Supplemental Materials

Inventory:

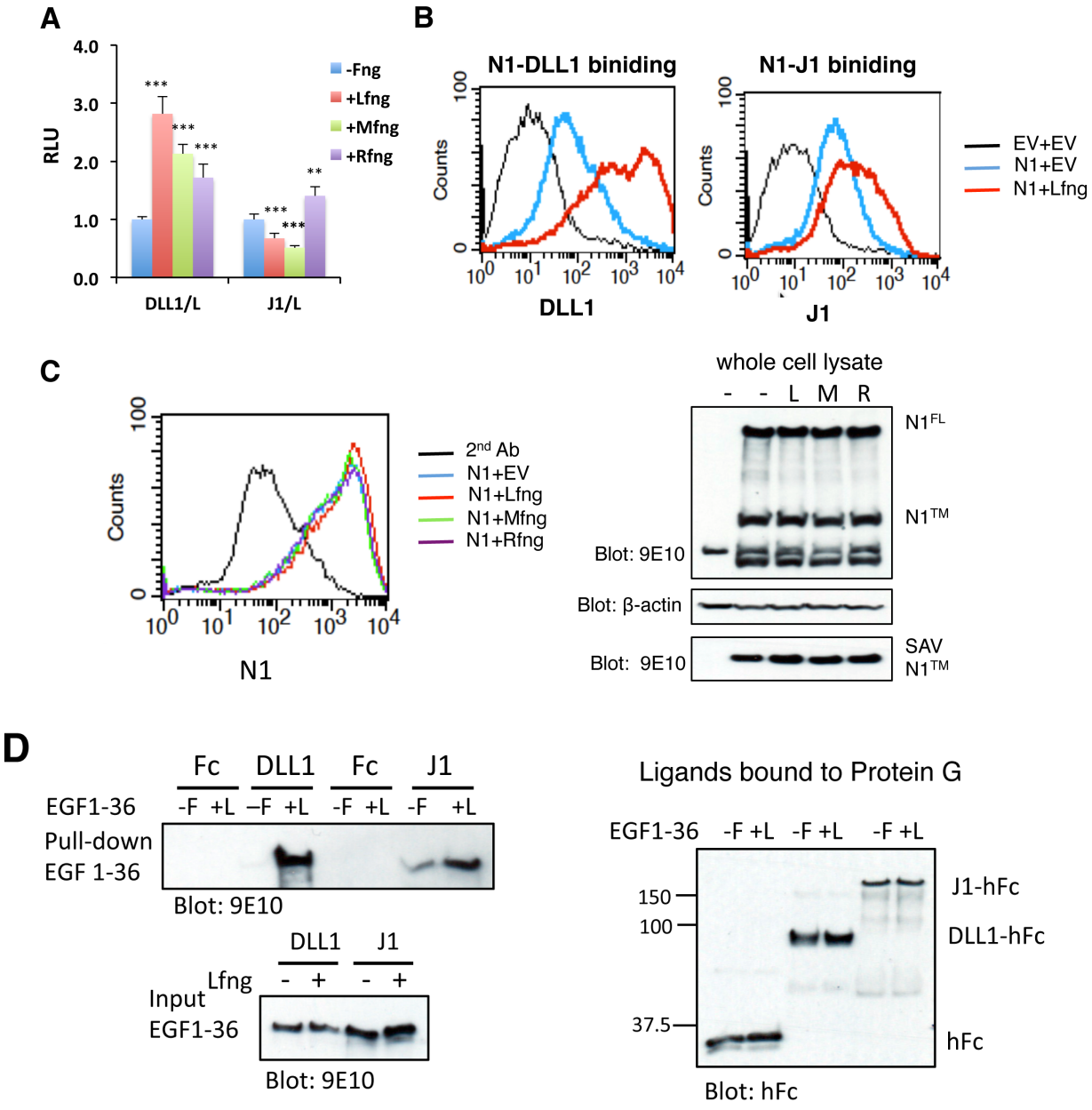
Supplemental Figure Legends

Supplemental Figure S1: Experiments related to Figure 1. (A) HEK293T cells showed the same activation patterns of N1 by three Fringes as in NIH3T3 cells. Cell-based co-culture N1 activation assays were performed as described in Supplemental Experimental Procedures. HEK293T cells were co-transfected with plasmids encoding human N1-Gal4 and one of the three Fringes or an empty vector (-Fng) control. The transfected HEK293T cells were then co-cultured with ether L cells, or L cells stably expressing DLL1 or J1. RLU was obtained by calculating relative luciferase activity for ligand expressing cells over L cell controls. RLU of the –Fng samples was set to 1 for each ligand (-Fng DLL1/L = 1.94 ± 0.11: J1/L = 2.84 ± 0.08). Statistical significance of -Fng versus plus Fringe was determined using one-way ANOVA. Bar graph shows mean +/- SD; two independent experiments n = 6 were analyzed. ***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.01$. **(B)** Cell-based N1-ligands binding histograms of Fig. 1E using 12 nM DLL1 and 7 nM J1. **(C)** Fringes do not affect cell surface expression of N1. HEK293T cells were co-transfected with pcDNA-N1 and one of the Fringes or empty vector (EV). Cell surface expression was analyzed by flow cytometric analysis with an anti-N1 ECD antibody (left) or by cell surface biotinylation followed by Western Blot (right) as described in Supplemental Experimental Procedures. **(D)** Purified N1 EGF1-36-Myc-His_6 expressed in the presence or absence of Lfng was incubated with Protein G-bound DLL1-Fc or J1-Fc as described in Supplemental Experimental Procedures. Bound proteins were eluted using 20 mM EDTA and detected by Western blot with the indicated antibodies.

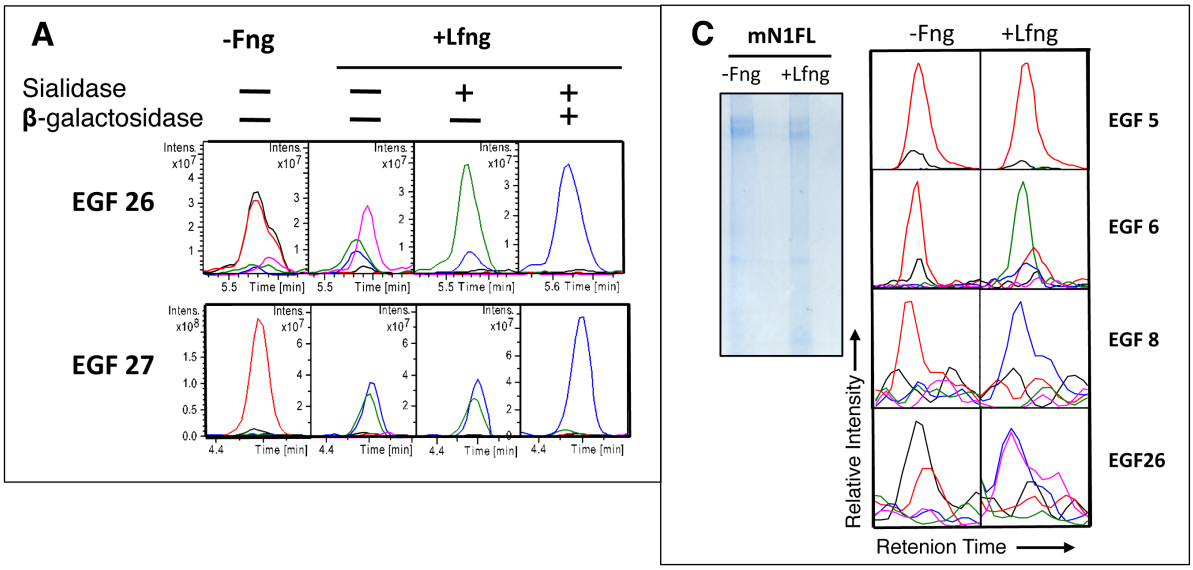
