Glycosylation of Specific Notch EGF Repeats by O-Fut1 and Fringe Regulates Notch Signaling in Drosophila

Graphical Abstract

Highlights

- Fringe regulates fly Notch signaling by adding GlcNAc to O-fucose on Notch EGF8, -9, and -12

- O-Fucose monosaccharide on Notch EGF12 is essential for fly embryonic neurogenesis

- Fringe-modified EGF8, -9, and -12 promote Delta-Notch signaling in wing vein formation

- Fringe-modified EGF8 and -12 prevent cis-inhibition of Serrate by Notch in vivo

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In Brief

POFUT1/O-Fut1 and Fringe glycosyltransferases regulate Notch signaling by adding fucose and GlcNAc, respectively, to Notch EGF repeats. Using in vitro and in vivo experiments, Pandey et al. define the critical target sites of these enzymes on Drosophila Notch and determine the distinct roles of each sugar in Notch-dependent processes.
Glycosylation of Specific Notch EGF Repeats by O-Fut1 and Fringe Regulates Notch Signaling in Drosophila

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SUMMARY

Fringe glycosyltransferases differentially modulate the binding of Notch receptors to Delta/DLL versus Serrate/Jagged ligands by adding GlcNAc to O-linked fucose on Notch epidermal growth factor-like (EGF) repeats. Although Notch has 22 O-fucosylation sites, the biologically relevant sites affecting Notch activity during animal development in vivo in the presence or absence of Fringe are not known. Using a variety of assays, we find important roles in Drosophila Notch signaling for GlcNAc-fucose-O glycans on three sites: EGF8, EGF9, and EGF12. O-Fucose monosaccharide on EGF12 (in the absence of Fringe) is essential for Delta-mediated lateral inhibition in embryos. However, wing vein development depends on the addition of GlcNAc to EGF8 and EGF12 by Fringe, with a minor contribution from EGF9. Fringe modifications of EGF8 and EGF12 together prevent Notch from cis-inhibiting Serrate, thereby promoting normal wing margin formation. Our work shows the combinatorial and context-dependent roles of GlcNAc-fucose-O glycans on these sites in Drosophila Notch-ligand interactions.

INTRODUCTION

Notch signaling is one of the evolutionary conserved signaling pathways required for development and tissue homeostasis in metazoans (Artavanis-Tsakonas and Muskavitch, 2010). Notch receptors and their canonical ligands from Delta (D)/DLL and Serrate (Ser)/Jagged (JAG) families are transmembrane proteins with multiple epidermal growth factor-like (EGF) repeats in their extracellular domains (Rebay et al., 1991). The interaction of Notch receptors with ligands from adjacent cells activates Notch signaling, or trans-activation (Bray, 2006). In addition, the interaction between Notch receptors and ligands from the same cell usually (but not always; Nandagopal et al., 2019) results in the inhibition of signaling, and these so-called cis-inhibitory interactions affect the outcome of Notch signaling in some contexts in vivo (Baek et al., 2018; Cordes et al., 2004; de Celis and Bray, 1997; Doherty et al., 1996; Geffers et al., 2007; Henrique et al., 1997; Jacobsen et al., 1998; Klein et al., 1997; Micchelli et al., 1997; Sprinzak et al., 2010). Given the broad roles that Notch signaling plays in animal development and human disease, understanding the molecular mechanisms that modulate the activity of this pathway in various contexts is of great interest (Masek and Andersson, 2017; Siebel and Lendahl, 2017).

Most EGF repeats of Notch receptors harbor glycosylation sites for one or more forms of O-linked glycans, which play multiple roles in Notch signaling by affecting the folding, trafficking, ligand binding, and/or cleavage of Notch receptors (Haltom and Jafar-Nejad, 2015; Stanley and Okajima, 2010; Takeuchi and Haltiwanger, 2014). One of the major forms of Notch O-glycosylation is O-fucosylation, which is the addition of an O-fucose (O-fuc) residue onto Notch EGF repeats by the enzyme protein O-fucosyltransferase 1 (POFUT1; O-Fut1 in flies) (Moloney et al., 2000b; Okajima and Irvine, 2002; Wang et al., 2001; Wang and Spellman, 1998). O-Fucosylation takes place between the second and third conserved cysteine residues on a serine or threonine in EGF repeats with a C2X4–5(S/T)C3 consensus sequence (Shao et al., 2003). The loss of O-fucosylation results in embryonic lethality and phenotypes resembling the loss of Notch signaling in flies and mammals, demonstrating that O-fucosylation is critical for Notch signaling (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003).

An N-acetylgalactosamine (GlcNAc) residue can be added to O-fuc on some EGF repeats by β1,3N-acetylgalactosaminyltransferase enzymes of the Fringe (Fng) family (Brückner et al., 2000; Harvey et al., 2016; Kakuda and Haltiwanger, 2017; Moloney et al., 2000a). Drosophila has a single Fng protein encoded by fng, while mammals have three homologs: Lunatic Fng (LFNG),
Manic Fng (MFNG), and Radical Fng (RFNG) (Cohen et al., 1997; Irvine and Wieschaus, 1994; Johnston et al., 1997). The loss of fng in flies and of Lfng in mice recapitulate some but not all of the phenotypes associated with the loss of Notch signaling (Correia et al., 2003; Evrard et al., 1998; Irvine and Wieschaus, 1994; Zhang and Gridley, 1998). The enzymatic activity of Fng proteins on Notch receptors differentially regulates the activation of Notch by DI/DLL versus Ser/JAG ligands (Brückner et al., 2000; Hicks et al., 2000; Moloney et al., 2000a; Panin et al., 1997). GlcNAcylation of the fly Notch by Fng enhances DI-Notch binding and decreases Ser-Notch binding (Brückner et al., 2000; Xu et al., 2007). Therefore, GlcNAc residues added to one or more of the many Notch EGF repeats that have an O-fucosylation site must mediate the effects of Fng proteins on Notch signaling.

Biochemical and cell-based assays have made important progress toward the identification of those Notch EGF repeats whose glycosylation by PO Fut1 and Fng proteins can modulate Notch binding and activation by DI/DLL and Ser/JAG ligands (Kakuda and Haltiwanger, 2017; Xu et al., 2005). However, the use of soluble forms of Notch and ligands in many such assays does not provide sufficient information to distinguish the effects of a given carbohydrate residue on trans- versus cis-interaction of Notch with ligands. Moreover, the overexpression of full-length Notch, ligand, and Fng proteins in cell culture assays may mask the effects of glycosylation on signaling at the endogenous levels of these proteins. Based on transgenic overexpression of the full-length fly Notch with mutations in individual O-fucosylation sites, a previous study concluded that Notch with an O-fuc site mutation in EGF12 is able to restore the DI-mediated embryonic neurogenesis in Notch null embryos (Lei et al., 2003). However, it remains to be seen whether this is the case when Notch is expressed at endogenous levels and whether this glycan acts together with O-fuc monosaccharides on other Notch EGF repeats. A knockin allele of the mouse Notch1 with an EGF12 O-fuc site mutation (Notch1[12f]) behaves as a hypomorphic allele (Ge and Stanley, 2008). Analysis of Notch1[12f] murine thymocytes indicated that the O-fuc glycan on EGF12 is required for optimal DLL-mediated activation of NOTCH1 in these cells. However, whether this effect is mediated by GlcNAc, fuc, or both is not known.

Here, we report our studies on the contribution of Fng-modified O-fuc glycans on specific Notch EGF repeats to Notch signaling and the Fng effect in Drosophila. Quantitative and saturable in vitro binding assays indicated a potential role for GlcNAcylation of O-fuc on Notch EGF8, EGF9, and EGF12 in mediating the effects of Fng on Notch-ligand binding. In vivo structure function and genetic interaction experiments, combined with cell-based aggregation assays, revealed both distinct and redundant roles for individual GlcNAc and/or fuc residues on EGF8, EGF9, and EGF12 in regulating the cis- and trans-interactions of Notch with DI and Ser ligands. Our data determine the functional importance of specific carbohydrate residues attached to Notch in regulating distinct Notch-ligand interaction modalities and establish the Notch EGF repeats that mediate the effects of Fng in fly Notch signaling.

RESULTS

A Modified Notch-Ligand Binding Assay Recapitulates Fng In Vivo Effects and Reveals Differential Mechanistic Effects on Notch Binding to DI and Ser

To investigate the mechanisms of how Fng modulates Notch-ligand binding, we modified a mammalian cell-based binding assay so that it could be used for Drosophila S2 cells (Kakuda and Haltiwanger, 2017). To simply and directly examine ligand binding, we used a Notch-CD2 (N-CD2) hybrid receptor that expresses EGF repeats 1–36 from the extracellular domain of fly Notch fused with the CD2 transmembrane protein (Figures 1A and S1A) (Brückner et al., 2000; Yamamoto et al., 2012). Ligand proteins used for binding assays were purified from the media of S2 cell lines stably expressing the extracellular domains of DI and Ser with C-terminal Myc-6xHis tags (Figures 1A and S1B). Before testing ligand binding, we confirmed the cell surface expression of N-CD2 using flow cytometry and also observed that it was not affected by Fng co-expression (Figure S1C). For these assays, it is advantageous that S2 cells do not exhibit endogenous Fng activity (Moloney et al., 2000a), and mass spectrometry on Notch isolated from S2 cells did not detect GlcNAc on O-fucosylated EGF repeats (Harvey et al., 2016). We then tested the increasing ratios of Fng co-transfected with N-CD2 into S2 cells incubated with ligand to examine the effect of Fng expression on the binding of N-CD2 to DI and Ser. We observed that increasing Fng co-expression increases DI binding (Figures 1B and S1D) but decreases Ser binding (Figures 1C and S1E). In both cases, the Fng effects were observed at very low Fringe:Notch ratios. To confirm that the effects on ligand binding in our assays were in fact due to Fng glycosyltransferase activity, we mutated the DXD motif to generate an enzymatically inactive Fng (DEE Fringe) (Moloney et al., 2000a). Western blots of transfected cell lysates showed that the enzymatically inactive Fng is expressed at levels similar to those of wild-type (WT) Fng (Figure S1F). We observed that while co-expression of N-CD2 with Fng increased DI binding and decreased Ser binding, co-expression with DEE Fng showed binding similar to that in the absence of Fng, demonstrating that Fng glycosyltransferase activity on N-CD2 specifically affects ligand binding (Figure S1G).

