Canonical Notch ligands and Fringes have distinct effects on NOTCH1 and NOTCH2

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Notch signaling is a cellular pathway regulating cell-fate determination and adult tissue homeostasis. Little is known about how canonical Notch ligands or Fringe enzymes differentially affect NOTCH1 and NOTCH2. Using cell-based Notch signaling and ligand-binding assays, we evaluated differences in NOTCH1 and NOTCH2 responses to Delta-like (DLL) and Jagged (JAG) family members and the extent to which Fringe enzymes modulate their activity. In the absence of Fringes, DLL4–NOTCH1 activation was more than twice that of DLL4–NOTCH2, whereas all other ligands activated NOTCH2 similarly or slightly more than NOTCH1. However, NOTCH2 showed less sensitivity to the Fringes. Lunatic fringe (LFNG) enhanced NOTCH2 activation by DLL1 and -4, and Manic fringe (MFNG) inhibited NOTCH2 activation by JAG1 and -2. Mass spectral analysis showed that O-fucose occurred at high stoichiometry at most consensus sequences of NOTCH2 and that the Fringe enzymes modified more O-fucose sites of NOTCH2 compared with NOTCH1. Mutagenesis studies showed that LFNG modification of O-fucose on EGF8 and -12 of NOTCH2 was responsible for enhancement of DLL1–NOTCH2 activation, similar to previous reports for NOTCH1. In contrast to NOTCH1, a single O-fucose site mutant that substantially blocked the ability of MFNG to inhibit NOTCH2 activation by JAG1 could not be identified. Interestingly, elimination of the O-fucose site on EGF12 allowed LFNG to inhibit JAG1-NOTCH2 activation, and O-fucosylation on EGF9 was important for trafficking of both NOTCH1 and NOTCH2. Together, these studies provide new insights into the differential regulation of NOTCH1 and NOTCH2 by Notch ligands and Fringe enzymes.

The Notch signaling pathway plays essential roles in development of metazoans, and defects in the Notch pathway result in a wide variety of congenital disorders and cancers (1–3). Notch receptors are transmembrane proteins with four homologs in mammals (NOTCH1–4). They can be activated by four canonical Notch ligands: Delta-like 1 and 4 (DLL1 and -4), and Jagged 1 and 2 (JAG1 and -2). Elimination of Notch1 or Notch2 in mice results in embryonic lethality (4, 5), whereas elimination of Notch3 or Notch4 does not cause any gross developmental phenotype (6, 7). Mutations in NOTCH1 in humans cause congenital heart defects (8) and Adams–Oliver syndrome (9), mutations in NOTCH2 cause Alagille syndrome 2 (10) and Hajdu–Cheney syndrome (11), and mutations in NOTCH3 cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (12). Mutations in all four Notch receptors are associated with a number of cancers (13).

Notch ligand interactions are regulated by O-linked carbohydrate modifications on the epidermal growth factor–like (EGF) repeats in the extracellular domain (ECD) of Notch receptors (14–16). The ECDs of both NOTCH1 and NOTCH2 contain 36 tandem EGF repeats, whereas NOTCH3 has 34 and NOTCH4 has 29 (1). Many of these EGF repeats contain consensus sequences for O-linked modifications, including O-linked fucose (17), O-linked glucose (two separate sites of modification) (18), and O-linked GlcNAc (19). All of these modifications are known to affect Notch activity, but O-fucose on EGF repeats 8 and 12 of NOTCH1 has been shown to directly interact with ligands (20, 21). Elimination of these sites alters Notch activity in vitro and in vivo (22–24). In particular, elimination of O-fucose on EGF12 results in embryonic lethality in both mice and flies (23, 24). Significantly, elongation of the O-fucose residues on Notch by the Fringe family of β3-N-acetylgalactosaminyltransferases modulates Notch activity, typically enhancing Notch signaling from Delta-family ligands while inhibiting signaling from Jagged ligands (14–16). Only one Fringe gene exists in flies, but mammals express three: Lunatic fringe (LFNG), Manic fringe (MFNG) and Radical fringe (RFNG). Elimination of Lfng in mice results in a severe somitogenesis defect (25, 26), and mutations in human LFNG cause a severe vertebral segmentation defect called spondylodiscal dysostosis type III (27). In this context, LFNG is regulating the activity of NOTCH1 (28). Although Mfng or Rfng-null mice display no significant developmental phenotypes (29), all three Fringes have been implicated in regulation of Notch activity in a variety of contexts, including angiogenesis (30), B and T cell maturation (31, 32), bile duct remodeling (33), ventricular chamber development (34), and kidney development (35). Fringes have also been implicated in a number of cancers (36–38). Apart from LFNG, which regulates NOTCH1 during somitogenesis, determining which Fringe modulates which Notch receptor in vivo is challenging due to the broad and overlapping expression patterns of the Notch receptors, Notch ligands, and Fringes in embryonic and adult tissues.

We and others have studied the effects of individual Fringe enzymes on discrete Notch-ligand pairs (39–45). In our recent study of NOTCH1 (22), we confirmed that all three Fringes enhance NOTCH1 activation by DLL1, and LFNG and MFNG inhibit activation by JAG1. In contrast, RFNG enhances
NOTCH1 activation by JAG1. Using mass spectral glycoproteomics methods, we demonstrated that the majority of predicted O-fucose consensus sequences are modified by protein O-fucosyltransferase 1 (POFT1) at high stoichiometry, that LFNG modifies O-fucose on a subset of these EGF repeats, and that MFNG and RFNG modify a subset of those EGF repeats modified by LFNG. Using cell-based NOTCH1 activation and ligand-binding assays, we showed that O-fucose on EGF8 and -12 of NOTCH1 are the major sites responsible for Fringe-mediated enhancement of NOTCH1 activation by DLL1. More recent studies show that the O-fucose residues on EGF8 and -12 are also important for Fringe-mediated enhancement of Drosophila Notch binding to Delta in vitro and activation during Delta-mediated wing vein development in vivo (23). These results are consistent with the importance of O-fucose on EGF8 and -12, as revealed by the recent co-crystal structures of the ligand-binding domain of NOTCH1 and fragments of DLL4 (20) or JAG1 (21). We also showed that LFNG and MFNG modification of O-fucose on EGF6 and -36 are mainly responsible for inhibition of NOTCH1 activation by JAG1 (22). In contrast, Fringe modifications of EGF8 and -12 of Drosophila Notch inhibit Serrate (Jagged ortholog in Drosophila) binding in vitro and affect Serrate-mediated wing margin formation in vivo, mainly through blocking the cis-inhibition of Serrate by Notch (23). These results suggest that Fringe modifications of O-fucose on EGF8 and -12 play a common role in enhancing Notch binding to Delta-family ligands, but there are differences in how Fringes inhibit signaling from Serrate/jagged family members.