Supplemental Figure S2: Experiments related to Figure 2. (A) Peptides bearing different *O*-fucose glycoforms ionize to similar extents. A plasmid encoding N1 EGF24-28 was co-transfected with Lfng (+Lfng) or empty vector (-Lfng) into HEK293T cells, the proteins were purified from the medium, digested with chymotrypsin and analyzed by nano-LC-MS/MS as in Figure 2. Note that when this short fragment is used instead of EGF1-36, the major glycoforms in the presence of Lfng on EGF27 are a mixture of di- and tri-saccharide. When the Lfng-elongated sample was digested with sialidase, the tetrasaccharide species on EGF26 disappeared, replaced with a trisaccharide species of equal intensity, indicating that these glycopeptides ionize to a similar extent. In addition, there was no change to the pattern from EGF27, consistent with the lack of sialic acid at this site. Further digestion with β-galactosidase converted all species to di-saccharide ions, again of similar intensity. **(B) Lfng shows preference for some EGF repeats over others.** Plasmid encoding N1 EGF1-5, 11-15, and 24-48 was co-transfected into HEK293T cells with increasing amounts of Lfng plasmid as indicated. The protein was purified from the medium and analyzed as shown in Figure 2. (**C) Analysis of *O*-fucosylation on full-length N1 expressed in HEK293T cells.** pcDNA-N1 was transfected into HEK293T cells in the presence (+Lfng) or absence (-Fng) of Lfng and purified as described in Supplemental Experimental Procedures (left, Gel code stain). Mass spectral analysis was performed as shown in Figure 2. EICs were generated for several sites (right). **(D) N1 EGF1-36 expressed in NIH3T3 cells shows similar patterns to those in Figure 3.** N1 EGF1-36 was expressed in NIH3T3 cells and purified from the medium (left, Gel code stain). Mass spectral analysis was performed as shown in Figure 2. EICs were generated for several sites (right).

