# Glycosylation of specific Notch EGF repeats by *O*-Fut1 and Fringe regulates Notch signaling in *Drosophila*

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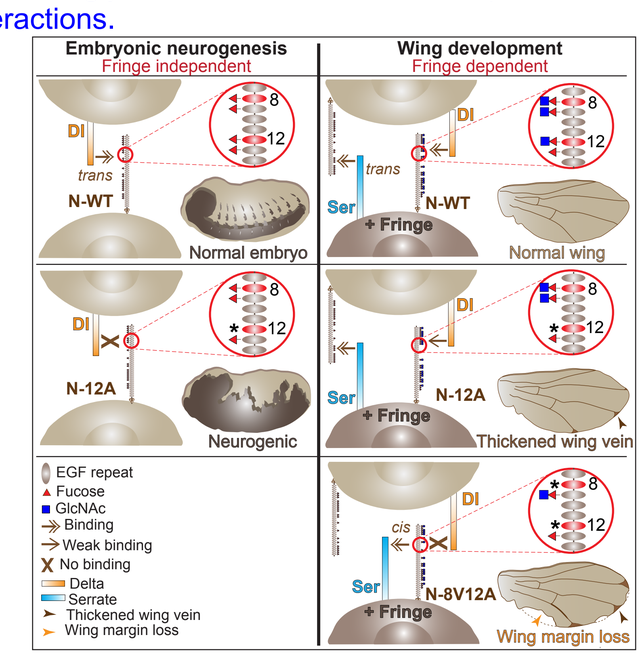
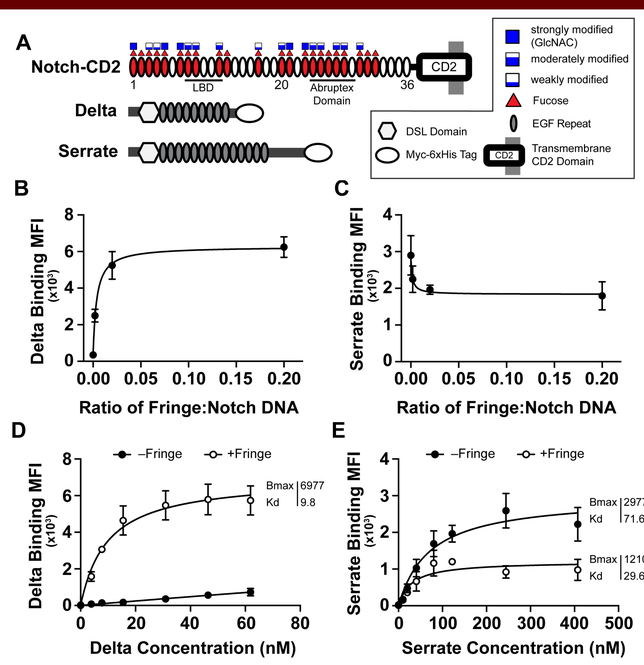
## Abstract

Fringe glycosyltransferases differentially modulate the binding of Notch receptors to Delta/DLL versus Serrate/Jagged ligands by adding N-acetylglucosamine (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. Here, we report important roles in Drosophila Notch signaling for GlcNAc-fucose-*O* glycans on three sites: EGF8, 9 and 12. *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 addition of GlcNAc to EGF8 and 12 by Fringe, with minor contribution from EGF9. Fringe modifications of EGF8 and 12 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.