Here we compare how all canonical ligands activate NOTCH1 and NOTCH2, and we examine how the Fringes modulate NOTCH2 activity. Early in vitro studies reported that Fringe differentially modulates JAG1 and DLL1 signaling from NOTCH1 and NOTCH2 (41). LFNG and MFNG were also shown to modify distinct regions of the NOTCH2 ECD (42). Here we compared NOTCH1 and NOTCH2 binding to and activation by canonical Notch ligands (DLL1, DLL4, JAG1, and JAG2), and we examined how LFNG, MFNG, and RFNG affect NOTCH2 binding to and activation by these ligands. We used semi-quantitative mass spectral glycoproteomics methods to identify O-fucose modifications on NOTCH2 EGF repeats and assess the relative abundance of Fringe modification at each site by the three Fringe enzymes. We mutated O-fucose sites to determine which are needed for the Fringes to mediate their effects on NOTCH2. Finally, we also examined how O-fucosylation at specific EGF repeats is important for trafficking of both NOTCH1 and NOTCH2. Together, these studies give new insights into the differential regulation of NOTCH1 and NOTCH2 by Notch ligands and Fringe enzymes.

**Results**

**NOTCH1 and NOTCH2 respond differently to Delta and Jagged ligands**

Although the extracellular domains of NOTCH1 and NOTCH2 both contain 36 tandem EGF repeats, the location of EGF repeats containing O-fucose consensus sequences is different (Fig. 1A). We compared NOTCH1 and NOTCH2 activities using ligand-coating Notch signaling assays in CHO-K1 cells with extracellular domains of the four canonical ligands: DLL1, DLL4, JAG1, and JAG2 (Fig. 1B and Fig. S1). DLL1 and JAG1 induced higher NOTCH2 activity than NOTCH1, but DLL4 induced NOTCH1 activity much more robustly than NOTCH2. JAG2 induced similar levels of NOTCH1 and NOTCH2 activity. Differences in the C-terminal tags (Fc or His) or species (human, mouse, or rat) of the ligands had little effect on NOTCH1 or NOTCH2 activity (Fig. S1). DLL3 did not activate NOTCH1 or NOTCH2 in trans (Fig. S1E), as reported previously (46). Relative binding of soluble ligands to NOTCH1- or NOTCH2-expressing HEK293T cells was consistent with their effects on NOTCH1 and NOTCH2 activity (Fig. 1C), suggesting that the ligands have similar effects on NOTCH1 and NOTCH2 in both CHO-K1 and HEK293T cells. NOTCH2 had a higher affinity for DLL1 than NOTCH1, whereas NOTCH1 had both a higher affinity and binding maximum for DLL4 than NOTCH2. These results are in line with the relative affinities of NOTCH1 and NOTCH2 for DLL1 and DLL4 reported previously (47, 48).

In addition, NOTCH2 had higher affinity for JAG1 than NOTCH1, whereas NOTCH1 and NOTCH2 showed similar affinity for JAG2 (Fig. 1C).

**LFNG enhances NOTCH2 activation by DLL1 and -4, and MFNG inhibits activation by JAG1 and -2**

Using cell-based co-culture assays with NIH3T3 cells, we previously reported that LFNG and MFNG enhance NOTCH1 activation from DLL1 and inhibit activation from JAG1, whereas RFNG enhances NOTCH1 activity from both ligands (22). We have reproduced the same effects of Fringes on NOTCH1 using ligand-coating assays, also using NIH3T3 cells (Fig. 2A). We and others have previously shown that NIH3T3 cells, HEK293T cells, and CHO-K1 cells express low levels of Fringes and respond similarly in Notch activation assays to expression of the three Fringe enzymes (22, 45). To be complete, we also examined how the Fringes affect NOTCH1 activation by DLL4 and JAG2 (Fig. 2A). Both LFNG and MFNG inhibited JAG2-NOTCH1 activity, similar to their effects on JAG1-NOTCH1. In contrast to DLL1, none of the Fringes caused significant enhancement of DLL4-NOTCH1 activation. These results are consistent with our prior results showing that the Fringe modification of NOTCH1 EGF11-13 significantly enhances binding to DLL1 but not to DLL4 (49).

To examine how Fringes modulate NOTCH2 activity, similar assays were performed using NIH3T3 cells transiently transfected with a NOTCH2 plasmid with or without a Fringe plasmid (Fig. 2B). The effect of all three Fringes on NOTCH2 was unexpectedly weak compared with NOTCH1. For example, LFNG resulted in a 9-fold increase in DLL1-mediated NOTCH1 activation but only a 3-fold increase in DLL1-mediated NOTCH2 activation. All three Fringes enhanced DLL1-NOTCH2 signaling less than DLL1-NOTCH1 signaling, and only MFNG significantly inhibited JAG1-NOTCH2 signaling. LFNG enhanced both DLL1- and DLL4-induced NOTCH2 signaling, and MFNG inhibited both JAG1- and JAG2-induced NOTCH2 signaling (Fig. 2B), indicating that the effects of LFNG and MFNG on NOTCH2 are consistent within ligand families. RFNG had very little effect on NOTCH2 signaling from any ligand.
Comparing the efficacy of Fringes on modulation of NOTCH1 and NOTCH2 activity

To evaluate whether different levels of Fringe enzymes relative to Notch protein have differential effects for NOTCH1 and NOTCH2, we measured Notch activity by changing Fringe/Notch expression ratios in the ligand-coating assays (Fig. 2, C and D). At a ratio of 0.5 LFNG/Notch, DLL1-NOTCH1 signaling increased to a maximum of about 9-fold, whereas DLL1-NOTCH1 signaling gradually increased with the MFNG or RFNG/NOTCH1 ratio. This observation is consistent with LFNG having a higher enzymatic activity than either MFNG or RFNG (50). LFNG showed the most significant increase in DLL1-NOTCH2 signaling, which reached a maximum at a ratio of 0.1 LFNG/NOTCH2. Increasing expression levels for any of the Fringes had no additional effect on DLL1-NOTCH2 activity. Surprisingly, compared with a gradually increasing effect of LFNG on DLL1-NOTCH1, JAG1-NOTCH1 signaling was maximally inhibited at the lowest LFNG/NOTCH1 ratio tested (0.02) and did not change with increasing levels of LFNG (Fig. 2D). In contrast, inhibition of JAG1-NOTCH2 activity only occurred minimally even at high LFNG/NOTCH2 levels (Fig. 2D). MFNG showed significant inhibition of JAG1-NOTCH1 and JAG1-NOTCH2 signaling at similar ratios. Finally, RFNG increased both DLL1-NOTCH1 and JAG1-NOTCH1 signaling as expected (22) but did not significantly increase NOTCH2 activation by either ligand at any RFNG/NOTCH2 ratio. Interestingly, high levels of RFNG began to inhibit JAG1-NOTCH1 signaling, suggesting that at high levels, RFNG may begin modifying the NOTCH1 inhibitory sites, EGF6 and/or EGF36 (22). Clearly, Fringe enzymes have different efficiencies in regulating NOTCH1 and NOTCH2 activities.