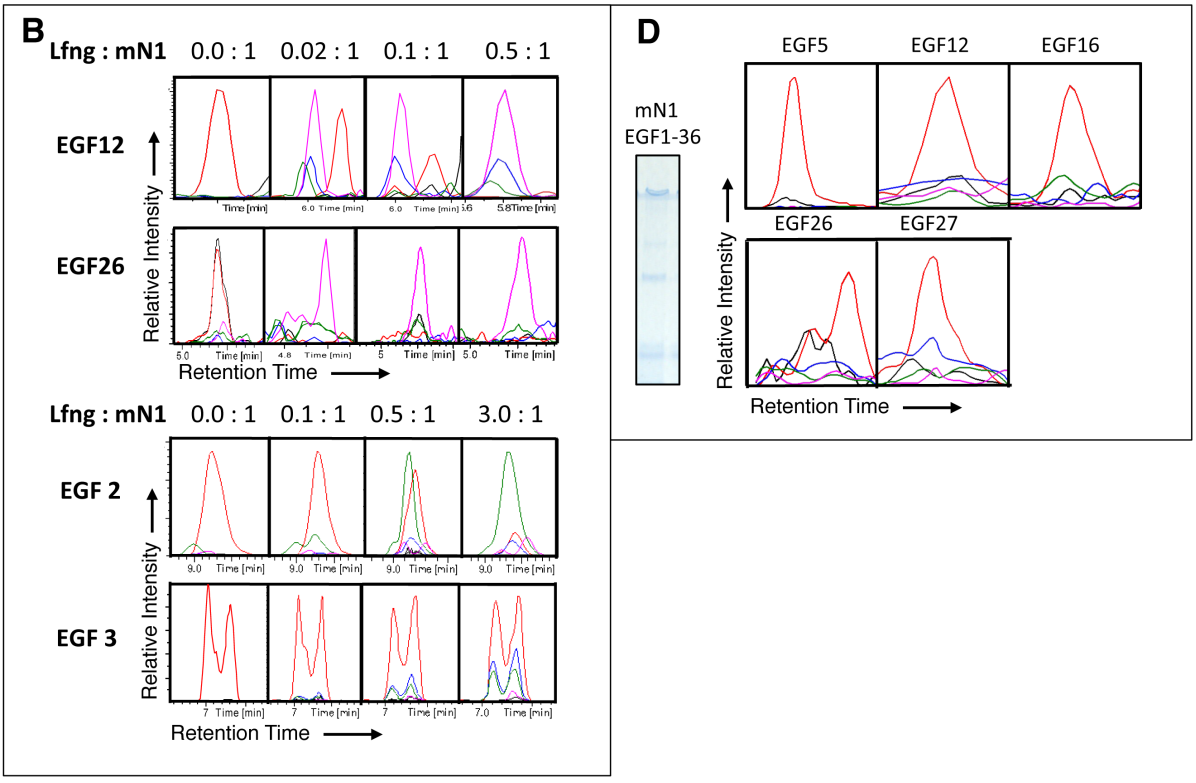
Supplemental Figure S3: Experiments related to Figure 3. (A) To determine whether *O*-fucose site mutations affect activation of N1 by ligands in the absence of any Fringe, we used cell-based co-culture N1 activation assays to assess the response of N1 to ligand (similar to Fig. 1D). Statistical significance of mutants compared to WT N1 was determined using one way ANOVA. Bar graph shows mean +/- SD; three independent experiments n = 9 were analyzed. ($p < 0.01$ *, $p<0.001$ **, $p<0.0001$ ***). **(B)** To determine whether *O*-fucose site mutations affect ligand binding to N1 in the absence of any Fringe, we used the cell-based N1-ligand binding assays described in Supplemental Experimental Procedures. Data were from three independent experiments. Bar graph shows mean +/- SD; Statistical significance of mutants compared to WT N1 was determined using one-way ANOVA ($p < 0.05$ *, $p<0.005$ **, $p<0.0005$ ***). **(C)** Cell surface expression of N1 mutants was detected via flow cytometry (as described in Supplemental Experimental Procedures). MFI shows relative amount of cell surface N1. Data were from three independent experiments. Bar graph shows mean +/- SD; Statistical significance of mutants compared to WT was determined using one-way ANOVA ($p < 0.05$ *, $p<0.005$ **, $p<0.0005$ ***). **(C and D)** Cell-based co-culture N1 activation assays in NIH3T3 cells with mutations in all *O*-fucose sites elongated by any Fringe (Fig. 2C) and were performed in the absence (-Fng) or presence of individual Fringes (+Lfng, +Mfng, or +Rfng) as described in Fig. 1D. Bar graph shows mean +/- SD; Statistical significance of -Fng versus +Fng (either +Lfng , +Mfng or Rfng) was determined using one-way ANOVA. Data are mean +/- SD; three independent experiments n = 9 were analyzed. ***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.01$. (**C)** Co-culture with DLL1-expressing L cells. (**D)** Co-culture with J1-expressing L cells.