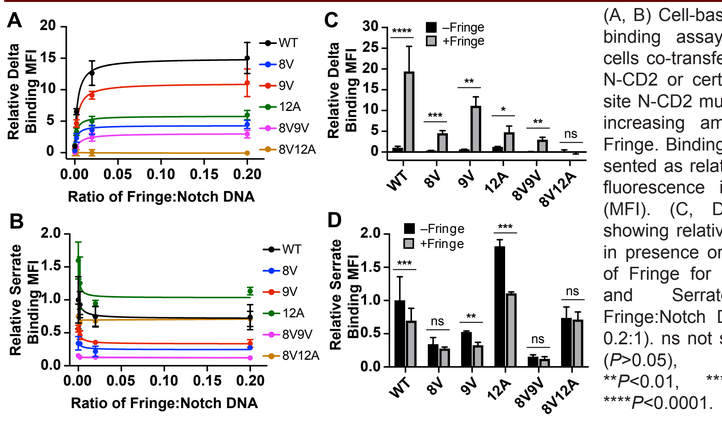

## Results

### 1. A modified Notch-ligand binding assay recapitulates Fringe *in vivo* effects and reveals differential mechanistic effects on Notch binding to Dl and Ser




(A) Schematic of Notch-CD2 containing 1-36 EGF repeats from *Drosophila* Notch fused to a rat CD2 protein. EGF repeats having an *O*-fucose consensus sequence (C2X4-5(S/T)C3) are filled red. *O*-fucose: red triangle, GlcNAc: blue square. (B-C) Cell-based ligand binding assays of S2 cells co-transfected with N-CD2 and increasing amounts of Fringe. Cells were incubated with 15.5 nM Dl-Myc-6xHis (B) or 203.7 nM Ser-Myc-6xHis (C) pre-clustered with PE-conjugated anti-myc antibody. MFI: Mean fluorescence intensity. (D-E) Ligand binding assays of S2 cells co-transfected with N-CD2 and empty vector (–Fringe) or a Fringe expression vector (+Fringe, Fringe:Notch DNA ratio 1:1) incubated with varying amounts of Dl-Myc-6xHis (D) or Ser-Myc-6xHis protein (E).

### 2. *O*-fucosylation sites in Notch EGF8, 9 and 12 are important for Fringe-mediated modulation of Notch-ligand binding *in vitro*

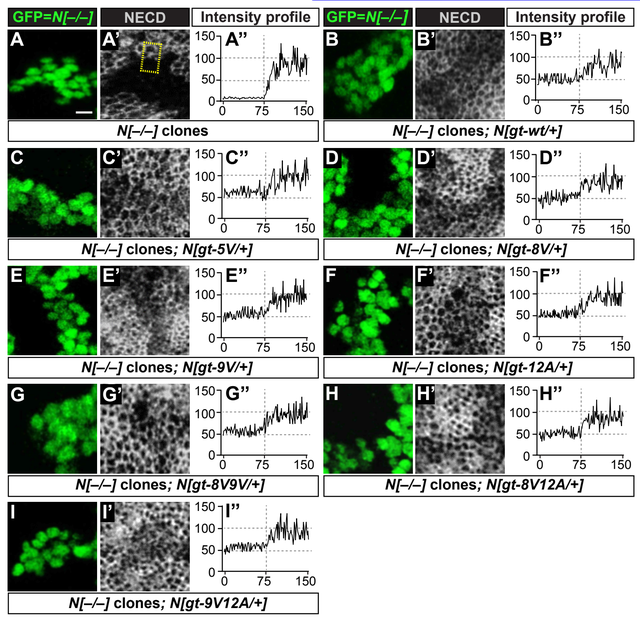



(A, B) Cell-based ligand binding assays of S2 cells co-transfected with N-CD2 or certain *O*-fuc site N-CD2 mutants and increasing amounts of Fringe. Binding is represented as relative mean fluorescence intensities (MFI). (C, D) Graph showing relative binding in presence or absence of Fringe for Delta (C) and Serrate (D). Fringe:Notch DNA ratio 0.2:1). ns not significant ($P$>0.05), *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001.

