P. Stanley, submitted for publication.

**EGF fragments, the QuikChange site-directed mutagenesis protocol (Stratagene) was used. All constructs were sequenced to confirm nucleotide sequence.**

To express and metabolically radiolabel the fragments, each construct was transiently transfected into Lc1 cells using Geneporter (Gene Therapy Systems) essentially as described previously (16). Following transfection (24 h), the medium was replaced with fresh medium containing 20 μCi/ml [\(^6\)\(^{3}\)H]Fucose. After 48 h, the medium was collected, and the fragments were purified by rotating the medium with Ni\(^2+\)-nitrilotriacetic acid-agarose (30 mg of beads/100-mm plate; Qiagen) for 1 h at 4 °C. After extensive washing (five times with 50 ml Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS), the fragments were eluted with 100 mM EDTA, pH 8.0.

**Analysis of O-Fucose Saccharide Structures—** Release of O-fucose saccharides from the fragments by alkaline-induced β-elimination and subsequent analysis by gel filtration chromatography on a Superdex peptide column was done essentially as described (8, 16, 22). Tetrasaccharide and trisaccharide forms of O-fucose were confirmed using exoglycosidase digests essentially as described (8), although the N-acetylenuraminidase I (α2,3-specific sialidase) (10 milliunits) and β-galactosidase (Diplococcal sialidase) digests were done concurrently. The disaccharide GlcNAc1,3Fucitol was confirmed using high pH anion exchange chromatography as described previously (8).

**Fringe Assays—** Fringe assays were performed as described (16) using His\(_6\)-tagged Drosophila Fringe as the enzyme, UDP-[\(^6\)\(^{3}\)H]GlcNac
Expression and O-fucosylation of EGF fragments from mouse Notch1. Plasmids encoding fragments (EGF 1–5, 11–15, 16–18, 19–23, 24–28, and 29–36) of mouse Notch1 extracellular domain were transfected into Lec1 cells, the cells were metabolically radiolabeled with [3H]fucose, and the expressed fragments were purified from medium as described under "Experimental Procedures." Equivalent amounts of each purified fragment (based on radioactivity) were analyzed by SDS-PAGE and fluorography (left) or Western blot analysis with anti-Myc antibodies (right). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (left). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (right). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (left). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (right). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (left). To more clearly detect the protein fragment, a second Western blot was performed with a secondary antibody (right).

RESULTS

Expression and O-Fucosylation of Notch1 Extracellular Domain Fragments—The mouse Notch1 extracellular domain contains 36 tandem EGF repeats, 10 of which contain the O-fucose consensus sequence C²XXGG(S/T)C³ (EGF repeats 3, 5, 16, 18, 20, 24, 26, 27, 31, and 35) and five of which are evolutionarily conserved (EGF repeats 3, 20, 24, 26, and 31) (Fig. 1b). To simplify the analysis of O-fucose and Fringe modification, we divided the mouse Notch1 extracellular domain into fragments. Constructs encoding EGF 1–5, 6–10, 11–15, 16–18, 19–23, 24–28, and 29–36 were generated from mouse Notch1 PCR. The desired fragments were obtained by PCR and cloned into a mammalian expression vector encoding a signal sequence for secretion and C-terminal Myc epitope and His₉ tags (see "Experimental Procedures"). Each of the fragments was expressed transiently in Lec1 cells and metabolically radiolabeled with [3H]fucose. Although several constructs encoding EGF 6–10 were generated, no protein was detected when transfected into cells, so no further experiments were performed with these constructs. In Lec1 cells, no complex-type N-glycans are synthesized (24), and the majority of [3H]fucose is incorporated into O-fucose structures (25). Thus, the presence of [3H]fucose on a fragment suggests O-fucosylation (as shown previously (8)). Notch extracellular fragments were purified from medium using Ni²⁺-nitrilotriacetic acid-agarose and analyzed by SDS-PAGE and fluorography (Fig. 2). Each of the fragments expressed in Lec1 cells was labeled with [3H]fucose, indicating that they were all modified with O-fucose. Interestingly, the extent of fucosylation varied from fragment to fragment. For instance, much more of the EGF 16–18 fragment needed to be loaded onto a gel than any of the other fragments to be readily detected by Western blot (Fig. 2), indicating that EGF 16–18 was more heavily radiolabeled than the other fragments. These results suggest that the stoichiometry of O-fucosylation on different EGF repeats may differ.