Supplemental Figure S1

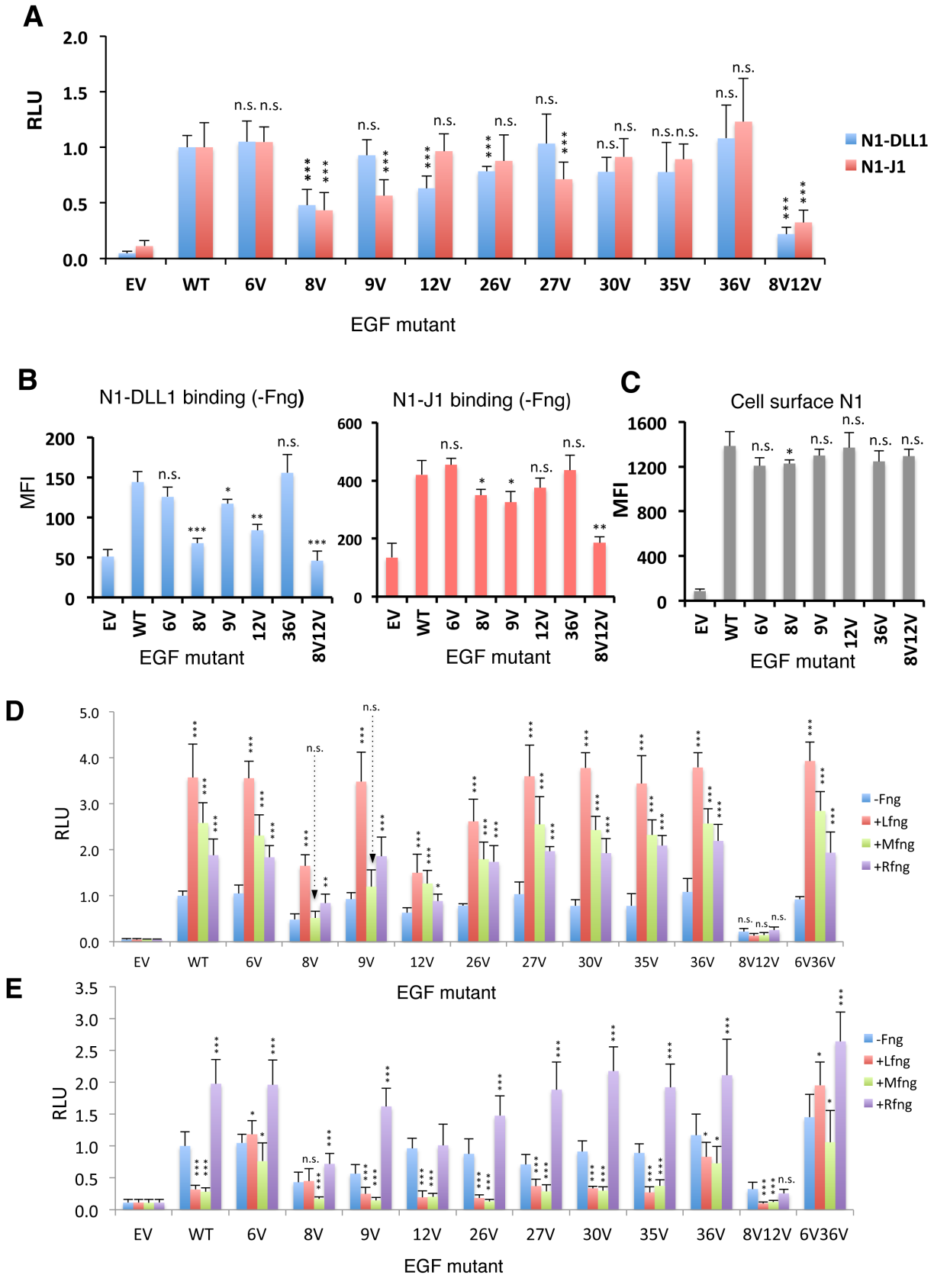


Supplemental Figure S2





Supplemental Figure S3



Data S1: Mass spectra for O-fucose glycoforms of peptides from the 20 EGF repeats containing an O-fucose consensus sequence in mouse N1 EGF1-36 (Fig. 1C), related to Figure 3. Samples were generated in HEK293T cells co-transfected with plasmid encoding EGF1-36 and EV (-Fng) or any of the Fringes (+Lfng, +Mfng or +Rfng)) as described in Experimental Procedures. Upper panels show an MS spectrum at a specific time, with the ion corresponding to the m/z of the peptide (red triangle above black bar) containing an O-fucose consensus sequence indicated. In the absence of any Fringe, this peptide is modified with O-fucose monosaccharide (red triangle). In the presence of Fringe samples (+Lfng, +Mfng or +Rfng), the indicated ions in the MS scan correspond to the m/z for O-fucose monosaccharide or Fringe elongated glycoforms of the same peptide from the EGF repeat as in the absence of Fringe. Lower panels show the MS/MS spectra confirming the identity of corresponding glycopeptides based on the presence of peptide-specific b- and/or yions. Tables (right) show the m/z used for the EIC searches for glycoforms of the peptide as in Fig. 2 (unmodified, mono-, di-, tri- and tetra-saccharide glycoforms). Key: black bar, unmodified peptide; red triangle, fucose; blue square, GlcNAc; yellow circle, galactose; purple diamond, sialic acid. Summaries of results are shown in Fig. 4.

EGF	Sequence Consensus sequence: CXXXXS/TC	Parent Ion ($M+H^+$)	Deglycopro-duct ($M+H^+$)	Mass Δ	Predicted Mass ($M+H^+$)	*O*-Glyco-form