### 3. *O*-Fucosylation site mutations in individual EGF repeats or pairwise combinations do not affect the cell surface level of Notch *in vivo*

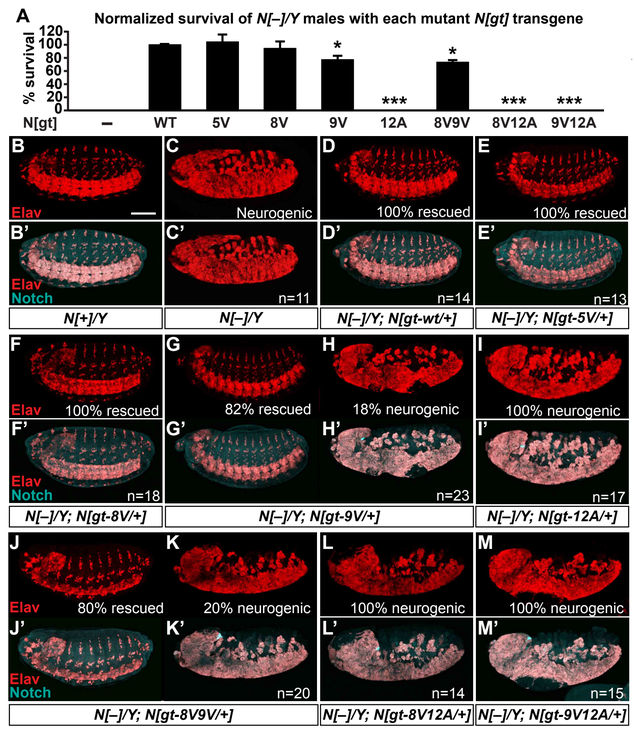
Mutant *Drosophila* Notch transgenes generated

| | |
|---|---|
| EGF 5(5V) | EGF 8&9(8V9V) |
| EGF 8(8V) | EGF 9&12(9V12A) |
| EGF 9(9V) | EGF 8&12(8V12A) |
| EGF 12(12A) | |




Clonal analysis in wing imaginal discs of third instar Drosophila larvae raised at 30°C are shown. Nuclear GFP (green) marks MARCM clones of the *N[54l9]* null allele (*N[–/–]*). In A-I, *N[–/–]* 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 genomic transgenes. Detergent-free surface staining is performed using anti-NECD antibody (Gray in A'-I').

### 4. Mutation in the Notch EGF12 O-fucosylation site abolishes its ability to rescue lethality and neurogenic phenotype in Notch null males

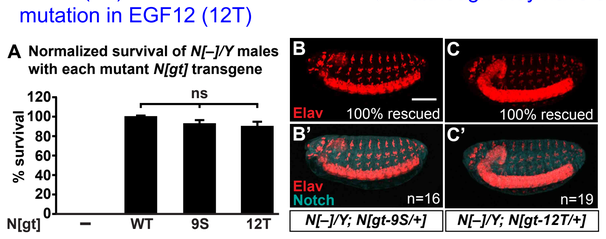



(A) Graph showing the survivability of Notch hemizygous males to adulthood in the presence of wild-type and mutant Notch transgenes. Data presented as mean ± SD, *$P$<0.05, ***$P$<0.001 (One-way ANOVA followed by Dunnett's post hoc test). (B-M') Elav (red) and Notch (Magenta) staining is shown in the indicated genotypes. Scale bar in (B) is 50 µm and applies to all panels.