To examine the differential effects of Fng on Notch-ligand binding in more detail, we analyzed the concentration dependence of ligand binding. DI bound minimally to N-CD2 cells in the absence of Fng, with only moderate increases in binding upon increased DI concentrations (Figure 1D, –Fringe). The Notch-DI binding did not reach saturation in the absence of Fng and therefore precluded the estimation of either Bmax or Kd. However, Fng greatly increased DI binding, such that DI binding was saturable, which allowed for the determination of estimated Bmax and Kd values (Figure 1D, +Fringe). These data suggest that Fng dramatically decreases the Kd for Notch binding to DI. In contrast, Ser binding to N-CD2 was decreased in the presence of Fng compared to the absence of Fng (Figure 1E). Ser binding was also saturable. We found that Fng reduced both the Bmax and the Kd of Notch binding to Ser (Figure 1E). These observations indicate that Fng mostly
affects the affinity (Kd) of Notch for DI, while it alters both the number of binding sites (Bmax) and the affinity between Ser and Notch. These data reveal distinct mechanisms for how Fng mediates its differential effects on Notch interactions with DI and Ser ligands.

**Cell-Based Assays Suggest That O-Fucosylation at Notch EGF8, EGF9, and EGF12 Are Important for Fng to Modulate Notch-Ligand Binding**

To gain further insight into the roles of specific Fng-elongated O-fuc sites of Notch on modulating ligand binding, we performed site-directed mutagenesis of 19 of the 22 O-fuc sites of fly Notch. To abolish O-fucosylation without dramatically changing the amino acid size, we introduced threonine-to-valine (T-to-V) and serine-to-alanine (S-to-A) mutations and named the mutant EGF repeats accordingly (e.g., EGF8V, EGF9V, EGF12A). Mutants of EGF2, EGF31, and EGF32 O-fuc sites were not generated since they are not efficiently modified by Fng (Figure 1A; Harvey et al., 2016). Although O-fucosylation on EGF13 and EGF28 was also poorly modified by Fng (Harvey et al., 2016), these sites were mutated due to their proximity to the ligand-binding domain (Rebay et al., 1991) and location within the Abruptex domain (Kelley et al., 1987), respectively (Figure 1A). We performed flow cytometry and western blotting to show that each N-CD2 O-fuc site mutant was expressed on the cell surface and at expected molecular weights (Figures S2A and S2B). We then analyzed the binding of each N-CD2 O-fuc site mutant to DI and Ser in the presence and absence of Fng (Figures S2C and S2D; Table S2). Mutation in O-fuc sites of EGF8, EGF9, and EGF12 mediated distinct effects on DI and Ser binding (Figure 2). These O-fuc site mutants were tested in binding assays with varied levels of Fng expression (Figure 2A). Mutating the O-fuc site on EGF9 slightly reduced Fng-dependent DI binding, while mutating the O-fuc sites at EGF8 and EGF12 had larger effects (Figure 2A). N-CD2 with mutations in both EGF8 and EGF9 (8V9V) showed an additive effect, and N-CD2 with mutations in both EGF8 and EGF12 (8V12A) was completely unable to bind DI with any amount of Fng (Figure 2A). Although the 8V, 8V9V, and 8V12A mutants showed slightly reduced cell surface expression (Figure S2A), this was not sufficient to account for the effect seen on ligand binding. Additional binding assays at a single Fringe:Notch ratio corroborated these results (Figures 2B and S2C). These data suggest that Fng modification of O-fuc on EGF8, EGF9, and EGF12 contributes to DI binding, and mutation of O-fuc on EGF8 and EGF12 is critical for Fng enhancement of Notch-DI binding.

Different effects on Notch-Ser binding were observed upon using varying levels of Fringe:Notch ratios with the EGF8, EGF9, and EGF12 O-fuc site mutants (Figure 2C). Most striking
was the effect of the EGF12 mutant, which showed enhanced Ser binding in the absence of Fng, suggesting an inhibitory role of O-fuc at this site (Figures 2C and 2D). The mutations at EGF8 and EGF9 had reduced Ser binding in the absence of Fng, suggesting that O-fuc at these sites contributes to Ser interactions with Notch, and mutation of both EGF8 and EGF9 (8V9V) almost eliminated binding (Figures 2C and 2D). Fng co-expression with the EGF9 and EGF12 mutants still reduced Ser binding, suggesting that O-fuc on EGF9 and EGF12 is not essential for the Fng effects on Ser (Figures 2C and 2D). All of the mutants containing an EGF8 O-fuc site mutation (8V, 8V9V, 8V12A) showed no significant reduction in Ser binding with Fng co-expression, suggesting that Fng modification of the O-fuc on EGF8 plays a major role in inhibiting Ser binding (Figures 2C, 2D, and S2D). These results reveal distinct roles of the O-fuc modifications on EGF8, EGF9, and EGF12 for Fng to modulate Notch binding to DI and Ser.

**O-Fucosylation Site Mutations in Individual Notch EGF Repeats or Their Pairwise Combinations Do Not Affect the Notch Cell Surface Levels in Drosophila**

Based on our cell-based data, we sought to obtain an in vivo functional assessment of the GlcNAc-fuc-O glycans on EGF8, EGF9, and EGF12 of fly Notch. We used a previously established gap-repair mutagenesis strategy (Leonardi et al., 2011; Leonardi and Jafar-Nejad, 2014) to generate Notch genomic transgenes with T-to-V or S-to-A mutations that prevent O-fucosylation in EGF8, EGF9, or EGF12 [N[gt-8V], N[gt-9V], and N[gt-12A], respectively] and in their pairwise combinations (N[gt-8V9V], N [gt-8V12A], and N[gt-9V12A]). Notch EGF5 shows a high level of O-fucosylation and GlcNAcylation (Figure 1A; Harvey et al., 2016) but does not show altered cell surface levels or ligand binding in vitro upon the loss of the GlcNAc-fuc-O glycan (Figure S2). Therefore, we also generated N[gt-5V] as a control for the in vivo studies.

Our S2 cell-based assays indicate that single O-fuc site mutations do not dramatically impair the surface expression of the Notch-CD2 hybrid protein (Figures S2A and S2B). However, the T-to-V and/or S-to-A mutations may affect the folding and cell surface trafficking of the full-length Notch in fly tissues independently of its glycosylation. Therefore, before performing functional studies, we examined the cell surface levels of mutant Notch proteins in vivo, as reported previously (Leonardi et al., 2011). Since N[54I9] is a protein null allele, N[54I9] homozygous clones do not show Notch extracellular domain (NECD) staining (Figures S3A–A”; Leonardi et al., 2011). Comparison of the relative signal intensities revealed no significant change in the cell surface level of any of the examined mutant Notch proteins compared to the WT Notch from N[gt-wt] (Figures S3B–S3J). We conclude that the loss of O-fucosylation from these Notch EGF repeats does not affect the cell surface level of Notch in vivo.

**Loss of O-Fucosylation of Notch EGF12 Impairs DI-Notch Signaling during Drosophila Embryonic Neurogenesis**

Having assessed the Notch cell surface level upon mutating the Fng-modified O-fucosylation sites on the selected Notch EGF repeats, we examined their effect on Notch signaling in vivo. Fly Notch is an X-linked gene, and hemizygous males of Notch null alleles show embryonic lethality (Artavanis-Tsakonas et al., 1983). The lethality of hemizygous males (N[–]/Y) can be rescued
by the addition of one copy of the WT $N[gt-wt]$ (Figure 3A) (Leonardi et al., 2011). To examine whether mutation in the O-fucosylation sites of the selected Notch EGF repeats (8V, 9V, 12A, 8V9V, 8V12A, and 9V12A) can rescue $N[-]/Y$ lethality, we compared the survival of $N[-]/Y$ males harboring one copy of mutant Notch transgenes with those harboring one copy of $N[gt-wt]$. The
addition of one copy of N[gt-5V] and N[gt-8V] fully rescued the lethality of N[–]/Y males (Figure 3A). However, N[gt-9V] and N[–]/Y showed a partial rescue compared to control animals (Figure 3A; 77% and 73%, respectively). Moreover, N[gt-12A], N[–]/Y, and N[gt-9V12A] did not rescue the N[–]/Y’s male lethality at all (Figure 3A). These data suggest an essential role for the O-fuc monosaccharide modification on Notch EGF12 and a minor but significant role for the O-fuc monosaccharide modification on EGF9 during fly development.