To investigate which EGF repeats actually bear O-fucose residues, site mutants were generated at sites predicted using both the original consensus (C²XXGG(S/T)C³) and the broadened consensus (C²X₃₋₅(S/T)C³) (Fig. 3a). EGF 19–23 has one C²XXGG(ST)C³ consensus site at EGF 20 (C²VNGGTGC³). Mutation of the threonine to alanine at EGF 20 (T20A) caused significant reduction, but not the elimination, in the fucosylation of this fragment (Fig. 3, b and c), suggesting that the broader sites are utilized. Mutation of the site at EGF 23 (S23A) also resulted in a reduction in fucosylation (Fig. 3, b and c). A double mutant where the sites at both 20 and 23 were eliminated (20A/23A) lost the majority of the fucosylation, although some residual fucosylation remained. The residual radioactivity on the double mutant is probably on EGF 21 (C²LNQGTC³). Attempts at expressing and analyzing the triple mutant (EGF 20A/21A/23A) were unsuccessful. These data demonstrate that both the C²XXGG(ST)C³ consensus sites and the broader sites (C²X₃₋₅(S/T)C³) are modified with O-fucose. To further refine the broader consensus sites, mutants were generated on EGF 11–15. Although no C²XXGG(ST)C³ consensus sites exist in this fragment, both EGF 12 and 15 contain C²X₃₋₅(S/T)C³ sites (Fig. 3a). Mutation of the site at EGF 12 completely eliminated the fucosylation of the fragment (Fig. 3, b and c), suggesting that EGF12 is modified with O-fucose. Interestingly, the site in EGF 12, believed to be a portion of the ligand binding site (26), is conserved in all Notch homologues in data bases (Fig. 1b and data not shown). These data also suggest that the site at EGF 15 is not modified with O-fucose. Since the site at EGF 15 has only three amino acids between the second cysteine and the modified serine or threonine (Fig. 3a), these results indicate that a minimum distance in this space is necessary for efficient fucosylation. Thus, the O-fucose consensus may be refined to C²X₃₋₅(S/T)C³ (Fig. 1).

Localization of O-Fucose Residues on Mouse Notch1 Modified by Fringe—To analyze which EGF repeats contain O-fucose...
that can be modified by Fringe, the mouse Notch1 extracellular domain fragments (Fig. 2) were expressed in Lec1 cell lines stably expressing Mfng or the corresponding empty vector (16). These cell lines were used previously in cell-based Notch signaling assays to demonstrate that Mfng inhibits Notch activation by Jagged1 (16, 19). Interestingly, CHO cells (including Lec1) have been shown to possess transcripts encoding Lfng and Rfng but not Mfng (19, 27). The presence of endogenous Lfng and/or Rfng explains the presence of elongated O-fucose structures seen previously on Notch isolated from Lec1 cells (8). Plasmids encoding the different fragments from mouse Notch1 were transiently transfected into both Lec1 cell lines, the cells were metabolically radiolabeled with [3H]fucose, and the fragments were purified from the medium using Ni₂⁺-elimination and analyzed by gel filtration chromatography analysis (see “Experimental Procedures”) (8). The denatured EGF-O-fucose was a very poor substrate for Fringe compared with the folded EGF-O-fucose (Fig. 5a). The denatured EGF-O-fucose was unfolded by reduction and alkylation (see “Experimental Procedures”), and both the native and unfolded EGF-O-fucose were analyzed as substrates for Fringe in an in vitro assay (Fig. 5a).}