Fringes modify O-fucose on more EGF repeats of NOTCH2 than NOTCH1 but less efficiently on EGF12

To better understand why the Fringes had less of an effect on NOTCH2 than NOTCH1, we examined which EGF repeats of NOTCH2 are modified by the three Fringes. Mouse NOTCH2 EGF1-36-Myc-His6 was expressed in HEK293T cells with or without each Fringe, purified from the medium by nickel-nitrilotriacetic acid agarose chromatography (Fig. S2A), and digested with proteases, and the resulting peptides were
analyzed by nano-LC–MS/MS (Dataset S1). The site occupancy and relative amount of Fringe-mediated elongation of O-fucose at each fucosylation site were determined by generating extracted ion chromatograms for each glycoform of a peptide (Dataset S1). A summary of these results is shown in Fig. 3 and Table S1. We identified peptides containing all 21 predicted O-fucosylation sites, 20 of which were modified with fucose at high stoichiometries, similar to what we have seen on mouse NOTCH1 (22) and Drosophila Notch (51). EGF24 was unmodified, similar to EGF24 of NOTCH1 (22). The consensus sequence in both cases is C$_2$XXXXSC$_3$, suggesting that serine may not be a good substrate for POFUT1. As with NOTCH1 (22), in the absence of overexpressed Fringes, none of the O-fucose residues were appreciably elongated past the monosaccharide, confirming the low expression levels of endogenous Fringe enzymes in HEK293T cells (Dataset S1).

As with mouse NOTCH1, MFNG and RFNG modified a subset of EGF repeats modified by LFNG (Fig. 3). Of the 20 O-fucosylated EGF repeats on NOTCH2, LFNG fully or partially modified 15, whereas MFNG and RFNG only modified nine each. In contrast, LFNG modified nine EGF repeats of NOTCH1, MFNG modified eight, and RFNG modified three (22). Eight sites on NOTCH2 were at least partially modified by all three Fringes (EGF5, -8, -9, -18, -25, -27, -30, and -31). Only NOTCH2 EGF18 was at least partially elongated to tetrasaccharide by all three Fringes. Surprisingly, NOTCH2 EGF12 was only partially modified by LFNG. In contrast, EGF12 of NOTCH1 was fully modified by LFNG and elongated to tetrasaccharide (22). MFNG did not

Figure 2. Fringes differentially regulate NOTCH1 and NOTCH2 activation. A and B, ligand-coating Notch signaling assays with the indicated ligands in NIH3T3 cells co-transfected with NOTCH1 (A) or NOTCH2 (B) plasmids with or without Fringes. RLU were calculated as in Fig. 1B. Statistical significance of controls (−FNG) versus +Fringe was determined using one-way ANOVA. The bar graph shows mean ± S.D.; three independent experiments (n = 9) were analyzed. *** p < 0.0001; n.s., not significant. C and D, ligand-coating NOTCH1 or NOTCH2 signaling assays with DLL1-Fc–coated (C) or JAG1–Fc–coated (D) plates co-transfected with different ratios of Fringe/NOTCH expression plasmids as indicated. RLU compared with controls (−FNG) normalized to 1 for each sample were calculated as above. Error bars, S.D.; three independent experiments (n = 9) were analyzed.

Figure 3. LFNG modifies more O-fucose sites than either MFNG or RFNG on NOTCH2. NOTCH2 EGF1-36-Myc-His$_6$ protein was produced in the absence or presence of each of three Fringes (at a Fringe/NOTCH2 ratio of 0.5) in HEK293T cells as described in the supporting Experimental procedures. The purified proteins were reduced, alkylated, digested with proteases, and analyzed by nano-LC–MS/MS as described in the supporting Experimental procedures. O-Fucosylated peptides were identified (Table S1), and the O-fucose glycoforms for each peptide were quantified by extracted ion chromatograms (Dataset S1). Red triangles, fucose; blue squares, GlcNAc; yellow circles, galactose; purple diamonds, sialic acid. The symbols that are half-white indicate <50% modification with that monosaccharide.
**Fringe effects on NOTCH2**

**Figure 4. LFNG modification of O-fucose on NOTCH2 EGF8 and -12 enhances NOTCH2-DLL1 binding and signaling.** A, HEK293T cells were co-transfected with full-length NOTCH2 and LFNG or EV (−FNG) as described under "Experimental procedures" and incubated with different concentrations of soluble DLL1-Fc as indicated. Binding was analyzed by flow cytometry, and the binding curves were generated by determining the MFI at increasing ligand concentrations. B, HEK293T cells transfected with full-length DLL1 were incubated with 16.5 nM NOTCH2 EGF fragments with or without LFNG modification as described under "Experimental procedures." Histograms from flow cytometry analysis are shown. Blue line, NOTCH2 fragment without LFNG; red line, NOTCH2 fragment with LFNG; black line, second antibody only. C, HEK293T cells transfected with full-length DLL1 were incubated with increasing concentrations of WT NOTCH2 EGF7–12 with or without LFNG modification, EGF7–12 8V mutant modified with LFNG, or EGF7–12 12V mutant modified with LFNG. Binding was analyzed by flow cytometry, and the binding curve was generated by determining the MFI from each concentration. D, WT full-length NOTCH2 or O-fucosylation site mutants 8V, 12V, and 8V12V or EV were tested in a NOTCH2 activation assay using plates coated with DLL1-Fc in the presence (red) or absence (blue) of LFNG as described under "Experimental procedures." The bar graph shows mean ± S.D. (error bars); statistical significance of the enhancement of activation relative to −FNG for each mutant was determined using one-way ANOVA. Three independent experiments (n = 9) were analyzed. ***p < 0.0001; n.s., not significant.

significantly modify EGF12 of NOTCH2 (Fig. 3) or NOTCH1 (22). Taken together, these results indicate that all three Fringes modify more O-fucose sites on NOTCH2 than NOTCH1 but LFNG modified EGF12 of NOTCH2 less efficiently.