N[–]/Y embryos show a fully penetrant neurogenic phenotype (Figures 3C and C’) due to impaired lateral inhibition, which is mediated by DI-Notch signaling during Drosophila neurogenesis (Heitzler and Simpson, 1991). Of note, Notch signaling does not depend on fng at this stage, as fng mutant animals do not exhibit a neurogenic phenotype (Haines and Irvine, 2003). We examined the ability of these transgenes to rescue the N[–]/Y neurogenic phenotype in embryos by performing ELAV staining, which marks a neuron-specific nuclear protein (Robinow and White, 1991). A full rescue of the neurogenic phenotype in N[–]/Y male embryos was observed upon the addition of a copy of N[gt-wt], N[gt-5V], or N[gt-8V] (Figures 3D–3F’). The addition of one copy of N[gt-9V] or N[gt-8V9V] showed a partial rescue of the neurogenic phenotype, in agreement with the partial rescue of N[–]/Y lethality at all (Figure 3A). These data suggest an essential role for the O-fuc monosaccharide modification on Notch EGF12 and a minor but significant role for the O-fuc monosaccharide modification on EGF9 during fly development.

Fng Modification of EGF8 and EGF12 Plays a Major Role in DI-Notch Signaling during Wing Vein Formation

DI-Notch signaling in Drosophila embryonic neurogenesis is independent of Fng (Haines and Irvine, 2003; Ishio et al., 2015). Therefore, to examine the role of GlcNAc added to O-fucosylated Notch EGF repeats by Fng, we analyzed the O-fuc mutant Notch transgenes in two additional contexts in which Fng plays important roles: wing vein development and wing margin development (Correia et al., 2003). Fly wing vein development is primarily regulated by DI-Notch signaling (de Celis et al., 1997; de Celis and Bray, 2000; Hupert et al., 1997), with a major contribution from Ser-Notch signaling, which is only revealed when both ligands are mutated (Zeng et al., 1998). Adult wings from Notch heterozygous females (Nfng+/–) show a fully penetrant wing vein-thickening phenotype (Figures 4A and 4J). The addition of one copy of N[gt-wt] rescues this phenotype (Figures 4B and 4J; Leonardi et al., 2011). Similarly, the addition of one copy of N[gt-5V] or N[gt-8V] fully rescued this phenotype (Figures 4C, 4D, and 4J). The addition of one copy of N[gt-9V], N[gt-12A], or N[gt-9V12A] partially rescued the phenotype in all Nfng+/– flies (Figures 4E, 4F, 4I, and 4J), suggesting that the Fng modification of O-fuc on EGF9 and EGF12 contributes to DI-mediated Notch signaling during normal wing vein development. In contrast, the addition of one copy of N[gt-8V9V] only showed a partial rescue in 22% of the animals and no rescue in the remaining 78% (Figures 4G and 4J). N[gt-9V12A] did not rescue the wing vein-thickening phenotype in any of the Nfng+/– flies (Figures 4H and 4J). These data are in agreement with our in vitro assays (Figure 2B) and indicate that GlcNAc-fuc-O on EGF8, EGF9, and EGF12 contribute to the modulation of DI-Notch signaling by Fng during wing vein development. The data also suggest that the glycans on EGF8 and EGF12 are more important than the glycan on EGF9 for this process.

Thus far, our data indicate that the impact of EGF12 O-fuc site mutation on embryonic neurogenesis (–Fringe; Figure 3) is dramatically different from its impact on wing vein development (+Fringe; Figure 4). To test whether Fng modification of EGF8 and EGF9 underlies this difference, we examined whether modulating the fng gene dosage affects the ability of N[gt12A], N[gt-8V12A], or N[gt-9V12A] transgenes to rescue the wing vein-thickening phenotype in Nfng+/– flies. As shown in Figure 4J, adding one genomic copy of fng improved the ability of all three transgenes in rescuing the Nfng+/– wing vein phenotype. Moreover, removing one copy of fng decreased the functionality of N[gt12A] and N[gt-9V12A] transgenes in this assay (Figure 4J). Note that N[gt-8V12A] did not show any rescue of this phenotype in an fng+/+ or fng+/– background (Figure 4J). Adding or removing one copy of fng in a WT background does not have any wing vein phenotypes by itself (Figure 4J). These observations support the notion that the addition of GlcNAc to EGF8 and EGF9 partially masks the effects of the loss of the O-fucose glycan from EGF12.

We next decided to more directly assess the contribution of each sugar (unmodified O-fuc versus GlcNAc-extended O-fuc) on these EGF repeats to DI-Notch interactions. We could not accurately measure DI-Notch binding in the absence of Fng using our flow cytometric binding assays because the level of soluble DI-Myc-6xHis binding was too low and saturation was
Figure 4. Wing Vein Thickening and Wing Margin Loss Phenotypes of N[+/-] Flies in the Absence or Presence of Wild-Type and Mutant Notch Genomic Transgenes

All of the animals were raised at 25°C.

(A) A wing from an N[+/-] fly showing wing vein thickening (red asterisk) and wing margin loss (red arrowhead) phenotypes. Scale bar, 0.5 mm (applies to all of the panels).

(B–I) N[+/-] wings with one copy of wild-type (B) or mutant (C–I) Notch genomic transgenes, as shown in each panel.

(J) Percentages of each class of wing vein-thickening phenotype (no rescue, partial rescue, and full rescue) in (A)–(I) and upon removing (1X) or adding (3X) one copy of fng

PBac[CH321-87O20]VK37 and fngL73 strains, respectively, were used.
Therefore, we used a well-established S2 cell-based aggregation assay to reveal the effect of O-fuc site mutations in the above-mentioned EGF repeats on Notch-ligand binding in the presence and absence of Fng (Fehon et al., 1990; Lee et al., 2017; Pandey and Jafar-Nejad, 2018).

We performed aggregation assays between stable S2-DI cells and S2 cells transiently transfected with inducible plasmids expressing full-length WT Notch (S2-N-WT) or mutated Notch (S2-N-12A, S2-N-8V, or S2-N-8V12A), with or without co-transfection of an Fng-expression construct. Untransfected S2 cells, which do not express Notch and DI (Fehon et al., 1990; Lee et al., 2017), were used as experimental controls. As reported previously, mixing S2-DI cells with S2 cells transfected with N-WT resulted in the formation of a number of aggregates (Figure 5A; Fehon et al., 1990). In the absence of Fng, we observed a significant decrease (>50%) in the number of aggregates between S2-DI and S2-N-12A compared to controls (Figures 5A and 5B). In contrast, an O-fuc mutation in EGF8 did not affect the aggregation of S2-N-8V cells with S2-DI cells (Figure 5C).

Moreover, S2-N-8V12A cells, which express Notch molecules with EGF8 and EGF12 mutations, showed the same level of decrease in aggregation with S2-DI as the single mutant S2-N-12A cells (Figure 5D). These observations indicate that in the absence of Fng, O-fuc on EGF12 plays a major role in promoting DI-Notch interactions and that O-fuc on EGF8 cannot compensate for the loss of O-fuc from EGF12.

Upon co-expression of Fng, both S2-N-WT and S2-N-12A cells showed a significant increase in the number of aggregates with S2-DI cells (Figures 5A and 5B), suggesting that the addition of GlcNAc to R1 Notch EGF repeats other than EGF12 can at least partially compensate for the decrease in DI-Notch interaction caused by the O-fuc mutation in EGF12. However, Fng overexpression failed to significantly increase the number of aggregates between S2-N-8V12A and S2-DI cells, although a weak trend toward increased aggregation was observed (Figure 5D). Fng overexpression also significantly enhanced the number of aggregates between S2-N-8V and S2-DI cells (Figure 5C). These results provide strong evidence that the Fng-mediated enhancement of DI-Notch-12A interaction is primarily mediated by GlcNAcylation of O-fucosylated EGF8. They are also consistent with our flow cytometric binding assay (Figures 2A and 2B).
the interaction between Ser and trans Notch (30%), we would conclude that the relative aggregation between S2-N and S2-Ser cells (i.e., relative aggregation 30%) of Notch on Ser. For example, if the number of aggregates between S2-N and S2-Ser-N cells transiently transfected with an Ser expression vector alone (S2-Ser) or co-transfected with WT or mutant Notch (S2-Ser-N8V12A). The relative aggregation between S2-N and S2-Ser cells in the presence and absence of cis-Notch was used as an indication of the degree of the cis-inhibitory effect of Notch on Ser. For example, if the number of aggregates between S2-N and S2-Ser cells transiently transfected with an Ser expression vector alone (S2-Ser) or co-transfected with WT or mutant Notch (S2-Ser-N8V12A). The relative aggregation between S2-N and S2-Ser cells transiently transfected with an Ser expression vector alone (S2-Ser) or co-transfected with WT or mutant Notch (S2-Ser-N8V12A). The relative aggregation between S2-N and S2-Ser cells transiently transfected with an Ser expression vector alone (S2-Ser) or co-transfected with WT or mutant Notch (S2-Ser-N8V12A).