Interestingly, O-fucose was elongated by Mfng to some extent on most EGF repeats (EGF 11–15, 16–18, 19–23, 24–28, and 29–36) but not on EGF 1–5 (Fig. 4). The extent of elongation varied significantly, from 15% on EGF 19–23 to 80% on EGF 29–36. These results suggest that Fringe shows a preference for O-fucose on some EGF repeats relative to others. In addition, several fragments showed significant elongation in the absence of Mfng (EGF 16–18, 24–28, and 29–36), presumably due to the action of the endogenous Fringes. Significantly, O-fucose on EGF 11–15 was elongated to a small extent by the endogenous Fringes but significantly by Mfng. Since EGF 12 is the only site bearing O-fucose in EGF 11–15 (Fig. 3), these results suggest that EGF 12 is a substrate for Mfng modification.

Signals for Fringe Recognition Are Embedded within the Sequence of EGF Repeats—The data in Fig. 4 suggest that Fringes show a preference for O-fucose on some EGF repeats over others. To begin addressing the basis of this preference, we first analyzed whether Fringe recognizes O-fucose in the context of a simple primary amino acid sequence or in the context of a properly folded EGF repeat. Bacterially expressed EGF repeat-1 from factor VII modified with O-fucose was unfolded by reduction and alkylation (see “Experimental Procedures”), and both the native and unfolded EGF-O-fucose were analyzed as substrates for Fringe in an in vitro assay (Fig. 5a).

To examine whether the determinants for this preference are encoded within individual EGF repeats, Fringe-mediated elongation of O-fucose on individual EGF repeats expressed in Lec1 cells was analyzed. EGF repeats 24 and 26 from mouse Notch1 were chosen because both contain evolutionarily conserved C²XGG(S/T)/C³-type O-fucose sites (see Fig. 1) and are within a fragment (EGF 24–28) showing partial elongation of O-fucose even in the absence of Mfng (Fig. 4). The partial modification suggested that the endogenous Fringes (Lfng and Rfng) may be modifying O-fucose on one of the EGF repeats but not the other. This indeed turned out to be the case. As before, plasmids expressing EGF 24 or 26 were transiently transfected into Lec1 cells, the cells were metabolically radiolabeled with [3H]fucose, and the EGF repeats were purified from the medium using Ni²⁺-nitriiotriacetic acid-agarose chromatography. In contrast to what was seen with EGF 24–28, the only sugar structure detected on EGF 24 was a monosaccharide (Fig. 5b), whereas both the tetra- and monosaccharide forms of O-fucose were seen on EGF 26 (Fig. 5c). Thus, the endogenous Fringes (Lfng and Rfng) can discriminate between EGF 26 and EGF 24 based solely on the sequence information in the individual EGF repeats. The O-fucose on EGF 24 is elongated when expressed in Lec1 cells with Mfng (data not shown), indicating that there may be some differences in the specificity of the individual Fringes. Nonetheless, since the Mfng is expressed at a higher level than the endogenous Fringes, this effect may be due to overexpression rather than specificity differences. Thus, a properly folded EGF repeat modified with O-fucose appears to be the basic unit of Fringe recognition. Although we have not
y determined the specific signals for Fringe recognition, these data suggest that such signals are embedded within the EGF sequence itself.