**LFNG modification of NOTCH2 EGF8 and -12 enhances DLL1-NOTCH2 binding and signaling**

We previously reported that O-fucose sites on NOTCH1 EGF8 and -12 are the major sites responsible for Fringe-mediated enhancement of DLL1 binding to and activation of NOTCH1 (22). Whereas LFNG increased DLL1-NOTCH1 binding nearly 10-fold (22), LFNG increased DLL1-NOTCH2 binding only slightly (Fig. 4A), consistent with the smaller effects on DLL1-NOTCH2 signaling (Fig. 2B). Because the effects of LFNG on DLL1-NOTCH2 binding were so much lower than on DLL1-NOTCH1, and NOTCH2 had a higher affinity for DLL1 than NOTCH1 (Fig. 1C), we examined which regions of the NOTCH2 ECD are responsible for binding to DLL1. We generated expression constructs that divided the 36 EGF repeats of NOTCH2 into six equal-sized Myc-His6–tagged fragments: EGF1–6, EGF7–12, EGF13–19, EGF20–25, EGF25–30, and EGF31–36. Each of these was expressed in HEK293T cells in the presence or absence of LFNG, purified from the medium, and used for binding to DLL1-expressing cells (Fig. 4B). Only the EGF7–12 fragment bound to DLL1-expressing cells, and the binding was greatly increased when EGF7–12 was modified by LFNG (Fig. 4, B and C).

To determine which EGF repeats in EGF7–12 are responsible for this enhanced binding by LFNG, we eliminated the O-fucose sites in EGF8 or EGF12 (O-fucose on EGF9 is discussed below) by mutating the modified threonine (T) to valine (V) and co-expressing the fragments with LFNG. Elimination of the O-fucose sites in EGF8 or EGF12 (8V or 12V) significantly reduced the ability of LFNG to enhance binding of EGF7–12 to DLL1 (Fig. 4C), indicating that Fringe modification of both EGF8 and -12 is important for enhancing DLL1-NOTCH2 binding. To confirm that these two sites are also important for LFNG-mediated enhancement of DLL1-NOTCH2 signaling, we made the same O-fucose mutants in EGF8 and EGF12 (8V, 12V, and double mutant 8V12V) in full-length NOTCH2 and performed DLL1 ligand-coating NOTCH2 signaling assays (Fig. 4D). Compared with WT NOTCH2, both 8V and 12V diminished NOTCH2 activity ~50% in the presence or absence of LFNG, suggesting the O-fucose at these sites are equally important for DLL1-NOTCH2 signaling and for LFNG-mediated enhancement of DLL1-NOTCH2 signaling as for NOTCH1 (22). Mutations in both EGF8 and EGF12 (8V12V) showed lower NOTCH2 activity that could not be enhanced by LFNG (Fig. 4D), confirming the importance of LFNG modifications at EGF8 and -12 for enhancement of DLL1-NOTCH2 binding and signaling. None of these mutations had a significant effect
Figure 5. LFNG modification of O-fucose on EGF12 prevents it from inhibiting JAG1-NOTCH2 signaling. A, WT NOTCH2 or O-fucosylation site mutants (or EV) were tested in a NOTCH2 activation assay using plates coated with JAG1-Fc in the absence (blue) or presence of MFNG (green) as described under “Experimental procedures.” The bar graph shows mean ± S.D. (error bars) for two independent experiments (n = 6). ***, p < 0.0001; *, p < 0.01; n.s., not significant. The asterisk or n.s. above each column compares that mutant with or without MFNG to WT with or without MFNG, respectively. The triple asterisk or n.s. above the line compares −FNG with +MFNG. B, Notch activation assays with WT NOTCH2 or O-fucosylation site mutants were performed as in A but in the absence (blue) or presence of LFNG (red) or MFNG (green). The bar graph shows mean ± S.D. for three independent experiments (n = 9). Statistical significance of the enhancement of activation relative to −FNG for each mutant was determined using one-way ANOVA. Three independent experiments (n = 9) were analyzed. ***, p < 0.0001; n.s., not significant. The triple asterisk or n.s. above the line compares −FNG with +LFNG or +MFNG.

Lack of modification on EGF12 is critical for MFNG inhibition of JAG1-NOTCH2 signaling

We previously showed that LFNG and MFNG modification of O-fucose residues at NOTCH1 EGF6 and EGF36 were largely responsible for inhibition of JAG1-NOTCH1 signaling (22). To determine which EGF repeats are responsible for MFNG inhibition of JAG1-NOTCH2 (Fig. 2B), we performed site-directed mutagenesis (T to V) of O-fucose sites in EGF repeats modified by MFNG (EGF1, -5, -8, -9, -18, -25, -27, -30, and -31) and measured their activity in the presence or absence of MFNG (Fig. 3). We also mutated EGF6 and EGF36 because they are involved in regulating JAG1-NOTCH1 signaling (22) as well as a few additional sites modified by LFNG (EGF2, -12, and -34). In contrast to what we observed for LFNG and MFNG inhibition of JAG1-NOTCH1 signaling, no single site mutant had a significant effect on the ability of MFNG to inhibit JAG1-NOTCH2 signaling (Fig. 5A). In contrast, mutation of the sites on EGF27 and -31 resulted in stronger inhibition. These results suggest that a combination of Fringe-modified EGF repeats contribute to MFNG inhibition of JAG1-NOTCH2.

It was surprising that LFNG did not inhibit JAG1-NOTCH2 signaling because LFNG modifies all of the EGF repeats modified by MFNG (Fig. 3). This suggested that some of the additional sites modified by LFNG might prevent inhibition of JAG1-NOTCH2, so we examined the above mutants in a JAG1-NOTCH2 signaling assay in the presence or absence of LFNG or MFNG (Fig. S6). Unexpectedly, elimination of the O-fucose site on EGF12 allowed LFNG to inhibit JAG1-NOTCH2 signaling similar to MFNG inhibition of WT JAG1-NOTCH2 signaling (Fig. 5B and Fig. S6). Because LFNG modified EGF12 whereas MFNG did not (Fig. 3, Fig. S4, and Dataset S1), LFNG modification of EGF12 may enhance binding of JAG1 to NOTCH2 such that it prevents inhibition. To explore this possibility, we bound soluble JAG1 or JAG2 to NOTCH2-expressing cells with or without co-expression of MFNG or LFNG. LFNG slightly enhanced binding, whereas MFNG had no effect on cell-surface presentation of NOTCH2 (Fig. S3). MFNG and RFNG enhanced DLL1-NOTCH2 less than LFNG (Fig. 2B), presumably due to the absence of modification of EGF12 by MFNG and less efficient modification of EGF8 by RFNG (Fig. 3 and Fig. S4).