**DISCUSSION**

Fng proteins play important roles in Notch signaling by adding GlcNAc to O-fucosylated EGF repeats on Notch receptors (Harvey and Halliwanger, 2018; Varshney and Stanley, 2018). Since Fng proteins differentially regulate the binding and response of Notch to the Dl/Dll versus Ser/Jag ligands, identification of the Notch EGF repeat(s) whose glycosylation mediates the Fng effects can provide important insight into Notch-ligand interactions. For many years, EGF11 and EGF12 were thought to make up the ligand-binding domain of Notch receptors (de Celis et al., 1993; Rebay et al., 1991), although it was suggested that additional EGF repeats may also contribute to ligand binding (Lawrence et al., 2000). More recent biochemical, structural,
mammalian cell culture, and fly genetic studies have provided evidence for a more extended ligand-binding domain spanning EGF8–EGF12 (Figure 1A; Andrawes et al., 2013; Kakuda and Haltiwanger, 2017; Luca et al., 2017; Yamamoto et al., 2012). Using in vitro and in vivo analyses, we report here that GlcNAc-fucose O-glycans on EGF8, EGF9, and EGF12 play important roles in the regulation of fly Notch signaling. While the importance of Fng modifications of O-fuc on EGF8 and EGF12 is consistent with recent work on mouse NOTCH1, we cannot exclude the potential contribution of other EGF repeats to the Fng effect in flies, as recently suggested for mouse NOTCH1 based on studies in a mammalian cell line (Kakuda and Haltiwanger, 2017). Nevertheless, our data demonstrate that glycosylation of the extended ligand-binding domain of Notch by O-Fut1 and Fng plays critical roles in fly Notch signaling.

Previous studies have provided strong evidence that the inhibitory effect of mammalian Fng proteins on JAG1-mediated NOTCH1 signaling is not mediated at the level of JAG1-NOTCH1 binding (Hicks et al., 2000; Kakuda and Haltiwanger, 2017; Taylor et al., 2014; Yang et al., 2005). In fact, so far, biochemical and cell-based assays indicate that Fng modifications enhance NOTCH1– JAG1 interactions (Kakuda and Haltiwanger, 2017; Taylor et al., 2014). This suggests that the negative regulation of JAG1– NOTCH1 signaling by Fng occurs at a step after the initial ligand binding (Kakuda and Haltiwanger, 2017). In contrast, our data indicate that Drosophila Fng modifications affect the binding of Notch with Ser in a manner that is consistent with its effect on signaling, as evidenced by decreased K\text{d} (affinity) and Bmax (number of binding sites) (Figure 1E). The reduction of Bmax suggests that Fng modifications could affect Notch conformation or promote Notch dimerization/multimerization. Regardless of the mechanism, this is in agreement with previous reports that showed that the co-expression of Fng with Notch in S2 cells impairs the binding of Notch to Ser (Oka-jima et al., 2003; Xu et al., 2007; Yamamoto et al., 2012). We conclude that despite the similarity between the in vivo roles of fly and mammalian Fng proteins in the regulation of Notch signaling, some differences are likely to exist in the molecular mechanisms through which Fng proteins regulate Drosophila versus mammalian Notch signaling.

The overexpression of fly Notch with an O-fucosylation site mutation at EGF12 was able to respond to Dl during embryonic neurogenesis and rescued the neurogenic phenotype of a Notch null allele (Lei et al., 2003), suggesting that the glycan decorating this site is dispensable for Dl-mediated Notch signaling. However, we find that when N[12A] is expressed at near-endogenous levels by N[gt-12A], it is not able to rescue the neurogenic phenotype in Notch null embryos (Figure 3). Since Fng does not play a role in embryonic neurogenesis (Haines and Irvine, 2003), this effect is likely to be due to the absence of fuc from EGF12. N[gt-12I], which changes the amino acid but still allows O-fucosylation at EGF12, completely rescues this phenotype (Figures S4D–S4F). We conclude that a single, unmodified fuc monosaccharide on EGF12 of Notch is essential for embryonic neurogenesis in Drosophila at endogenous levels of Notch. Notably, mice homozygous for Notch1[12f], which are equivalent to N[∗]/Y; N[gt-12A]/+, flies in our studies, exhibit impaired T cell development but do not show any abnormalities during embryonic development (Ge and Stanley, 2008). This suggests that the contribution of O-fuc glycans on individual Notch EGF repeats in each in vivo context depends not only on the specific site but also on other factors, including the levels of the Notch receptor and the presence or absence of Fng proteins.
N[gt-12A] showed a significant rescue of wing vein thickening in N[+/+] flies (full rescue in 57% of the wings, partial rescue in the remaining 43%; Figure 4). This is in contrast to the embryonic neurogenesis in N[−]/Y males, in which this mutation showed no rescue of the neurogenic phenotype. Multiple lines of evidence suggest that this difference is due to the Fng modification of EGF8 and also EGF9 to a lesser degree. First, unlike embryonic neurogenesis, Fng is involved in wing vein development (Correia et al., 2003; Ishio et al., 2015). Second, despite the significant decrease in DI binding of N-12A, our aggregation and in vitro assays indicate that Fng is able to robustly increase the binding of N-12A with DI (Figures 2 and 5), in agreement with previous in vitro binding assays (Lei et al., 2003). Third, the simultaneous loss of the O-fucosylation site in EGF8 and to some extent EGF9 decreases the ability of N-12A to rescue the wing vein phenotype of N[+/+] females (Figure 4). Fourth, the ability of N-8V12A to mediate the aggregation of S2 cells with S2-DI cells cannot be enhanced by Fng any longer (Figure 5). Fifth, the wing vein-thickening phenotype of N[+/−]; N[gt-12A], N[+/+]N[−]/Y, N[+/−]; N[gt-8V12A], and N[+/−]; N[gt-9V12A] animals can be modified by altering the fng gene dosage (Figure 4). These data suggest that (1) in the absence of Fng, O-fuc on EGF12 is essential for DI-Notch signaling required for embryonic neurogenesis and cannot be compensated for by O-fuc on other EGF repeats, and (2) in the presence of Fng, GlcNAc on O-fucosylated EGF12 contributes to DI-Notch signaling, but is largely redundant with GlcNAc-fuc-O on EGF8 and partially redundant with that on EGF9. These data are in agreement with the previous observation that the O-fuc modification and GlcNAc elongation of EGF12 and EGF8 together facilitate Fng-mediated increase in DLL1-NOTCH1 signaling in mammalian cell-based assays (Kakuda and Haltiwanger, 2017).

Quantitative assays in mammalian cells and genetic interaction studies in flies indicate that Fng proteins can affect the cis-interactions between Notch and ligands similar to their effect on Notch-ligand trans-interactions (promote Notch-DI/DLL1 cis-interactions, inhibit Notch-Ser/JAG cis-interactions) (LeBon et al., 2014). Moreover, it has been reported that in the fly wing imaginal discs, Notch cis-inhibits Ser and decreases its ability to activate signaling in neighboring cells (Becam et al., 2010). However, it was not known how Fng regulates this process. Our data indicate that upon mutating the O-fuc sites on both EGF8 and EGF12 (but not individually), Notch cis-inhibits Ser and impairs normal wing margin formation. Moreover, cell aggregation assays suggest that mutating these two sites impairs the ability of Fng to block the cis-interaction between Notch and Ser. Based on these data, we propose that during Drosophila wing margin formation, a key role of Fng is to reduce the Notch-mediated cis-inhibition of Ser by adding GlcNAc to O-fucosylated EGF8 and EGF12. It has recently been hypothesized that during inner ear hair cell development, LFNG and MFNG block the cis-inhibition of JAG1 and JAG2 by NOTCH1, thereby allowing these ligands to induce Notch signaling in neighboring cells (Basch et al., 2016). We suggest that a non-cell-autonomous loss of Notch signaling due to impaired ligand cis-inhibition by Notch should be considered as a possibility when interpreting Fringe loss-of-function phenotypes in vivo.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.027.