O-Fucosylation and Fringe Modification on Larger EGF Fragments—To determine whether analysis of small fragments of Notch is reflective of what happens in the intact molecule, we analyzed for O-fucose and Fringe modification on larger fragments. Although EGF 1–36 was modified with O-fucose and Mfng, individual point mutants caused undetectable changes in elongation (data not shown). Thus, fragments expressing EGF 1–18 and EGF 19–36 were prepared. To evaluate whether some of the same sites are modified in these larger fragments as in the smaller fragments, a mutation was introduced into EGF 1–18 at the O-fucose site on EGF 12, and a mutation was introduced into EGF 19–36 at the O-fucose site on EGF 26. These fragments were analyzed for Fringe-mediated elongation of O-fucose in the same manner as the smaller fragments in Fig. 4. (Table II). Mutation of serine on EGF 12 had no effect on the relative amounts of mono- and multisaccharide on EGF 1–18 in control cells, but it resulted in a significant decrease in the multisaccharide from the Mfng cell line, suggesting that EGF 12 is one of the major sites of Mfng action in this larger fragment. Similarly, mutation of serine on EGF 26 caused a significant decrease in the amount of multisaccharide on EGF 19–36 from control cells, indicating that EGF 26 is a major site for endogenous Fringe activity. These data indicate that the Fringe effects we observed on smaller fragments such as EGF 11–15 and EGF 24–28 are similar to those seen with these larger fragments. They also indicate that EGF 12 is a major target for Mfng and that EGF 26 is a major target for endogenous Fringes. The fact that we can observe similar Fringe effects from both the smaller and larger EGF fragments suggests that analysis of the small fragments of mouse Notch1 accurately predicts how Fringe will act on the whole Notch protein.

Abruptex Mutants Can Interfere with Fringe Action—Abruptex mutants are a class of Drosophila Notch missense mutations localized in EGF repeats 24–29 (29) that result in a hyperactivatable form of Notch (30). The Abruptex phenotype is believed to be caused by abolition of cell-autonomous inhibition of Notch by ligands (31). In addition, some of the Abruptex mutants are refractory to Fringe (31), suggesting some relationship between the mechanism of Fringe action and the Abruptex mutations. Within the Abruptex mutation region (EGF repeats 24–29), there are several O-fucose modification sites (Fig. 1). The O-fucose modifications in this region are also significantly elongated by Fringe (Fig. 4). We were interested to determine whether any of the Abruptex mutations would alter
the ability of Fringe to induce elongation of O-fucose and particularly if the mutants would be refractory to Fringe action. We chose two Abruptex mutants, \textit{Ax}^{992} (Asp948 → Val) and \textit{Ax}^{59b} (Cys972 → Gly), for study because the sites are well conserved across species (Fig. 6). Both of these Abruptex mutations occur within \textit{Drosophila} Notch EGF 24. We co-transfected mouse Notch EGF 24–28, bearing one or the other mutation, with mouse Mfng or Dfng. Sugar analysis showed that elongation of O-fucose on \textit{Ax}^{992} (Asp948 → Val) was not increased by Mfng, indicating that this mutation interferes with the ability of Mfng to recognize this fragment (Table III). Dfng could elongate O-fucose on \textit{Ax}^{992} (65% multisaccharide), but less extensively than the wild-type EGF 24–28 (78.5% multisaccharide) (Table III). In contrast, increases in elongation of O-fucose on \textit{Ax}^{59b} (Cys972 → Gly) were caused by both Fringes, similar to the wild-type EGF 24–28 (Table III). These results suggest that although some Abruptex mutations may be refractory to Fringe action (e.g. \textit{Ax}^{992}), the Abruptex phenotypes are not necessarily linked to a block in Fringe action (e.g. \textit{Ax}^{59b}).

\section*{DISCUSSION}
To begin understanding how Fringe-mediated alterations on O-fucose saccharide structure may modulate Notch function, we have analyzed sites of O-fucose and Fringe modification on mouse Notch1. During these studies, we identified O-fucose modifications on sites predicted using the C2X3(S/T)C3 consensus site proposed in earlier studies (10), demonstrating that this broader consensus site can be used to accurately predict O-fucose modification. The fact that O-fucose modifies a broader set of sites than previously predicted (summarized in Fig. 7) increases both the number of sites on the Notch receptors and the number of proteins predicted to bear this modification. Many of these sites are evolutionarily conserved on Notch (Fig. 1b). In particular, the site at EGF 12, believed to be essential for ligand binding (26), is conserved in the analogous EGF repeat in all Notch known homologues, including \textit{Glp1} and \textit{Lin12} from \textit{Caenorhabditis elegans}. We have also examined