The extent of the LFNG-induced increase in binding of EGF7–12 to DLL1-expressing cells was significantly greater than the enhanced binding of DLL1-Fc to NOTCH2-expressing cells (Fig. 4, compare A with C). The EGF7–12 fragment contains three O-fucosylation sites: EGF8, -9, and -12), all of which were modified by LFNG in EGF1–36 (Fig. 3). Semiquantitative mass spectral analysis of EGF8 or -12 glycopeptides showed that LFNG modified O-fucose on EGF8 at high efficiency in both EGF1–36 and EGF7–12, but the O-fucose on EGF12 was modified much more efficiently in EGF7–12 than in EGF1–36 (Fig. S5). At a 0.5 LFNG/EGF7–12 ratio, O-fucose on EGF12 in EGF7–12 was fully modified, even to tetrasaccharide, whereas it was only partially modified in EGF1–36. This indicates that the additional elongation on EGF12 in EGF7–12 is responsible for the enhanced binding of NOTCH2 to DLL1.

Fringe effects on NOTCH2

We previously showed that LFNG and MFNG modification of O-fucose residues at NOTCH1 EGF6 and EGF36 were largely responsible for inhibition of JAG1-NOTCH1 signaling (22). To determine which EGF repeats are responsible for MFNG inhibition of JAG1-NOTCH2 (Fig. 2B), we performed site-directed mutagenesis (T to V) of O-fucose sites in EGF repeats modified by MFNG (EGF1, -5, -8, -9, -18, -25, -27, -30, and -31) and measured their activity in the presence or absence of MFNG (Fig. 3). ***, p < 0.0001; n.s., not significant. The triple asterisk or n.s. above the line compares −FNG with +LFNG or +MFNG.
or slightly reduced binding (Fig. 6, A and B). To determine which portion of NOTCH2 binds to JAG1, we used the NOTCH2 EGF fragments used in Fig. 4B to bind JAG1-expressing cells (Fig. 6C). As with DLL1, only EGF7–12 bound to JAG1, and modification by LFNG slightly enhanced binding of EGF7–12 to JAG1 cells (Fig. 6, C and D), but much less so than LFNG enhanced binding of EGF7–12 to DLL1 cells (Fig. 4A). Elimination of the O-fucose site on EGF12 (12V) caused a much larger reduction on binding to JAG1 cells than elimination of the O-fucose site on EGF8 (8V) (Fig. 6D). Because LFNG, but not MFNG, modifies EGF12 (Fig. 3 and Fig. S4), these results suggest that LFNG modification of EGF12 enhances binding of NOTCH2 to JAG1 sufficiently to prevent the inhibition caused by MFNG modifications.

**O-Fucosylation of EGF9 affects the trafficking of NOTCH1 and NOTCH2**

Among all O-fucosylation site mutants of NOTCH1, we found that eliminating the O-fucose site within EGF9 (Thr to Ala mutant, EGF9A) significantly reduced cell-surface expression of NOTCH1, measured by both flow cytometry and Western blotting analysis (Fig. 7, A and B). Reverting the mutant to WT (EGF9A-T) rescued the phenotype (Fig. 7, A and B). Eliminating the O-fucose site within EGF9 (Thr to Val) in NOTCH2 also showed reduced cell-surface expression (Fig. 7, C and D), resulting in a small reduction in NOTCH2-JAG1 signaling (Fig. 5A). To confirm that the NOTCH1 EGF9A mutant accumulated in the ER, we co-transfected WT or EGF9A NOTCH1 with an ER marker (pEFires-P-mTagBFP-KDEL) into HEK293T cells. Immunofluorescent staining of permeabilized cells revealed primarily cell-surface localization for WT NOTCH1 and co-localization of NOTCH1 EGF9A with the ER marker (Fig. 7E). Together, these results suggest that O-fucose on EGF9 of NOTCH1 and NOTCH2 is important for Notch folding and trafficking.

**Discussion**

Here we used cell-based assays to show that NOTCH1 and NOTCH2 respond differently to canonical ligands and Fringe enzymes. We found that DLL4 bound and activated NOTCH1 more than NOTCH2, whereas the reverse was true for DLL1 and JAG1. NOTCH1 and NOTCH2 showed similar binding to and activation by JAG2. Therefore, the affinity between a given Notch-ligand pair is a good predictor for the degree of pathway activation mediated by the pair. LFNG and MFNG both enhanced DLL1-NOTCH2 activation, but the extent of enhancement was weak compared with NOTCH1. In contrast, none of the Fringes significantly enhanced DLL4-NOTCH1 activation, and only LFNG modestly enhanced DLL4-NOTCH2. Interestingly, only MFNG inhibited activation of NOTCH2 by JAG1 or JAG2, whereas both LFNG and MFNG inhibited activation of NOTCH1. RFNG had very little effect on NOTCH2 activation by any ligand but enhanced NOTCH1 activation by both DLL1 and JAG1.

There appear to be two contributing factors for the differential ability of the Fringes to enhance NOTCH1 or NOTCH2 activation by DLL1 or DLL4. The first is the difference in
binding affinity of DLL ligands to NOTCH1 or NOTCH2 in the absence of Fringe. DLL1 had a higher binding affinity for NOTCH2 than NOTCH1, but DLL1-NOTCH1 activity was enhanced more by the Fringes than DLL1-NOTCH2. In contrast, DLL4 bound more tightly to NOTCH1 than NOTCH2, but DLL4-NOTCH1 activity was not enhanced by any of the Fringes, whereas LFNG enhanced DLL4-NOTCH2 activity slightly. These results suggest that a higher binding affinity in the absence of Fringe results in more resistance to the influence of Fringe in enhancing Notch activity. 