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AUTHOR CONTRIBUTIONS

A.P., B.M.H., R.S.H., and H.J.-N. designed and conceived the project and interpreted the data. A.P. and B.M.H. performed the experiments. M.F.L. and A.I. contributed to the reagent preparations. A.P., B.M.H., R.S.H., and H.J.-N. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| mouse anti-CD2 (OX-34 clone) (1:100) | Bio-Rad | Product Code MCA154GA; RRID: AB_566608 |
| Mouse anti-Notch (F461.3B) | DSHB | RRID: AB_258409 |
| Rabbit anti-V5 | Sigma Aldrich | RRID: AB_528409 |
| Alexa Fluor680 rabbit anti-mouse | Invitrogen | Cat # A-21065; RRID: AB_2535728 |
| HRP-conjugated goat anti-mouse | Jackson ImmunoResearch Lab | Code 115-035-044; RRID: AB_2338503 |
| PE-conjugated goat anti-mouse | Life Technologies | Cat # P-852; RRID: AB_2539848 |
| PE-conjugated mouse anti-myc (9E10 clone) | R&D systems | Cat # IC3696P |
| IRDye700 goat anti-rabbit | Rockland Immunochemicals | Cat # 611-130-002; RRID: AB_1660969 |
| Goat anti-rat Cy3-conjugated 1:500 | Jackson ImmunoResearch Lab | RRID: AB_2338240 |
| Goat anti-mouse Alexa Fluor-488 | Jackson ImmunoResearch Lab | RRID: AB_2338400 |
| Mouse anti-Notch (C458.2H), 1:100 | DSHB | RRID: AB_258408 |
| Rat-Elav-7E8A10 anti-Elav, 1:200 | DSHB | RRID: AB_258218 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Blasticidin | Invitrogen | Cat# R210-01 |
| methotrexate | Sigma-Aldrich | M9929, CAS: 0133073731 |
| FuGENE HD | Promega | Cat # E2311 |
| Hygromycin B | Invitrogen | Cat# 10687010 |
| **Critical Commercial Assays** |        |            |
| Calcium Phosphate Transfection Kit | Invitrogen | Cat # K2780-01 |
| QuikChange II XL Site-Directed Mutagenesis Kit | Agilent Technologies | Cat # 200522 |
| Effectene Reagent | QIAGEN | Cat # 301425 |
| **Experimental Models: Cell Lines** |        |            |
| S2 cells | Invitrogen | Cat # R69007 |
| S2-Di-Myc-6xHis stable cell line | This study | N/A |
| S2-Ser-Myc-6xHis stable cell line | This study | N/A |
| S2-N stable cell lines | Drosophila Genomics Resource Center (DGRC) | Stock No:154 FBtc0000154 |
| S2-DI stable cell lines | DGRC | Stock No: 152 |
| S2-SerTom stable cells | Fleming et al., 2013 | FBtc0000152 |
| **Experimental Models: Organisms/Strains** |        |            |
| y w | Bloomington Drosophila Stock Center (BDSC) | BDSC6598 |
| w; nuc{Sco}/CyO; TM3, Sb1/TM6, Tb1 | Lee et al., 2013 | N/A |
| y w, N5{Se11}/FM7c, B1 | Leonardi et al., 2011 | N/A |
| y w, N4{FRT19A}/FM7c, B1 | Leonardi et al., 2011 | N/A |
| y w Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFP{Gus}/CyO, y+ | Leonardi et al., 2011 | N/A |
| y w; PBac[Nfr-wt]VK22 | Leonardi et al., 2011 | N/A |
| y w; PBac[Nfr-SV]VK22 | This study | N/A |
| y w; PBac[Nfr-SV]VK22 | This study | N/A |
| y w; PBac[Nfr-SV]VK22 | This study | N/A |
| y w; PBac[Nfr-SV]VK22 | This study | N/A |
| y w; PBac[Nfr-SV]VK22 | This study | N/A |
| y w; PBac[Nfr-SV12A]VK22 | This study | N/A |
| y w; PBac[Nfr-SV12A]VK22 | This study | N/A |

(Continued on next page)
**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hamed Jafar-Nejad (hamedjn@bcm.edu). Constructs and fly lines generated in this study will be shared upon request.

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| y w; PBac[Np-GV12A]VK22 | This study | N/A |
| y w; PBac[Np-GS]VK22 | This study | N/A |
| y w; PBac[Np-12T]VK22 | This study | N/A |
| y w; PBac[Ser9-wt]VK31 | This study | N/A |
| y w; P[Dp[mt]attP2 | LeBon et al., 2014 | N/A |
| fng-T/TM3.Sb1 | Correia et al., 2003 | N/A |
| PBac(CH321-87O20)VK37 | Genetvision | Stock ID#P12-A6 |

### Oligonucleotides

Primers used for Site-directed mutagenesis of N-CD2 and Fringe for binding assays are listed in Supplementary table (Table S1).

Primer pair for generating EGF8TV for cell-based aggregation assay 5' - CGGAGCGCTCTGTAACACACTACGGATCG-3' and 5' - CTGACAGACGGCTCCGATCGCAGCCG-3'.

Primer pair for generating EGF12SA for cell-based aggregation assay 5' - CGAGGGAGCTTGCCTGGATGATCCGG-3 and 5' - CAGGCAAGCTCCCTGTTCTGGCATG-3'.

### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pMT-Notch | DGRC | Stock number 1022 |
| pMT-Ser | Okajima et al., 2003 | Ken Irvine lab |
| pMT-fng | Bruckner et al., 2000 | Cohen lab |
| BAC clone attB-P[acman]-CmR-CH321-69C08 | Venken et al., 2009 | N/A |
| pMT-N-CD2 | Yamamoto et al., 2012 | From Shinya Yamamoto |
| pMT-V5His | Thermo Fisher | Cat# V412020 |
| pMT-Frg-V5His | This study | N/A |
| EGFP-N1 | Clontech | Cat #6085-1 |
| pMT-GFP | This study | N/A |
| pMT-Di-Myc-6xHis | This study | N/A |
| pMT-Ser-Myc-6xHis | This study | N/A |
| pMT-Di-AP | Okajima et al., 2003 | Ken Irvine lab |
| pMT-Ser-AP | Okajima et al., 2003 | Ken Irvine lab |

### Software and Algorithms

| SOFTWARE AND ALGORITHMS | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| FlowJo software | FlowJo | https://www.flowjo.com/solutions/flowjo/downloads |
| Amira 5.2.2 | Thermo Scientific | |
| ImageJ 1.47 | NIH | https://imagej.nih.gov/ij/ |
| Prism 6 | Graphpad | https://graphpad-prism.software.informer.com/6.0/ |
| Adobe Illustrator CS5.1 | Adobe | https://www.adobe.com/products/illustrator.html |
| Adobe Photoshop CS5 | Adobe | https://www.adobe.com/products/photoshop.html |
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Following Drosophila strains were used in the study: (1) y w (Bloomington Drosophila Stock Center); (2) w; nucSco/Cyo; TM3, Sb/C1; TM6, Tb (Lee et al., 2013); (3) y w, N5xe17/FM7c, B, (4) y w N5Sh/FRT19A/FM7c, B, (5) y w Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFP/Cyo, y and (6) PBac(NR-wt)VK22 (Leonardi et al., 2011); (7) PBac(NR-wt)VK22, (8) PBac(NR-RV)VK22, (9) PBac(NR-RV)VK22, (10) PBac(NR-RV)VK22, (11) PBac(NR-RV)VK22, (12) PBac(NR-RV)VK22, (13) PBac(NR-RV)VK22, (14) PBac(NR-RV)VK22, (15) PBac(NR-RV)VK22 and (16) PBac(Ser-RV)VK31 (this study), (15) P(Dmatt)attP2 (LeBon et al., 2014), (16) PBac(CH321-87020)VK37 (fng duplication strain, GenetVision), (17) fng+/−/TM3, Sb/C1 (BDSC#9416, Correia et al., 2003). All crosses in the study were performed on standard media.

METHOD DETAILS

Expression Plasmids Used for Cell-Based Binding Assays

The plasmid expressing EGF repeats 1-36 from Drosophila Notch fused to a C-terminal CD2 domain (N-CD2) was provided by Dr. Shinya Yamamoto (Baylor College of Medicine) and has been previously described (Brückner et al., 2000; Yamamoto et al., 2012). The plasmid expressing Drosophila Fng was generated by cloning Fng from a plasmid provided by Dr. Ken Irvine (Rutgers University; Okajima et al., 2003)) into the pMT-V5His plasmid (Thermo Fisher) using primers (forward) GGAATTCGATGATGAGCCT GACTGTGCTCTCGC and (reverse) GCTCTAGATTTCTGGGCGGGCAGAAGCT. Plasmids encoding the extracellular domains (amino acids 1-592 from DI and amino acids 1-1213 from Ser) of the ligands with C-terminal Myc-6xHis tags were generated by inserting sequences encoding the Myc-6xHis tag with a stop codon immediately after the respective extracellular domains in pMT-Di-AP and pMT-Ser-AP plasmids, also gifts from Dr. Ken Irvine (Rutgers University). To generate the plasmid expressing GFP used in the cell-based binding assays, EGFP was cloned from the EGFP-N1 plasmid (Clontech) and inserted into the pEFPI-N1 plasmid (Clontech) and inserted into pMT-V5His empty vector using primers (forward) GGAATTCGATGGTGAGCAAGGGCGA and (reverse) GCTCTAGACGCTTGTACAGCTCGTCCATGC.

Production and Purification Of DI and Ser Protein for Binding Assays

Stable S2 cell lines expressing DI or Ser ligands with C-terminal Myc-6xHis tags were generated by transfecting 20 μg of plasmid using the calcium phosphate transfection method as described in the Calcium Phosphate Transfection Kit (Invitrogen), although the buffers used were made in-lab. Stable cell lines expressing ligands were maintained with 50 μg/ml Blasticidin (Invitrogen) selection. To generate protein used in binding assays, cells were induced with 0.7 mM CuSO4 in complete medium for three days. Batches of ligand protein were purified from about 100 mL of clarified medium with 1 mL packed Ni-NTA resin (QIAGEN). Resin was washed with 10 mM imidazole, 0.5M NaCl in PBS and proteins were eluted with 250 mM imidazole in PBS. Elutions were concentrated and imidazole was diluted with PBS using Amicon centrifugal filters (Millipore). Protein was aliquoted and stored at −80 °C.

Site-Directed Mutagenesis of N-CD2 and Fng for Binding Assays

N-CD2 with mutant O-fucosylation sites in the EGF repeats of and the catalytically inactive Fringe-DEE mutant were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacture’s protocol. O-fuc site mutations were designed to change the modified threonine (T) or Serine (S) within the O-fucosylation consensus sequence (C2xxxx(S/T)C2 (Harvey et al., 2016; Rana and Haltiwanger, 2011)) to Valine (V) or Alanine (A), respectively. Mutations were confirmed by DNA sequencing. Primers used for mutagenesis are listed in Table S1.