### 5. 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

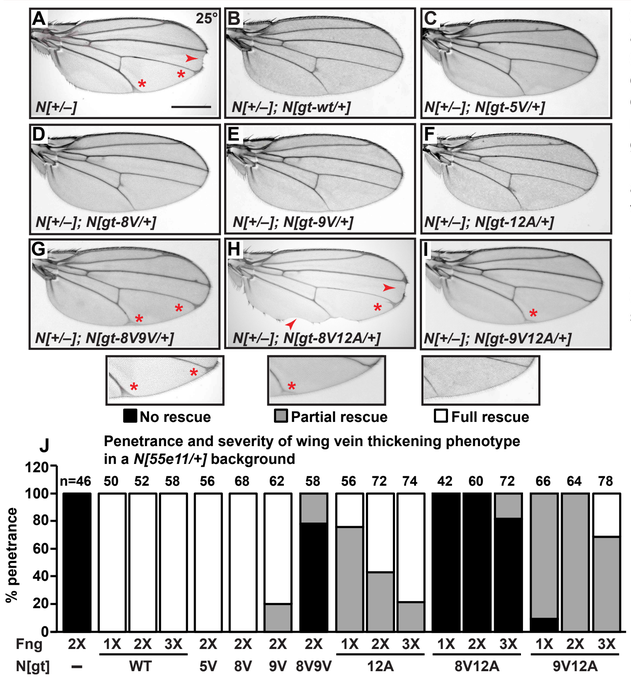
Effects of O-fucose site mutations in EGF9 (9V) and EGF12 (12 A)
- Loss of O-linked fucose monosaccharide **?**
- Altered configuration of these EGF repeats due to amino acid change **?**

Threonine-to-Serine mutation in EGF9 (9S) & Serine-to-Threonine mutation in EGF12 (12T) → Allow O-fucosylation of EGF9 and 12 although they have changed amino acid



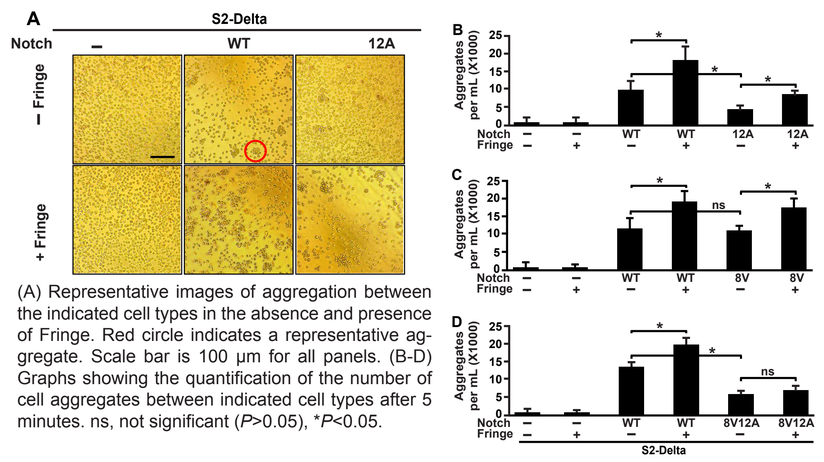

(A) survivability of Notch hemizygous males harboring a *N[gt-9S]* or *N[gt-12T]*. (B-C') Elav (red) and Notch (Magenta) stainings are shown for the indicated genotypes. Scale bar in (B) is 50 µm and applies to panels B-C'. ns not significant ($P$>0.05).

### 6. Fringe modification of EGF8 and 12 plays a major role in Dl-Notch signaling during wing vein formation



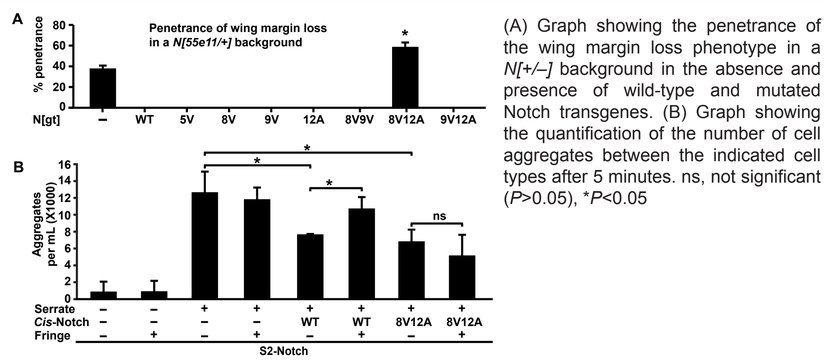

(A-I) Wing vein thickening (red asterisk) and wing margin loss (red arrowhead) phenotypes of *N[+/–]* flies in the absence or presence of wild-type and mutant Notch genomic transgenes. All animals were raised at 25ºC. (J) Percentages of each class of wing vein thickening phenotype (no rescue, partial rescue and full rescue) in panels A-I and upon removing (1X) or adding (3X) one copy of fringe (*fng*) in some genetic backgrounds.