The second factor is that the Fringes modify O-fucose on NOTCH2 EGF8 and EGF12 less efficiently than NOTCH1 (Figs. 3 and 8), the key sites for DLL1-NOTCH2 binding (Fig. 4C). NOTCH2 EGF12 was more efficiently modified with LFNG on EGF7–12 than on EGF1–36, resulting in a greater LFNG-induced increase in binding of EGF7–12 to DLL1. This suggests that something in the overall structure of the larger protein prevents more efficient modification of NOTCH2 EGF12. The less efficient level of modification of O-fucose on NOTCH2 EGF repeats, and the greater number modified, is more similar to Drosophila Notch than to NOTCH1 (Fig. 8). The reduced efficacy of the Fringe enzymes was also observed when the ratio of Fringes to NOTCH1 or NOTCH2 was varied in cell-based assays. Enhancement of DLL1-NOTCH2 signaling by all of the Fringes reached its maximum at a low Fringe/NOTCH2 ratio, consistent with the idea that something in the full-length protein blocks additional modification of EGF12. In contrast, the enhancement of DLL1-NOTCH1 continued to increase as the Fringe/NOTCH1 ratios increased.

The fact that O-fucose on some NOTCH2 EGF repeats were modified by Fringes whereas others were not lends support to our hypothesis that the sequences within individual EGF repeats determine whether it is modified (22, 50, 52). There are also differences between the EGF repeats modified by individual Fringes (Fig. 3). This may be due to differences in the binding pockets of the Fringe enzymes for O-fucosylated EGF repeats but may also be affected by the difference in their catalytic activity. In in vitro assays, LFNG has significantly higher activity than either MFNG or RFNG (50). This could explain why fewer NOTCH2 EGF repeats are modified by MFNG and RFNG and may also explain differences in elongation past the disaccharide. If LFNG rapidly modifies an O-fucose on a specific EGF repeat, it is more likely to be elongated with galactose and sialic acid as NOTCH2 moves through the Golgi apparatus. Whereas this is consistent with the pattern of elongation seen in Fig. 3, other explanations, such as changes in conformation of the NOTCH2 protein as it is modified by these enzymes, could also play a part.

Figure 7. O-Fucose on EGF9 affects trafficking of NOTCH1 and NOTCH2. A, HEK293T cells were co-transfected with NOTCH1, NOTCH1 EGF9A, revertant EGF9A-T, or EV along with GFP plasmid. Cell-surface NOTCH1 was stained with a PE-conjugated NOTCH1 extracellular antibody and analyzed by flow cytometry. WT NOTCH1 (light green), NOTCH1 EGF9A (purple), NOTCH1 EGF9A-T revertant (dark green), and EV control (black). B, Western blotting of whole-cell extracts of HEK293T cells transfected with NOTCH1, NOTCH1 EGF9A, NOTCH1 EGF9A-T revertant, or EV (control) for 24 h. Migration positions for full-length NOTCH1 (N1FL) and transmembrane subunit of NOTCH1 (N1TM) are indicated. C, HEK293T cells were co-transfected with WT NOTCH2, NOTCH2 EGF9V, or EV along with GFP plasmid. Cell-surface NOTCH2 was stained with a PE-conjugated NOTCH2 extracellular antibody and analyzed by flow cytometry. WT NOTCH2 (light green), NOTCH2 EGF9V (purple), or EV control (black). D, Western blotting of whole-cell extracts of HEK293T cells transfected with WT NOTCH2, NOTCH2 EGF8V, EGF9V, or EV for 24 h. Migration positions of full-length (N2FL) and transmembrane subunit (N2TM) of NOTCH2 are indicated. E, HEK293T cells were co-transfected with plasmids encoding WT NOTCH1 or NOTCH1 EGF9A and an ER marker. NOTCH1 was stained with anti-NOTCH1 extracellular domain antibody and FITC-conjugated anti-sheep IgG antibody. Arrows, co-localization with the ER marker and NOTCH1 EGF9A. Scale bar (bottom right), 10 μm.
The importance of the O-fucose modifications on EGF8 and -12 for binding to and activation by Delta ligands appears to be similar between NOTCH1, NOTCH2, and Drosophila Notch. Our finding that NOTCH2 EGF8 and -12 are the major sites responsible for Fringe-mediated enhancement of DLL1-NOTCH2 binding and activation is consistent with our previous report of the effects of Fringe on DLL1-NOTCH1 (22) and on Drosophila Notch-Delta interactions (23). The O-fucose monosaccharide (in the absence of Fringe) on EGF8 and -12 is important for both DLL1-NOTCH2 (Fig. 4) and DLL1-NOTCH1 signaling (22), whereas the O-fucose monosaccharide on EGF12 of Drosophila Notch is more important for Notch-Delta interactions than EGF8 (23). Recent results show that eliminating the O-fucose site on EGF12 of Drosophila NOTCH (23) or mouse NOTCH1 (24) in vivo results in embryonic lethality in flies and mice, respectively. Although there are no data on the effects of eliminating O-fucose on EGF8 of NOTCH1 in mice in vivo, eliminating O-fucose on EGF8 of Drosophila NOTCH enhances the effects of the EGF12 mutation in wing vein formation in flies, a Fringe-dependent event (23). Together, these results suggest that Fringe modification on both EGF8 and -12 of Notch proteins plays an important role in regulating interactions with Delta ligands both in vitro and in vivo.

The molecular mechanism for inhibition of Notch-Jagged/Serrate signaling by Fringes is more complicated. No individual O-fucose site mutation abrogated the ability of MFNG to inhibit JAG1-NOTCH2 activity but had no effect on MFNG (Fig. 5B), which does not modify EGF12. Binding assays showed that LFNG slightly enhanced binding of NOTCH2 to JAG1 or JAG2, whereas MFNG either had no effect or slightly reduced binding. Mutating the O-fucose site in EGF12 significantly reduced the binding of LFNG-modified NOTCH2 EGF7–12 to JAG1, indicating that LFNG modification on EGF12 enhanced JAG1-NOTCH2 binding sufficiently to prevent the inhibitory effects of modification at other sites. In NOTCH1, the inhibitory sites are EGF6 and -36, although mutations at these sites do not affect binding of NOTCH1 to JAG1 (22), so the mechanism for inhibition is unclear. The EGF8V mutation in Drosophila NOTCH abrogates the ability of Fringe to inhibit Notch-Serrate binding in vitro, and the EGF8V12V double mutant blocks cis-inhibition of Serrate by Notch in vitro and in vivo (23). In contrast, the EGF8V12V mutation in NOTCH2 can still be inhibited by MFNG (Fig. 5B). Thus, although Fringes reduce NOTCH1, NOTCH2, and Drosophila Notch activation by Serrate/Jagged ligands, the mechanisms responsible for these reductions appear to be different.