Cell-Based Ligand Binding Assays and Flow Cytometry

S2 cells were transfected with plasmids encoding N-CD2 or O-fuc site N-CD2 mutants for cell-based binding assays using Effectene Reagent (QIAGEN) following the manufacturer’s instructions. Briefly, 1 μg of Notch-CD2 or empty vector control was transfected with 0.2 μg of pMT-GFP plasmid in order to monitor transfection efficiency and allow us to gate out untransfected cells from analysis. Varying amounts of Fng plasmid or control empty vector were also transfected when we tested the effects of Fng. DNA was diluted in 150 μL of Effectene Buffer. Enhancer reagent was mixed in a 1:7 DNA to reagent ratio for 5 minutes, then Effectene was added in a ratio of 1:9 for 10 minutes. The DNA Effectene solutions were mixed with 1ml of complete medium then directly mixed with 1x10⁶ suspended cells in 1ml medium and plated in 35 mm dishes. Cells were induced with 0.7mM CuSO4 for 4 hours following the transfection and cultured for 3 days.

N-CD2 expressing S2 cells were collected and washed in cold binding buffer (HBSS, 1mM CaCl2, 1% BSA, 0.05% sodium azide). For detecting cell surface expression of N-CD2 or O-fuc site mutants, cells were labeled with anti-CD2 antibody, which recognizes the extracellular portion of the CD2 protein, (1:100) in 50 μL binding buffer for 1 hour at 4 °C. Cells were washed with 1 mL of binding buffer, then labeled with PE-conjugated goat anti-mouse antibody (1:100) in 100 μL binding buffer for 1 hour at 4 °C. Cells were washed twice with 1 mL binding buffer, then analyzed with flow cytometry.

For ligand binding assays, ligand protein was clustered with PE-conjugated mouse anti-myc (1:20) in 50 μL binding buffer for 1 hour at 4 °C (clustering of ligand enhances avidity, mimicking binding to cell-surface ligand). Clustered ligand solutions were then mixed with the N-CD2 expressing cells for 1 hour at 4 °C. After binding with PE-clustered ligands, cells were washed twice with 1 mL binding buffer, then analyzed with flow cytometry.
For flow cytometric analyses, cells were then resuspended in about 300-400 μL binding buffer and filtered into flow cytometry tubes with cell strainer caps (Falcon). Cellular debris were excluded by gating. GFP-positive cells were gated before analyzing cell surface expression or ligand binding. Data were acquired and analyzed using a BD FACS Calibur flow cytometer (BD Biosciences) with BD CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software. S2 cells transfected with control empty vector were generated for each experiment, and the mean fluorescence intensities of labeling from these control cells were subtracted from each data point.

**Antibodies for Flow Cytometric Analyses and Western Blotting**

Antibodies used for western blotting or labeling for flow cytometric analyses include: mouse anti-CD2 (OX-34 clone, Bio-Rad); mouse anti-Notch (F461.3B, DSHB); rabbit anti-V5 (Sigma); HRP-conjugated goat anti-mouse (Jackson ImmunoResearch); Alexa Fluor680 rabbit anti-mouse (Invitrogen); IRDye700 goat anti-rabbit (Rockland Immunochemicals); PE-conjugated goat anti-mouse (Life Technologies); PE-conjugated mouse anti-myc (9E10 clone, R&D systems). Western blots using fluorescent probes were visualized using the Odyssey system (LI-COR), while western blots with peroxidase conjugated probes were detected by enhanced chemiluminescence blotting substrate (Thermo Scientific) and film development.

**Site-Directed Mutagenesis of PMT-NOTCH for Cell Aggregation Assays**

The pMT expression plasmid encoding *Drosophila* Notch (pMT-Notch; *Drosophila* Genome Research Center stock number 1022, https://dgrc.bio.indiana.edu/product/View?product=1022) was used as template for making mutants eliminating the O-fucosylation site in EGF repeats 8 and 12.

For the mutant EGF8 TV, the corresponding two nucleotide mutations (underlined in primers) were introduced by PCR-based site-directed mutagenesis with this plasmid and the following primers: 5’-CGGAGCGCTGCTGACAAACACCTACGCGATCG-3’ and 5’-TGTACAGACCGCTCTGCTGGACACCGG-3’.

For the mutant EGF12 SA, the corresponding nucleotide mutation (underlined in primers) was introduced by PCR-based site-directed mutagenesis with this plasmid and the following primers: 5’-CGAGGGAGCTTGCCTGGATGATCCGG-3’ and 5’-CAGGCAAGCTCCCTGTTTCTGGCATGG-3’.

Double mutants were generated by iteration of the above. Successful incorporation of the mutation was confirmed by direct DNA sequencing.

**Generation of Genomic Transgenes**

To generate the O-fucosylation site mutant Notch transgenes, we used the gap-repair mutagenesis strategy, as described previously (Leonardi et al., 2011; Leonard and Jafar-Nejad, 2014). To generate the Notch-5V transgene, we used the N4,5/CS-attB-P[pacman]-Ap8 construct, in which a 213 bp region in the Notch genomic locus encoding EGF4 and EGF5 is replaced by the CAT/SacB (chloramphenicol acetyl transferase/sucrose sensitivity; CS) selection cassette (Leonardi et al., 2011; Leonard and Jafar-Nejad, 2014). The following primers were used to introduce a TV mutation in the O-fucosylation site of EGF5 in the LA-EGF4,5-RA-pCR-Blunt II-TOPO targeting vector (LA and RA are left and right homology arms, respectively):

- EGF5-TV-for: GCAAATACGGCGGCGTATGTGTCAACACCC
- EGF5-TV-rev: GGGTGTTGACACATACGCCGCCGTATTTGC
- EGF5-TV-for: GCAAATACGGCGGCGTATGTGTCAACACCC
- EGF5-TV-rev: GGGTGTTGACACATACGCCGCCGTATTTGC

Recombineering was then used to replace the CAT/SacB cassette in the N4,5/CS-attB-P[pacman]-Ap8 construct with the 213 bp insert from the pCR-Blunt II-TOPO-EGF4,5V targeting vector resulting from the above-mentioned mutagenesis. The final construct (N[gt-5V]-attB-P[acman]-Ap8) was verified by EcoRI fingerprinting and sequencing of all exons and exon-intron junctions.

For EGF9 and EGF12 single mutants (EGF9V, EGF9S, EGF12A and EGF12T), a similar strategy was used, with N10-35/CS-attB-P [pacman]-Ap8 as the template construct (Leonardi et al., 2011; Leonard and Jafar-Nejad, 2014). The coding sequence for EGF8 and EGF9 are located in the left homology arm of this construct and can be targeted. The following primers were used to introduce the intended mutations in the LA-EGF9,10.5-RA-pCR-Blunt II-TOPO targeting vector:

- EGF9-TV-for: GTTTCTACGGAGCGGTGTGCATCGATGGCG
- EGF9-TV-rev: CGCCATCGATGCACGAGGCTCCGTAGAAAC
- EGF9-TS-for: GTTTCTACGGAGCGGTGTGCATCGATGGCG
- EGF9-TS-rev: CGCCATCGATGCACGAGGCTCCGTAGAAAC
- EGF12-SA-for: TGCCAGAACGAGGGAGCTTGCCTGGATGATC
- EGF12-SA-rev: GATCATCCAGGCAAGCTCCCTGTTTCTGGCA
- EGF12-ST-for: TGCCAGAACGAGGGGGACTTGCCTGATGGATC
- EGF12-ST-rev: GATCATCCAGGCAAGCTCCCTGTTTCTGGCA

A 13-bp sequence immediately 3’ to the coding sequence for EGF8 O-fucosylation site is identical to another 13-bp sequence in the coding sequence for EGF7. This duplicated sequence prevented us from performing a conventional site-directed mutagenesis on the O-fucosylation site in EGF8 in the LA-EGF10.35-RA-pCR-Blunt II-TOPO targeting vector. We therefore used PCR to generate...
two fragments of the insert in the above-mentioned vector, each containing one of the two copies of the 12-bp region. We then used site-directed mutagenesis to introduce a TV mutation in EGF8 and performed a triple-ligation to assemble the two fragments and the vector backbone into the LA-EGF10.35-EGF8V-RA-pCR-Blunt II-TOPO targeting vector. The following primers were used:

3’-16EGF-XbaI: CCCTCTAGAGCGATTGGGATAGCTACGTAGCGAAAGCTATC
5’-16EGF-KpnI: CCGTACCTTAATCGAGGAGGGAGGATGAG
16EGF-Bsal-for: ACAGGTCTCCACGTGCTGTCGAG
16EGF-Bsal-rev: ACAGGTCTCCAGGTAAGTCGGAGATCCATC
EGF8-TV-for: TGCCAGAAGCGCGCTTGTACATTAC
EGF8-TV-rev: GTGAGTGTGTACAGACGCGCTCCGTTGGCA

Similar strategies were used to generate double mutant versions of the LA-EGF10.35-RA-pCR-Blunt II-TOPO targeting vector. Recombineering was then used to exchange the CAT/SacB cassette in the N10-35/CS-attB-P[pacman]-ApR FRT19A/FM7c or vector backbone into the site-directed mutagenesis to introduce a TV mutation in EGF8 and performed a triple-ligation to assemble the two fragments and the two fragments of the insert in the above-mentioned vector, each containing one of the two copies of the 13-bp region. We then used of surface Notch in Notch protein, as reported previously (Leonardi et al., 2011). Since null MARCM clones, To examine the effects of Bar eye and curly wing or Tubby phenotypes. Since temperature to obtain N55e11/FM7; Ngt/CyO, females were crossed with y w Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFPnls/CyO, y+ males were crossed with y w/Y; Ngt/CyO, y+ males were crossed to y w N54l9 females and were then transferred to 30°C. Then, after, detergent-free immunostainings was performed to examine the cell surface level of Notch protein, as reported previously (Leonardi et al., 2011). Since N54l9 is a protein null allele (Figures S3A–S3A’), the only source of surface Notch in N54l9 clones will be that expressed from wild-type or mutant Notch transgenes. Considering the potential batch-to-batch variations in staining intensity, for each sample we generated the plot profiles of the signal intensity for a box covering both clonal area and the neighboring control area equally and calculated the relative signal intensity of the clone to control area in each sample as a measure of the cell surface level of Notch (Figures S3B–S3I’). Plot profile intensity was measured using ImageJ 1.47. Confocal images were scanned using a Leica TCS-SP5 microscope and processed with Amira5.2.2. Images were processed with Adobe Photoshop CS5; Figures were assembled in Adobe Illustrator CS5.