2	^{70}NAGTCHVVDHGGTVDYACSCPLGFSGPLCLTPLDNACLANPCR^{112}	4853.4	4707.0	146.0	4707.3	Fuc
3	^{113}NGGTCDLLTLTEYK^{126}	1731.4	1585.4	146.0	1585.7	Fuc
5	^{182}CSQNPGLCRHGGTCHNE^{198}	2132.2	1986.2	146.0	1985.1	Fuc
6	^{217}LPYVPCSPSPCQNGGTCRPTGDTTHE^{242}	3037.0	2890.0	147.0	2890.2	Fuc
8	^{302}MPNACQNGGTCHNTHGGYNCVCNGW^{327}	3112.9	2966.8	146.1	2967.2	Fuc
9	^{346}QGATCHDRVASFYCECPHGRTGL^{368}	2823.4	2677.3	146.1	2679.6	Fuc
12	^{456}CISNPCQNDATCLDQIGE^{473}	2668.0	2095.9	572.1	2096.2	Fuc+GXX
16	^{616}GTCQDRDNSYLC^{627}	1634.2	1488.1	146.1	1488.5	Fuc
18	^{682}CAGSPCHNGGTCEDGIAGFTCRCPE^{706}	2916.2	2770.2	146.0	2771.2	Fuc
20	^{739}CDCAPGWSGTNCDINNNECESNPCVNGGTCK^{769}	4122.6	3550.2	572.4	3548.8	Fuc+GXX
21	^{781}EGFSGPNCQTNINECASNPCLNQGTCIDDVAGYK^{814}	4597.8 4729.8	3792.6 3792.6	805.2 937.8	3793.1 3793.1	GalGlcNAc+Fuc+GX GalGlcNAc+Fuc+GXX
23	^{862}QGQTCEVDINECVKSPCRHGASCQNTNGSY^{891}	3485.6	3485.6	0.0	3485.7	NP
24	^{900}TGRNCESDIDDCRPNPCHNGGSCTDGINTAF^{930}	3851.0 3499.0	3499.0 3499.0	349.2 0.0	3499.6 3499.6	Fuc+GlcNAc NP
26	^{994}NGGTCVDGINSF^{1005}	1378.6 1241.6	1241.6 1241.6	146.0 0.0	1241.3 1241.3	Fuc NP
27	^{1021}DVNECDSRPCLHGGTCQDSY^{1040} OH	2533.3	2387.2	146.1	2371.5	Fuc
30	^{1142}CEDEVDECSPNPCQNGATCTDYL^{1164} OH	2894.8	2751.0	145.8	2734.0	Fuc
31	^{1189}SQPCQNGGTCIDL^{1201}	1594.8	1449.0	145.8	1450.0	Fuc
32	^{1238}CFNNGTCVDQVGGYTCTCPPGFVGER^{1263}	2951.0	2951.0	0.0	2952.8	NP
35	^{1357}CLNGGTCISGPR^{1368}	1438.6	1292.6	146.0	1292.5	Fuc
36	^{1369}SPTCLCLGSFTGPECQFPASSPCVGSNPCYNQGTCEPTSENPFYR^{1413}	5633.0	5121.4	511.6	5121.6	GlcNAc+Fuc