Recent results from our laboratory suggest that POFUT1 participates in a noncanonical ER quality control pathway for the folding of Notch EGF repeats (53). The addition of O-fucose to folded EGF repeats results in stabilization of the folded form, enhancing cell-surface expression of the Notch protein. Here we demonstrated that the EGF9A or EGF9V mutation in NOTCH1 or NOTCH2, respectively, results in a trafficking defect, suggesting that the presence of the O-fucose monosaccharide is particularly important for folding of EGF9 from either Notch, adding support to this hypothesis.

The presence of four Notch receptors, four canonical ligands, and three Fringes in mammalian systems already reveals the tremendous diversity of the Notch signaling pathway. The
variety of phenotypes upon elimination of any of these components (or mutations in human diseases) demonstrates that elimination of some components has effects in multiple systems, whereas elimination of others causes more specific effects. The data we present here show that O-fucosylation of the Notch ECD provides an additional layer of diversity in regulation of Notch signaling, at least in the cell systems evaluated. Further work needs to be done to confirm these results in vivo. It will be interesting to see how other forms of O-glycosylation on Notch ECD (O-glucose (18) and O-GlcNAc (19)) contribute to the amazing fine tuning of this crucial pathway.

**Experimental procedures**

**Antibodies and protein reagents**

*Primary antibodies*—Primary antibodies were as follows: sheep polyclonal mouse Notch-1 antibody (R&D Systems, AF5267), anti-c-Myc antibody (9E10, Stony Brook University Cell Culture/Hybridoma Facility), anti-FLAG antibody (M2), Sigma (F3165), PE-conjugated anti-mouse NOTCH1 antibody (BioLegend, catalog no. 130607), and PE-conjugated anti-mouse NOTCH2 antibody (BioLegend, catalog no. 130707).

*Secondary antibodies*—Secondary antibodies were as follows: FITC-conjugated anti-sheep IgG (Invitrogen 818611), PE-conjugated goat anti-mouse IgG (Invitrogen P-852); PE-conjugated anti-human IgG (Jackson ImmunoResearch 109115-098), and PE-conjugated anti-Myc (R&D Systems, catalog no. IC3696P).

**Recombinant soluble notch ligands generously supplied by R&D systems**

The following recombinant soluble notch ligands were generously supplied by R&D Systems: human Dll1-His (1818-DL-050), mouse DLL1-Fc (5026-DL-050), human DLL4-His (1506-D4-050), mouse DLL4-His (1389-D4-050), mouse DLL4-Fc (10089-D4-050), human JAG1-Fc (1277-JG-050), rat JAG1-His (9907-JG-050), rat JAG1-Fc (599-JG-100), human JAG2-Fc (1726-JG-050), mouse JAG2-Fc (4748-JD-050), human JAG2-Fc (10089-JG-050), human JAG1-Fc (1277-JG-050), rat JAG1-His (9907-JG-050), rat JAG1-Fc (599-JG-100), human JAG2-Fc (1726-JG-050), mouse JAG2-Fc (4748-JD-050), human DLL3-His (9749-DL-050).

**Plasmids**

The plasmid encoding full-length mouse NOTCH2 (pTracer-NOTCH2-FLAG) was generously provided by Dr. Shigeru Chiba (Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo) (54). The plasmid encoding full-length mouse DLL1, pTracer-DLL1-FLAG, was generously provided by Dr. Achim Gossler (55), and plasmid encoding full-length mouse JAG1, pTracer-JAG-V5, was generously provided by Dr. Hamed Jafar-Nejad (56). Fringe-expressing plasmids SEAP (EV), LFNG-AP, MFNG-AP, and RFNG-AP were described previously (40). The TP1-1 luciferase reporter construct (Ga981-6) was a gift from Dr. Georg Bornkamm (Munich, Germany), and the gWIZ B-galactosidase construct was from Gene Therapy Systems. A plasmid expressing GFP (pEGFP-N1) was from Clontech. Note that “N1” in this plasmid name refers to a NotI restriction site following the GFP coding region. pEFIREs-p-mTagBFP-KDEL was from Addgene.

To subclone full-length mouse NOTCH1 into pTracer to generate pTracer-NOTCH1-FLAG, we used the TAKARA In-Fusion® HD Cloning Plus system. For amplification of full-length mouse NOTCH1, we used pcDNA1-NOTCH1-Myc as template (57) with the following primers: 5’-AGTGGTGTGGAATTCCAGCAGGCAATGCCAGGGCT-3’ and 5’-GTCGAGGGCTGATCAGCACACACTCTGTATTATAATTATG-3’. For amplification of pTracer-CMV (Invitrogen), we used the following primers: 5’-CTGATACGCCTGACTGTG-3’ and 5’-GAAATTTCCACACACTGGACATG-3’. After subcloning, we mutated the mouse NOTCH1 stop codon into Ser (TAA – TCA) and added a FLAG tag followed by a stop codon using the primers 5’-GACTCAAAAGACGATGCAGATTAAATGAGAGATGATGTCG-3’ (italized and underlined sequence encodes the FLAG tag) and 5’-TGGTTTAATGCCTTCGGAAT-3’ (underlined sequence is the mutated site). Successful insertion of mouse NOTCH1 and FLAG tag was confirmed by direct DNA sequencing.

Plasmids encoding mouse NOTCH2 fragments (EGF1–36 (amino acids 23–1421 based on UniProt O35516), EGF1–6 (amino acids 23–261), EGF7–12 (amino acids 258–494), EGF13–18 (amino acids 492–723), EGF19–24 (amino acids 718–952), EGF25–30 (amino acids 947–1190), and EGF31–36 (amino acids 1185–1421)) were created by amplifying the sequences encoding the indicated amino acids using pTracer-NOTCH2-FLAG as template and subcloning the products between the HindIII and XhoI sites in pSecTag2C (Invitrogen), in frame with the N-terminal Igκ signal peptide and C-terminal Myc-His6 tag. All pSecTag-NOTCH2 EGF fragment plasmids were confirmed by DNA sequencing.