In Vivo Clonal Analysis for Cell Surface Expression Level of Notch
To examine the effects of O-fuc mutations on the surface expression of Notch in vivo, we used the MARCM system (Lee and Luo, 2001) to generate animals with clones of the Notch null allele N[54l9], with or without a copy of wild-type or mutant N[gt]. To generate Notch null MARCM clones, y w Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFPnls/CyO, y+ males were crossed to y w N54l9 females and were then transferred to 30°C. Then, after, detergent-free immunostainings was performed to examine the cell surface level of Notch protein, as reported previously (Leonardi et al., 2011). Since N54l9 is a protein null allele (Figures S3A–S3A’), the only source of surface Notch in N54l9 clones will be that expressed from wild-type or mutant Notch transgenes. Considering the potential batch-to-batch variations in staining intensity, for each sample we generated the plot profiles of the signal intensity for a box covering both clonal area and the neighboring control area equally and calculated the relative signal intensity of the clone to control area in each sample as a measure of the cell surface level of Notch (Figures S3B–S3I’). Plot profile intensity was measured using ImageJ 1.47. Confocal images were scanned using a Leica TCS-SP5 microscope and processed with Amira5.2.2. Images were processed with Adobe Photoshop CS5; Figures were assembled in Adobe Illustrator CS5.

Dissections, Staining, Image Acquisition and Processing
Dissection and staining were performed using standard protocols. Embryos were collected at stage 12-14 (10-13 hours). Wing imaginal discs were dissected from late third instar larvae. Notch surface staining was performed without using detergent. Antibodies were mouse anti-NECD 1:100 and rat anti-Elav 1:200 (Developmental Studies Hybridoma Bank), goat anti-mouse Alexa Fluor-488 and goat anti-rat Cy3-conjugated 1:500 (Jackson ImmunoResearch Laboratories). Confocal images were scanned using a Leica TCS-SP5 microscope and processed with Amira5.2.2. Images were processed with Adobe Photoshop CS5; Figures were assembled in Adobe Illustrator CS5.

S2 Cell Culture and Cell Aggregation Assays
S2 cells (Invitrogen) were cultured in Schneider’s Drosophila Medium (Lonza) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL). For S2-N and S2-Di stable cell lines (DGRC, Bloomington, USA), 200 nM methotrexate (Sigma-Aldrich) was added. For S2-SerTom stable cell line, 100 μg/mL hygromycin B was used. Cells were cultured for 24 hours at 24 ± 1°C prior to induction with CuSO4 (0.7 mM). Cells were incubated with CuSO4 for 1–2 days and then used in aggregation assays. For each aggregation assay, 2.5 × 10^5 of the stable S2-Di or S2-N cells were mixed with 5 × 10^5 of S2 cells transiently transfected with 2 μg total of either pBluescript (control) or pBluescript and pMT-Notch (S2-N) and pBluescript and pMT-Ser (S2-Ser) or pBluescript, pMT-Ser and pMT-Notch (S2-Ser-N) and pMT-Ser, pMT-Notch and pMT-fng (S2-Ser-N-Fng) using 3 μL of FuGENE
HD (Promega). For all transiently transfected S2 cells expressing trans-Notch, one μg of pMT-Notch was used. For the S2 cell expressing Ser and cis-Notch, 1.2 μg of pMT-Ser and 0.6 μg of pMT-Notch were used. A ratio of 1:2 was used for Fng and Notch co-expression all aggregation assays. After mixing, cells were gently shaken at 150 rpm to allow aggregation. Images of aggregate formation were taken after 5 minutes. The number of cell aggregates was quantified using a hemocytometer after 5 minutes of aggregation. Each assay was repeated at least three times. P-values were determined either by Student’s t test or by One-Way ANOVA followed by Dunnett’s post hoc test.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters including the sample sizes and replicates, data plotted (mean ± SD), P values, and statistical tests used are mentioned in Figure Legends. Statistical analyses were performed using Graphpad Prism 6. Data were analyzed by Student’s t test or by one-way ANOVA with the Dunett’ Post hoc multiple comparisons test.

DATA AND CODE AVAILABILITY

This study did not generate/analyze any datasets/code.
Glycosylation of Specific Notch EGF Repeats
by O-Fut1 and Fringe Regulates
Notch Signaling in Drosophila

Ashutosh Pandey, Beth M. Harvey, Mario F. Lopez, Atsuko Ito, Robert S. Haltiwanger, and Hamed Jafar-Nejad
Supplementary Data

Figures S1-5

Table S1

Table S2
Figure S1. Fringe glycosyltransferase activity directly modulates Notch ligand binding in a cell-based assay. Related to Figure 1.

(A) Western blot shows Notch-CD2 (N-CD2) expression in S2 cell lysates detected by anti-CD2 and anti-Notch antibodies. (B) Coomassie-stained protein gel of purified Delta-Myc-6xHis and Serrate-Myc-6xHis proteins used in the ligand binding assays. (C) Histograms of N-CD2 cell surface expression detected by flow cytometry of anti-CD2 antibody gated on cells co-transfected with GFP. Gray profiles are S2 cells transfected with empty vector control instead of N-CD2. Profiles with solid lines represent cells transfected with Fringe (+Fringe, Fringe:Notch DNA ratio 0.2:1), while dashed lines represent cells transfected with an empty vector control instead of Fringe (−Fringe). Relative mean fluorescence intensities (MFI) of N-CD2 are shown in the panel to the right (mean ± SD of three independent experiments). ns not significant (P>0.05, Student’s t-test). (D, E) Representative flow cytometry histograms of ligand binding. Cells were incubated with (D) 61.9 nM Delta-Myc-6xHis or (E) 407.4 nM Serrate-Myc-6xHis. Gray profiles are S2 cells transfected with empty vector control instead of N-CD2. Profiles with solid lines represent cells transfected with Fringe (+Fringe), while dashed lines represent cells transfected with an empty vector control instead of Fringe (−Fringe). (F) Western blot of S2 cells expressing N-CD2 co-transfected with empty vector control (−Fringe), wild-type Fringe, or enzymatically inactive Fringe (DEE) (Fringe:Notch DNA ratio, 0.2:1), detecting N-CD2 with anti-CD2 antibody and using anti-V5 antibody to confirm Fringe expression. (G) Delta-Myc-6xHis (15.5 nM) and Serrate-Myc-6xHis (203.7 nM) binding to S2 cells expressing N-CD2 co-transfected with empty vector control (−Fringe), wild-type Fringe, or enzymatically inactive Fringe (DEE).
Binding is represented as MFI (mean ± SD of three independent experiments). ns not significant ($P>0.05$), **$P<0.01$, ****$P<0.0001$; one way ANOVA followed by Dunnett's Post hoc test compared to empty vector control for each ligand.
Fig S2
Figure S2. Expression and ligand binding of O-fucose site mutants of N-CD2.

Related to Figure 2.

(A) Cell surface expression of O-fucose site mutants of N-CD2 detected by anti-CD2 labeling and flow cytometry analyses shown as relative mean fluorescence intensities (MFI). Mean ± SD of three independent experiments is shown. **P<0.01, ***P<0.001, ****P<0.0001; one way ANOVA followed by Dunnett’s Post hoc test compared to wild-type N-CD2 for each mutant. (B) Western blots of S2 cell lysates expressing wild-type N-CD2 or each O-fucose site mutant. (C, D) Cell-based ligand binding assays of S2 cells co-transfected with wild-type N-CD2 or O-fucose site N-CD2 mutants and empty vector control (–Fringe) or Fringe (+Fringe, Fringe:Notch ratio 0.2:1). Cells were incubated with (C) 15.5 nM Delta-Myc-6xHis or (D) 203.7 nM Serrate-Myc-6xHis pre-clustered with PE-conjugated anti-myc antibody, and ligand binding was detected by flow cytometric analysis. Binding is represented as relative mean fluorescence intensities (MFI) ± SD of three independent experiments. WT, 8V, 9V, 12A, 8V9V and 8V12A data are highlighted in Figure 2.
Fig S3

Normalized signal intensity per unit mutant area

% relative intensity

N[gt]  WT  5V  8V  9V  12V  8V9V  8V12A  9V12A

0  20  40  60  80  100
Figure S3. Mutations in O-fucosylation sites do not affect the cell surface level of Notch in vivo. Related to Figure 3.