Supplemental Table S1: Related to Figure 2. Peptides from N1 identified with *O*-fucose , *O*-Glucose, *O*-GlcNAc glycan and β-hydroxylation modifications. Consensus sites for *O*-fucose (red), *O*-glucose (blue), and *O*-GlcNAc (green) are indicated. β-hydroxylated D is indicated by OH. Spectra confirming these assignments are in Data S1. All masses were converted to [$M+H^+$]. GXX, *O*-glucose trisaccharide; GX, *O*-glucose disaccharide; NP, naked peptide. For each glycopeptide, the mass of the parent ion, the fully deglycosylated product (lacking all sugars), and the difference between these corresponding to the mass of the modifications is shown. The predicted mass of the unglycosylated peptide is also shown. All peptide masses are adjusted for carboxyamidomethylation of Cysteines. For peptides with a mass below 2000 Da, monoisotopic masses were used to calculate predicted masses, while for those above 2000 Da, average masses were used.

Supplemental Experimental Procedures

Plasmids

Plasmids encoding mouse Notch1 (N1) EGF1-36, EGF1-5, EGF11-15 and EGF24-28 with C-terminal Myc-His_6 tags (pSecTag2-Hygro, Invitrogen) were described previously (Rana et al., 2011). The plasmid encoding full-length mouse Notch1 (pcDNA1-N1-myc) was generously provided by Dr. Jefferey Nye (Nye et al., 1994). Fringe expressing plasmids SEAP (EV), LfngAP, MfngAP and RfngAP were previously described (Moloney et al., 2000). The TP1-1 luciferase reporter construct (Ga981-6) was a gift from Dr. Georg Bornkamm (Munich, Germany), and the gWIZ β–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 Not1 restriction site following the GFP coding region. Plasmids encoding soluble Notch ligands, rat DLL1-hFc (pD1FC1) and rat Jagged1-hFc (Jagged1-FC/pIRES2-EGFP), were generously provided by Dr. Pamela Stanley (Albert Einstein College of Medicine) (Stahl et al., 2008). The control hIg-Fc expression plasmid (hIgG-pRK5) was described previously (Hsieh et al., 1999).

Cell culture

HEK293T and NIH3T3 cells (NIH3T3 CRL-1658) were obtained from the American Type Culture Collection (Manassas, VA) and L cells stably expressing Jagged1 (J1) or Delta-like 1 (DLL1) were a kind gift of Dr. Gerry Weinmater (UCLA). All cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% bovine calf serum at 37 ^{o}C in a humidified incubator at 5% CO_2.

Antibodies and Protein reagents

The following primary antibodies and recombinant soluble Notch ligands were used for Western blotting and Flow Cytometry:

Primary antibodies:

Sheep Polyclonal Mouse Notch-1 antibody, R&D systems (AF5267)

Anti c-Myc antibody (9E10), Stony Brook University Cell Culture/Hybridoma Facility

Secondary antibodies:

PE-Anti-sheep lgG, Santa Cruz SC-3757

PE-Goat-anti-mouse IgG, Invitrogen P-852

PE-Anti-human IgG, Jackson Immuno Research 109115-098

Recombinant soluble Notch ligands:

Rat DLL1-mouse Fc fusion protein, R&D systems 3970-DL-050

Rat J1-human Fc fusion protein, R&D systems 599-JG-100

Production of N1 EGF 1-36 (or fragments) in the absence or presence of Lfng, Mfng or Rfng.