**Mutagenesis of O-fucosylation sites in pTracer-NOTCH2-FLAG or pSecTag-EGF7–12**

Site-directed mutagenesis of O-fucosylation sites in EGF repeats of the pTracer-NOTCH2-FLAG or pSecTag-EGF7–12 plasmid was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s protocol. Mutations were designed to change the modified threonine within the O-fucosylation consensus sequence (C²GXXX(S/T)C³) to valine. A list of primers used for mutagenesis is shown in *Table S2*. All mutants were confirmed via DNA sequencing.

**Cell culture**

HEK293T, NIH3T3 (NIH3T3 CRL-1658), and CHO (CHO K-1) cells were obtained from the American Type Culture Collection (Manassas, VA), and L cells stably expressing JAG1 were a kind gift of Dr. Gerry Weinmaster (UCLA). HEK293T, NIH3T3, and L cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen), and CHO cells were grown in minimal essential medium α (Invitrogen), both supplemented with 10% bovine calf serum at 37°C in a humidified incubator at 5% CO₂.

**Ligand-coating Notch signaling assay (for Fig. 1B and Fig. S1)**

CHO cells (2.0 × 10⁴) were seeded in a 96-well culture plate previously coated with the indicated concentrations of Notch ligand at RT for 2 h. After 16–18 h, the cells were co-transfected
with 0.02 μg of pTracer with or without a full-length Notch receptor (empty vector (EV), pTracer-NOTCH1-FLAG, or pTracer-NOTCH2-FLAG), 0.03 μg of TP1-1 luciferase reporter construct, and 0.02 μg of gWIZ β-gal construct for transfection efficiency normalization using 0.4 μl of 1 mg/ml polyethylenimine (PEI) (22) as a transfection reagent for each well. After 4 h, the medium was changed to growth medium for another 24 h. Cells were lysed, and luciferase and β-gal assays were performed based on the manufacturer’s instructions (Luciferase Assay System, Promega) as described previously (22). Relative luciferase units (RLU) were determined by dividing luciferase activity by β-gal activity.

**Ligand-coating Notch signaling assay with Fringes (for Figs. 2, 4D, and 5)**

NIH3T3 cells (1 × 10^4) were seeded in a 96-well tissue culture plate previously coated with 2 μg/ml DLL1-Fc or JAG1-Fc at RT for 2 h. After 16–18 h, the cells were co-transfected with 0.03 μg of pTracer-NOTCH1 or pTracer-NOTCH2, 0.015 μg of Fringe plasmid (SEAP (EV), LFN-G-AP (LFNG), MFNG-AP (MFNG), and RFNG-AP (RFNG)), 0.03 μg of TP1-1 luciferase reporter construct, and 0.02 μg of gWIZ β-gal construct for transfection efficiency normalization using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. For different Fringe/Notch ratio experiments (Fig. 2, C and D), total amount of transfected Fringe plasmid was normalized by SEAP (EV) plasmid. After 4 h, the medium was changed to growth medium for another 24 h. Cells were lysed, and luciferase and β-gal assays were as described above. RLU were determined by dividing luciferase activity by β-gal activity.

**Notch-expressing cell-soluble ligand-binding assay (for Figs. 1C, 4A, and 6 (A and B))**

HEK293T cells (8.5 × 10^5) were seeded in a 3.5-cm culture plate, and after 16–18 h, the cells were co-transfected with 1.5 μg of pTracer-DLL1-FLAG or pTracer-JAG1-V5 and 0.4 μg of pEGFP-N1. PEI was used as a transfection reagent at a ratio of 1 μg of DNA/6 μl of PEI mixed with 100 μl of Opti-MEM (Invitrogen) at RT for 15 min. At 28–30 h post-transfection, the cells were dissociated with cold PBS, pH 7.4, containing 1% BSA and resuspended in binding buffer (1 mM CaCl2, 1% BSA, and 0.05% NaN3 in Hanks’ balanced salt solution, pH 7.4 (Gibco)). Purified NOTCH2 fragments (16.5 nm for DLL1 cells and 66 nm for JAG1 cells in Figs. 4B and 6C or different concentrations as indicated in Figs. 4C and 6D) were preincubated with 10 μl of PE-conjugated anti-Myc antibody in 100 μl of binding buffer at 4°C for 30 min and added to cells (0.5–1 × 10^6). After incubating at 4°C for 1 h, the cells were washed with binding buffer and analyzed by flow cytometry as described above for the Notch-expressing cell-soluble ligand binding assay.

**Western blotting and immunofluorescence microscopy**

Western blotting for NOTCH1 and NOTCH2 were performed as described (22). For immunofluorescent localization studies, 0.5 μg of WT NOTCH1 or EGF9A NOTCH1 expression plasmid along with 0.5 μg of ER maker plasmid (pEFIRESP-mTagBFP-KDEL) were transfected into HEK293T cells onto coverslips in a 3.5-cm plate using Lipofectamine 2000 according to the manufacturer’s instructions. After 24 h, cells were fixed with 4% paraformaldehyde in PBS buffer for 10 min at RT, and permeabilized with cold methanol for 5 min at −20°C. Cells were blocked for 30 min at RT with PBS containing 3% BSA. To detect NOTCH1, cells were incubated with anti-NOTCH1 antibody (10 μg/ml) for 1 h at room temperature and washed three times with PBS, incubated with FITC-conjugated rabbit anti-sheep secondary antibody (1:100) for 30 min at RT, and washed again with PBS. Cells stained on coverslips were mounted onto microscope slides (Vector H-1000) and then imaged on a Zeiss 510 Meta NLO confocal microscope.

**Statistical analysis**

Statistical significance of the Fringe effect (−Fng versus +Fng for each sample) was determined using one-way ANOVA. Significance levels are indicated in the legends of the relevant figures.
**Data availability**

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (58) partner repository with the dataset identifier PXD020431.

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**Author contributions**—S. K. and R. K. L. data curation; S. K. and R. S. H. formal analysis; S. K., R. K. L., and A. I. investigation; S. K. and A. I. methodology; S. K. writing-original draft; S. K., R. K. L., and A. I. review and editing; A. I. and R. S. H. resources; R. S. H. conceptualization; R. S. H. supervision; R. S. H. funding acquisition; R. S. H. project administration.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: DLL, Delta-like ligand; JAG, Jagged; LFNG, Lunatic fringe; MFI, mean fluorescence intensity; MFNG, Manic fringe; POFUT1, protein O-fucosyltransferase 1; RFNG, Radical fringe; EGF, epidermal growth factor–like; ECD, extracellular domain; ER, endoplasmic reticulum; PE, phycocerythrin; RT, room temperature; EV, empty vector; RLU, relative luciferase units; ANOVA, analysis of variance.

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