Confocal images of wing imaginal discs of third instar Drosophila larvae raised at 30°C are shown. Nuclear GFP (green) marks MARCM clones of the N\textsuperscript{54l9} null allele. In A-I, N\textsuperscript{54l9/54l9} MARCM clones are generated in the absence (A, A') or presence (B-I') of one copy of wild-type (B, B') or mutant (C-I') Notch transgenes. Detergent-free surface staining is performed using anti-NECD antibody (Gray in A'-I'). (A, A') Lack of NECD staining in the clone confirms that N\textsuperscript{54l9} is a protein-null allele. (A-I) Projection of three consecutive basolateral optical sections showing nuclear GFP positive clone cells. (A'-I') Projection of three consecutive apical optical sections from the same datasets showing cell surface NECD staining. The dotted yellow box in A' is an example of the boxes that were used for generating the plot profiles for each genotype. Scale bar in (A) is 5 μm and applies to all panels. (A''-I'') Plot profiles showing the Notch surface level in terms of intensity/unit in wild-type and mutant clones. (J) Graph showing the average plot intensities for indicated Notch transgenes in clone area normalized by their corresponding wild-type area (n=3 for each genotype). Data presented as mean±SD.
D Normalized survival of *N[–]/Y* males with each mutant *N[gt]* transgene

| N[gt] | % survival |
|-------|------------|
| –     | 120        |
| WT    | 100        |
| 9S    | 80         |
| 12T   | 60         |

ns

E Elav 100% rescued

F Elav 100% rescued

E' Notch

F' Notch

n=16

N[–]/Y; N[gt-9S/+]

n=19

N[–]/Y; N[gt-12T/+]
Figure S4. *Notch* transgenes with a threonine-to-serine mutation in EGF9 or a serine-to-threonine mutation in EGF12 fully rescue the lethality and neurogenic phenotype of *N[–]/Y* males. Related to Figure 3.

(A, B) Nuclear GFP marks clones of the *N*549 protein-null allele harboring one copy of *N[gt-9S]* (A) and *N[gt-12T]* (B). (A’, B’) Surface staining of the apical sections of the same clones as in A, B with an antibody against NECD. Scale bar in (A) is 5 μm and applies to panels A-B’. (A”, B”) Plot profiles showing the Notch surface level in terms of intensity/unit. Compare to wild-type in Fig S3B”. (C) Graph showing the average plot intensities for indicated *Notch* transgenes in clone area normalized by their corresponding wild-type area (n=3 for each genotype). Data presented as mean± SD. (D) Graph showing survivability of *Notch* hemizygous males harboring a *Notch* transgene with threonine-to-serine mutation in EGF 9 or a serine-to-threonine mutation in EGF12. Data presented as mean ± SD. (E-F’) Elav (red) and Notch (Magenta) stainings are shown for the indicated genotypes. E-F show Elav staining only; E’-F’ show Elav, Notch double-staining. Scale bar in (E) is 50 μm and applies to panels E-F’. ns not significant (*P*>0.05).
Fig S5

Aggregates per mL (X1000)

| Notch | Fringe | WT   | WT   |
|-------|--------|------|------|
|       |        | –    | +    |
| –     | –      | –    | +    |
| –     | –      | +    | –    |

S2-Serrate

* indicates significance.
Figure S5. Co-expression of *Fng* with Notch in S2 cells decreases trans-binding of Notch with Ser in aggregation assays. Related to Figure 6C.

Graph showing the quantification of the number of cell aggregates between the indicated cell types after 5 minutes. WT= Wild-type Notch cDNA. Data presented as mean ± SD. *P<0.05 (Student’s t-test).
Table S1. Primers used for Site-directed mutagenesis of N-CD2 and Fringe for binding assays are listed below with mutated residues underlined. Related to STAR Methods (Site-Directed Mutagenesis of N-CD2 and Fng for Binding Assays).

|    |    |    |
|----|----|----|
| EGF1 T→V_F | GCCAGAATGGCGGCGGTATGCCTACACAAC |
| EGF1 T→V_R | GTTGTGTAACGCATACGCCGCCCATTCTGGC |
| EGF3 T→V_F | CCTCAATGGAGGCCCTCTGTCAGCTAAAGACAC |
| EGF3 T→V_R | GTGTCTTTAGCTGACAGACGCCCTCCATTGAGG |
| EGF4 T→V_F | CGGAATGGAGGCCCTCTGCACCACCTTTCG |
| EGF4 T→V_R | CAAAGCAGGTGCAGACCGCTCCATTCC |
| EGF5 T→V_F | GCAAATACGGGCGGTATGTGTCACAAACCCC |
| EGF5 T→V_R | GGGTGTGAAAGACGCCGCCGTATTTCG |
| EGF7 T→V_F | CAGAAGAGGAGGCTAGCTGACAGGCTCCATT |
| EGF7 T→V_R | GATGCCATCGATGACACTCCTCCATT |
| EGF8 T→V_F | GCCGAACGGAGCCCTCTGTACAACACACTC |
| EGF8 T→V_R | GAGTGTGTTGTACAGACGCTCCATTTCAG |
| EGF9 T→V_F | GTTTCTACGGAGCCGCTTGAGCTGACAG |
| EGF9 T→V_R | GCCATCGATGCACACGGCTCCGCTGAAAC |
| EGF12 S→A_F | CCAGAAACGGAGCCTTGATGATGATC |
| EGF12 S→A_R | GATCATCCAGGCAAGCTCCATTGAG |
| EGF13 T→V_F | CTGGTGAACGATGAGAGGTGGATAGCT |
| EGF13 T→V_R | CATTGATCTTGTCGAGCTCCATT |
| EGF17 T→V_F | CAACAATGGTGCCGTCTGCATCGGTGAAAAC |
| EGF17 T→V_R | GATGCCATCGATGACACGGCACCATTG |
| EGF20 T→V_F | GCCAGCATGGTGGCGTCTGTTATGCTGCTG |
| EGF20 T→V_R | GAGCTTATACACGAGGGCCACCATGCTGATG |
| EGF21 T→V_F | GCGGAATGGAGGCGCTCTGCATTGACAAG |
| EGF21 T→V_R | CCTTGTCAATGCAGACGCCTCCATTTCAG |
| EGF23 S→A_F | CTGTCGGAATGGAGGCCTTGTTTGAATGTTTC |


| EGF23 S→A_R | GGAACATTCAAACAGGCAGCTCCATTCCGACAG |
| EGF24 T→V_F | CAGAACGTTGGAAGTCTGTCTGGATGGGATC |
| EGF24 T→V_R | GATCCCATCCAGACAGACTCCACCCTTCTG |
| EGF25 T→V_F | GCCAGAATGGAGCCGTGTCTGGATGCAGATGGC |
| EGF25 T→V_R | CACATACTGACTACACACGGCTCCATTCTGGC |
| EGF26 S→A_F | CTTAACGGTGCGCTTTGATCGATGGCATC |
| EGF26 S→A_R | GATGCCATCGATGCAAGCGCCACCGTTTAAG |
| EGF27 T→V_F | GTTTGAACGGAGCCGTCCTGCTGACCCGAGCAAAAC |
| EGF27 T→V_R | GTTTTGCTCGTGGCAGACGGCTCCGTTCAAAC |
| EGF28 T→V_F | GCGAGAATGGAGCCGTCTGCCACGAGCAAAAC |
| EGF28 T→V_R | CATCTGGCTACAGACGGCTCCATTCCAGAGCCG |
| EGF30 T→V_F | CCAGAATGGTGGAAGTGTGCSAGATCTCATC |
| EGF30 T→V_R | GATGAGATCTCGGCACACACCCACCATTCTGG |
| Fringe DEE_F* | GTGGTTCTGTCACTTCCGACGAAGAAATATGTCAATGGC |
| Fringe DEE_R* | CGGCACATTGACATGTTTCTTTCGAGTGACAGAACCAC |

*(D236-D237-D238 to D236-E237-E238)*
Table S2: Average MFI and Standard Deviations for –Fng data in Figure S2C. Related to Figure 1.

| O-fucose site mutant | Average MFI (–Fng) | SD  |
|----------------------|--------------------|-----|
| WT                   | 1                  | 0.379 |
| 1V                   | 1.206              | 0.323 |
| 3V                   | 1.360              | 0.246 |
| 4V                   | 1.106              | 0.265 |
| 5V                   | 1.027              | 0.797 |
| 7V                   | 1.412              | 0.335 |
| 8V                   | 0.321              | 0.055 |
| 9V                   | 0.603              | 0.078 |
| 12A                  | 1.208              | 0.148 |
| 13V                  | 1.294              | 0.480 |
| 17V                  | 0.840              | 0.053 |
| 20V                  | 1.429              | 0.365 |
| 21V                  | 0.817              | 0.349 |
| 23A                  | 0.943              | 0.235 |
| 24V                  | 0.459              | 0.265 |
| 25V                  | 1.077              | 0.209 |
| 26A                  | 1.085              | 0.327 |
| 27V                  | 1.069              | 0.098 |
| 28V                  | 1.062              | 0.100 |
| 30V                  | 1.131              | 0.262 |
| 8V9V                 | 0.055              | 0.074 |
| 8V12A                | 0.024              | 0.484 |