HEK293T cells were co-transfected with plasmids encoding mouse N1 EGF 1-36 (or EGF fragments) (2 μg) and either SEAP (EV), LfngAP (Lfng), MfngAP (Mfng) or RfngAP (Rfng) (1 μg) in a 10-cm plate using 18 μl of PEI reagent (polyethylenimine) mixed with 300 ml of OPTI-MEMl (Invitrogen). After 4 days, the proteins were purified from media using Ni-NTA resin (Qiagen) and eluted with 250 mM imidazole as described previously (Kakuda and Haltiwanger, 2014).

Mutagenesis of *O*-fucosylation sites in N1

Site-directed mutagenesis of *O*-fucosylation sites in EGF repeats of in the pcDNA1-N1myc plasmid was performed using QuikChange II XL site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacture's protocol. Mutations were designed to change the modified threonine (T) within the *O*-fucosylation consensus sequence ($C^2xxxx(\underline{S/T})C^3$ (Rana and Haltiwanger, 2011)) to valine (V). The mutants were confirmed via DNA sequencing. A list of primers used for mutagenesis is shown below with mutated residues in capital letters:

EGF06_T232V_F: ccagaatggaggcGTctgccgtcctac

EGF06_T232V_R: gtaggacggcagACgcctccattctgg

EGF08_T311V_F: gccagaatggcggaGTctgccacaacac

EGF08_T311V_R: gtgttgtggcagACtccgccattctggc

EGF09_T349V_F: gtttccagggtgccGTttgccacgacc

EGF09_T349V_R: ggtcgtggcaaACggcaccctggaaac

EGF12_T466V_F: gtcagaatgatgccGTttgcctggaccagattgg

EGF12_T466V_R: ccaatctggtccaggcaaACggcatcattctgac

EGF26_T997V_F: ctgcttcaatggtggtGTctgtgtggatggtatc

EGF26_T997V_R: gataccatccacacagACaccaccattgaagcag

EGF27_T1035V_F: gtctgcacggtggtGTctgccaagacagc

EGF27_T1035V_R: gctgtcttggcagACaccaccgtgcagac

EGF30_T1159V_F: ctgccagaatggagctGTctgcactgactatc

EGF30_T1159V_R: gatagtcagtgcagACagctccattctggcag

EGF35_T1362V_F: ctcaacggtggtGTatgcatctcgggcc

EGF35_T1362V_R: ggcccgagatgcatACaccaccgttgag

EGF36_T1402V_F: gctacaatcagggcGTctgtgagcccacatc

EGF36_T1402V_R: gatgtgggctcacagACgccctgattgtagc

Analysis of cell surface N1 expression by flow cytometry

HEK293T cells were co-transfected with 1.5 μg of either EV or pcDNA-N1-Myc (WT or mutant) and 0.4 μg of GFP (pEGFP-N1) in a 3.5-cm plate using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. At 28-30 h post-transfection, the cells were dissociated with cold PBS, pH 7.4, containing 1% bovine serum albumin (BSA) and resuspended in binding buffer (1 mM $CaCl_2$, 1% BSA and 0.05% NaN_3 in Hanks' balanced salt solution, pH 7.4, Gibco). Cells (0.5-1 x 10^6) were incubated with 100 μl of mouse Notch-1 antibody 10 μg/ml for 1 h at 4°C. Cells were washed with the binding buffer and then incubated with PE-anti-sheep IgG (1:100) for 30 min at 4°C. After two washes with binding buffer, the cells then were analyzed with a FACSCalibur (BD, Bioscience) flow cytometer. The gate was set to collect the GFP positive population of 20,000 events for each sample and analyzed using CellQuest Pro Software (BD, Bioscience).

***In vitro* N1 EGF1-36 binding to DLL1-Fc or J1-Fc**

Purified N1 EGF1-36 (0.5 μg) expressed in the presence or absence of Lfng was incubated with 5 μl of Protein G-agarose (Roche) pre-bound with either human Fc, DLL1-hFc, or J1-hFc (produced in HEK293T cells) at 4°C for 2 hours. After washing with TBS, 0.1% NP-40, bound EGF1-36 was eluted with 20 mM EDTA, resolved by SDS-PAGE, transferred to PVDF, and probed with the appropriate antibodies.