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
Constructs transfected & Monosaccharide & Multisaccharide \\
\hline
EGF 1–18 control & 80.5 \pm 0.5 & 19.5 \pm 0.5 \\
EGF 1–18 (12A) control & 81 \pm 1 & 19 \pm 1 \\
EGF 1–18 + Mfng & 59.5 \pm 1.5 & 40.5 \pm 1.5 \\
EGF 1–18 (12A) + Mfng & 69 \pm 1 & 31 \pm 1 \\
EGF 19–36 control & 88.5 \pm 1.5 & 11.5 \pm 1.5 \\
EGF 19–36 (26A) control & 99 \pm 0 & 1 \pm 0 \\
EGF 19–36 + Mfng & 67 \pm 2 & 33 \pm 2 \\
EGF 19–36 (26A) + Mfng & 37.5 \pm 1.5 & 26.5 \pm 1.5 \\
\hline
\end{tabular}
\caption{Fringe modification of O-fucose on EGF 1–18 and EGF 19–36}
\end{table}

The ratio of radioactivity of the mono- or multisaccharide (includes di-, tri-, and tetrasaccharide species) to total radioactivity was determined as described in the legend to Fig. 4. Increase in multisaccharide indicates an increase in Fringe modification. The average of duplicate analysis is shown with the range of the data.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Fig. 5. Signals for recognition by Fringe are encoded within individual EGF repeats. a, Fringe assay was carried out as described previously (16), using either native or reduced and alkylated factor VII EGF-O-fucose (see “Experimental Procedures”) as substrate. b and c, plasmids encoding individual EGF 24 (b) or 26 (c) from mouse Notch1 were transfected into control Lec1 cells. The O-fucose saccharides derived from the expressed proteins were evaluated as described under “Experimental Procedures.” The migration position of tetrasaccharide (TS) and monosaccharide (MS) forms of O-fucose are shown.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Fig. 6. Conservation of sequences from EGF 24 sequence. Notch EGF 24 sequences from different species are listed. The positions of the \textit{Ax}^{992} and \textit{Ax}^{59b} Abruptex mutation sites (underlined) are shown.}
\end{figure}
ined the recognition of O-fucose by Fringe and shown that some EGF repeats in Notch extracellular domain are modified by Fringe, whereas others are not. Interestingly, some of the sites modified by Fringe overlap with functional regions of the Notch extracellular domain (summarized in Fig. 7). Each of these results points to a potential mechanism for how elongated O-fucose affects Notch activation.

Although our prior work on O-fucosylation of Notch ligands resulted in the proposal of a broader consensus site, \( C^2X_{\alpha,\beta,S/T/C}^3 \) (10), the results reported here allow us to further refine the consensus. We have demonstrated the presence of O-fucose on three EGF repeats from mouse Notch1 predicted to be modified using the broader consensus: EGFs 12, 21, and 23. Each of these new sites contains four amino acids between \( C^2 \) and the modified serine or threonine. The fact that the serine prior to \( C^3 \) in EGF 15 (\( C^3HYGS\)) was not modified suggests that more than three amino acids may be necessary between \( C^2 \) and the modified Ser/Thr for O-fucosylation, limiting the sites to \( C^2X_{\alpha,\beta,S/T/C}^3 \). Using this broader consensus site identifies 21 potential O-fucosylation sites on mouse Notch1 (13 evolutionarily conserved), instead of nine sites (five evolutionarily conserved) (Fig. 1). Work to further define the consensus site for O-fucose modification is currently being carried out in our laboratory.