### 7. Decreased Notch-Dl binding upon loss of *O*-fucosylation at EGF12 and abolished Fringe effect on Notch-Dl binding upon loss of the GlcNAc-fuc-*O* glycans at both EGF8 and 12



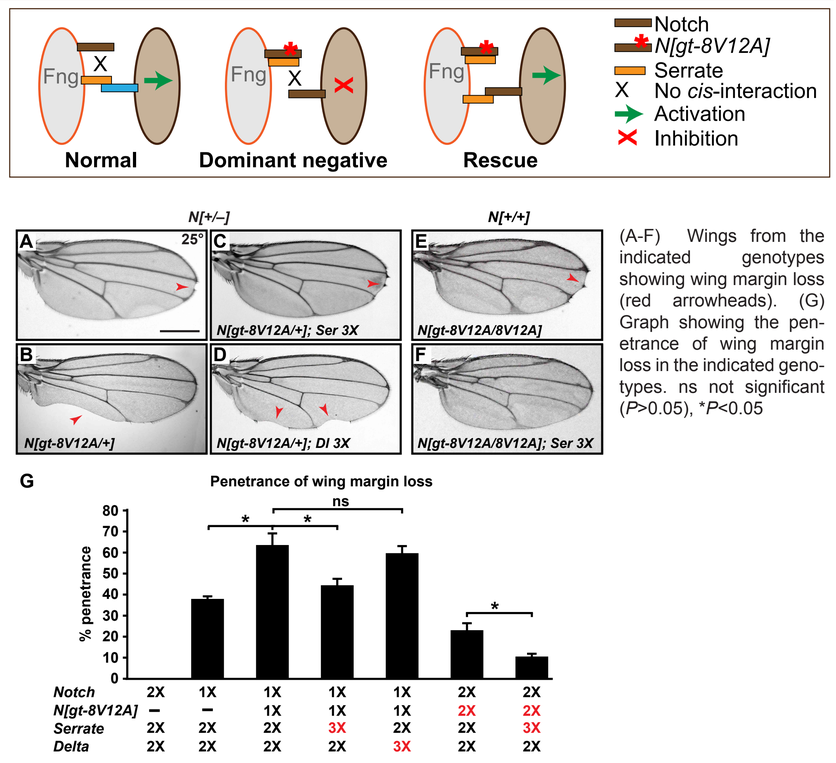

(A) Representative images of aggregation between the indicated cell types in the absence and presence of Fringe. Red circle indicates a representative aggregate. Scale bar is 100 µm for all panels. (B-D) Graphs showing the quantification of the number of cell aggregates between indicated cell types after 5 minutes. ns, not significant ($P$>0.05), *$P$<0.05.

### 8. *N[gt]* with *O*-fucose mutations in both EGF8 and 12 shows a dominant negative wing margin loss phenotype *in vivo* and enhances *cis*-binding with Ser in S2 cell-aggregation assays




(A) Graph showing the penetrance of the wing margin loss phenotype in a *N[+/–]* background in the absence and presence of wild-type and mutated Notch transgenes. (B) Graph showing the quantification of the number of cell aggregates between the indicated cell types after 5 minutes. ns, not significant ($P$>0.05), *$P$<0.05

### 9. Increasing *Ser* gene dosage suppresses the dominant negative wing margin loss phenotype exhibited by *N[gt-8V12A]*




(A-F) Wings from the indicated genotypes showing wing margin loss (red arrowheads). (G) Graph showing the penetrance of wing margin loss in the indicated genotypes. ns not significant ($P$>0.05), *$P$<0.05

## Conclusions

# Fringe regulates fly Notch signaling by adding GlcNAc to *O*-fucose on 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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