Cell-based N1-ligand binding assay

HEK293T cells (8.5 x 10^5) were seeded in a 3.5 cm culture plate and co-transfected with 1.5 μg of wild type or mutant pcDNA1-N1-myc, 0.4 mg of GFP plasmid, and 0.75 μg of either SEAP (EV), LfngAP (Lfng), MfngAP (Mfng) or RfngAP (Rfng) plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. At 28-30 h post-transfection, the cells were dissociated with cold PBS, pH 7.4, containing 1% bovine serum albumin (BSA) and resuspended in binding buffer (1 mM $CaCl_2$, 1% BSA and 0.05% NaN_3 in Hanks' balanced salt solution, pH 7.4, Gibco). Cells (0.5-1 x 10^6) were incubated with 100 μl of increasing concentrations of DLL1-Fc or J1-Fc as indicated for 1 h at 4°C. After binding, the cells were washed with binding buffer and then incubated with secondary antibodies PE-goat anti-mouse IgG (1:100) or PE-anti-human IgG (1:100) for 30 min at 4°C (Fig. 1E). In Figs. 3B and D we altered the ratio of the Fringe plasmids to the N1 plasmids to observe the effect of increasing Fringe on N1-ligand binding. Total plasmid was maintained at 0.75 μg by making up the difference with empty vector (SEAP). In these assays, we used a fixed concentration of soluble ligands in the linear range of the binding curve (3.6 nM DLL1-Fc or 2.1 nM J1-Fc) pre-incubated with PE-Anti-human or mouse IgG (1:100) for 30 min at 4°C. The clustered ligands were incubated with N1 expressing cells for 1 h at 4°C. Cells then were washed with binding buffer and analyzed with a FACSCalibur (BD, Bioscience) flow cytometer. The gate was set to collect the GFP positive population of 20,000 events for each sample and analyzed using CellQuest Pro Softwaer (BD, Bioscience).

Cell-based co-culture N1 activation assay using HEK293T cells

HEK293T cells (0.3 x 10^5) were seeded in a 48-well tissue culture plate and co-transfected (using PEI) with 0.05 μg of Flag-hN1-Gal4 in pcDNA5 FRT/TO and 0.05 μg of Gal4-firefly luciferase reporter construct (generously provided by Dr. Stephen C. Blacklow (Andrawes et al., 2013)), 0.025 μg of either SEAP (EV), LfngAP (Lfng), MfngAP (Mfng) or RfngAP (Rfng) plasmid, and 0.025 μg of gWIZ β-galactosidase construct for transfection efficiency normalization. After 4 h, either L cells or L cells stably expressing J1 or DLL1 were overlaid on the transfected HEK293T cells at a density of 1.2 x 10^5 cells/well for another 24 h. Cells were lysed and luciferase assays were performed based on the manufacture's instructions (Luciferase Assay System, Promega) as described previously (Rana et al., 2011; Yamamoto et al., 2012).

Statistical Analysis

Statistical significance of the Fringe effect (-Fng versus +Fng for each sample) was determined using one-way ANOVA. Significance levels: (***) for $p < 0.0001$, (**) for $P < 0.001$, (*) for $p < 0.01$ for cell-based N1 signaling assay, and (***) for $p < 0.0005$, (**) for $P < 0.005$, (*) for $p < 0.05$ for N1-ligand binding assays and cell surface N1 expression (Fig. S3B and C).

Exoglycosidase digestions

Purified N1 EGF24-28 was concentrated by acetone precipitation and resuspended in 50mM sodium phosphate, pH6.0. Sialidase A (10 mU, GLYKO) alone or both sialidase A and β1,4-galactosidase (2mU, *Streptococcus pneumoniae*, SIGMA) were added (or mock-digested without enzyme) and incubated for 2 h at 37 °C. The reaction was terminated by

acetone precipitation. The proteins were processed for in-gel digestion and mass spectral glycoproteomic analysis as described above.

Cell surface biotinylation

HEK293T cells were co-transfected with 2 μg of pcDNA-N1 WT and 1 μg of either SEAP (EV), LfngAP (Lfng), MfngAP (Mfng) or RfngAP (Rfng) plasmid per 6-cm dish using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. After 48 h, the cells were washed three times in cold phosphate-buffered saline (PBS) containing 0.3 mM $CaCl_2$ and them incubated in PBS containing 1 mg/ml of Sulfo-NHS-Biotin (Santa Cruz) for 45 min at 4˚C. Cells were washed 3 times with cold PBS and lysed with 1% NP-40 in 20 mM Tris, pH 7.4, 0.15 M NaCl, and protease inhibitor (Complete mini, EDTA free, Roche). Equal amounts of whole cell lysates (WCL) proteins, as determined by Dye Reagent Concentrate (Bio-RAD), were incubated with streptavidin at 4˚C for 2 h. The WCL and cell surface N1 pull-down by Streptavidin Agarose (Thermo) (SAV) were analyzed by Western blot. Samples were run on a 6% SDS-PAGE, transferred to PVDF membrane and detected using 9E10 (1:2000, Stony Brook University Cell Culture/Hybridoma) as a primary antibody and Goat anti-mouse IgG (Jackson) as a secondary antibody.

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