In addition to refining the O-fucose consensus site, we have localized the O-fucose residues on mouse Notch1 modified by Fringe. Analysis of Fringe-mediated elongation of O-fucose on fragments from mouse Notch1 extracellular domain revealed that none of the Fringes (endogenous Lfng and Rfng, exogenous Mfng) modified O-fucose residues on EGF 1–5. Within the fragments that were elongated by Mfng, dramatically different efficiencies of elongation were observed (e.g. O-fucose on EGF 24–28 was elongated to a much greater extent than O-fucose on EGF 19–23). Significantly, Fringe-mediated elongation of O-fucose was observed on regions of the mouse Notch1 extracellular domain corresponding to the ligand binding sites and the Abruptex region (Fig. 7). We showed that a properly folded EGF repeat was required for Fringe recognition, demonstrating that the enzyme recognizes specific features of the three-dimensional structure of an EGF repeat. Analysis of individual EGF repeats (24 and 26) in cells showed that the endogenous Fringes elongated O-fucose on EGF 26, but not on EGF 24. These results suggest that the basic unit of recognition is the EGF repeat and that signals required for Fringe modification are encoded within the individual EGF repeat.

A previous report showed that Dfng can modify O-fucose on a *Drosophila* Notch EGF 1–3 fragment *in vitro* (17). Although we found that mammalian Fringes do not modify O-fucose on EGF 1–5 in Lec1 cells, this may reflect the differences in specificity between mammalian and *Drosophila* Fringe or differences in *in vitro versus in vivo* assays. Additionally, Shimizu and co-workers (27) have previously compared Lfng and Mfng action on mouse Notch2 by analyzing the shift in migration of the protein on an SDS-PAGE caused by the Fringe-mediated change in sugar structure. Their data suggested that Lfng modifies O-fucose on mouse Notch2 EGF 1–15, whereas Mfng modifies O-fucose on mouse Notch2 EGF 23–29. Whereas analyzing changes in carbohydrate structure using a shift in migration on SDS-PAGE is less definitive than the analysis of Fringe-mediated alterations in O-fucose structures described here, it will be interesting to determine whether the Fringe effect we observed on EGF 12 is specific for Notch1 and Mfng.

Although it is clear that Fringe modulates Notch function by altering O-fucose structure (16, 17, 19), the mechanism by which the change in sugar structure affects Notch activity is unknown. We have previously proposed several potential models for how the change in sugar structure alters Notch function (for details, see Refs. 13 and 16). The present results offer support for two of these models. The first is based on the observation that Mfng modifies O-fucose on EGF 12. EGF repeats 11 and 12 of *Drosophila* Notch are both necessary and sufficient for interaction with Delta and Serrate (32). In this model, the O-fucose moiety is predicted to be directly involved in binding to ligand or to an accessory protein that influences ligand binding. Thus, the inhibition of Serrate/Jagged signaling by Fringe could be caused by a decrease in binding to Notch due to steric hindrance of the binding site by the elongated sugar structure. A direct role for the O-fucose in Notch-ligand binding interactions is supported by the finding that Notch signaling is reduced in Lec13 cells (which have reduced levels of fucosylation) (16, 19). The recent demonstration that elimination of protein O-fucosyltransferase using RNAi in *Drosophila*...
Notch activation is an area of continuing research. Direct effects of Fringe on Notch ligand interactions have been demonstrated in cell binding assays, although some of the results are conflicting. For instance, Shimizu et al. (27) showed that Mfng and Lfng cause a reduction in Jagged1 binding to Notch2 in cell-based binding assays, but other reports using different cell types showed no change in Serrate/Jagged binding caused by Fringe (17, 27, 33). Data from our recent demonstration that Fringe modifies Notch (27) showed that Mfng and Lfng cause a reduction in Notch interaction with its ligands. Direct effects of Fringe on Notch ligand interactions were affected by loss of \( \text{fucose} \) (17, 27, 33). Data from our recent demonstration that Fringe modifies Notch (27) showed that Mfng and Lfng cause a reduction in Notch interaction with its ligands. Direct effects of Fringe on Notch ligand interactions were affected by loss of \( \text{fucose} \) (17, 27, 33).
