What are the Real Functions of *O*-Glycan Modifications of Notch?

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Abstract

Notch is a transmembrane protein receptor that mediates direct cell–cell interactions and controls various cell-fate specifications in metazoans. The extracellular domain of Notch contains 36 tandem epidermal growth factor (EGF)-like repeats, most of which have *O*-linked glycan modifications: *O*-glucose, *O*-fucose, and *O*-GlcNAc. The function of these individual glycans in Notch signaling activation has been investigated by elimination of single modification sites and by knockout of individual glycosyltransferases. Single site mutants show weaker phenotypes compared with glycosyltransferases knockouts in Notch signaling activation. Thus, the collaboration between two or more glycan modifications appears to be essential for full Notch activation. In this review, we describe the history of Notch's glycan modifications, the individual functions of the modifications, and how glycan modifications might collaborate in regulating Notch function.

A. Introduction

The first notched wing in *Drosophila* was observed in Dr. John S. Dexter's laboratory in 1914 (1) and further analyzed in Dr. Thomas Morgan's laboratory in 1917 (2, 3). The gene name *Notch* is derived from this phenotype, since heterozygotes of *Notch* mutations have a “notched” wing (4). Molecular cloning of the gene encoding *Drosophila* Notch was originally reported by Dr. Spyros Artavanis-Tsakonas' laboratory, revealing that Notch is a 280 kDa transmembrane protein with 36 tandem Epidermal Growth Factor-like (EGF) repeats in its extracellular domain (5, 6). A novel gene, *fringe*, was identified as a segmentation gene (7) and modulator of Notch activity in flies (8). Fringe, encoded by *fringe*, was reported to share sequence similarity with several bacterial galactosyltransferases, suggesting it might alter Notch activity by altering glycan structures on the receptor (9). Soon afterwards, the Haltiwanger laboratory found that Notch is modified by *O*-glycan and *N*-linked glycan modifications (10) and that Fringe is a β -1,3-*N*-acetylglucosaminyltransferase (β 3GlcNAcT), modifying *O*-linked fucose on Notch EGF repeats (11, 12). After Fringe was identified, several other glycosyltransferases responsible for adding glycans to Notch were found, and knockouts of these enzymes in mice and flies revealed that they also affect Notch activity. Here we review what is known about the glycan structures on Notch and raise several questions regarding how they function.

B. Notch Signaling Activation

The Notch protein is a type I transmembrane receptor and its structure is well conserved from *Drosophila* to humans (13, 14). The Notch extracellular domain (NECD) is modified during biosynthesis by *O*-linked glycan modifications in the ER and Golgi (Fig. 1). Notch is cleaved by Furin protease (S1 cleavage)

in the *trans*-Golgi network, generating a heterodimer held together by non-covalent bonds (13–15). Notch is then transported to the plasma membrane where the NECD can bind to its ligands on adjacent cells. *Drosophila* Notch's ligands are Delta (mammalian Delta-like-ligand1, 3, 4) and Serrate (mammalian Jagged1, 2), which are also type I transmembrane proteins (13, 14, 16). Physical interaction between the NECD and Notch ligands occurs between apposing cell membranes (Fig. 1). Endocytosis of the ligand results in a pulling force on Notch, causing the Notch Negative Regulatory Domain (NRR) to unfold, exposing a protease site that can be cleaved by the cell-surface protease, Kuzbanian/ADAM10 (S2 cleavage) (13, 14, 17). This is followed by another cleavage by γ -secretase (S3 cleavage), releasing the Notch intracellular domain (NICD) from the endosome (13, 14, 18) (Fig. 1). The liberated NICD translocates to the nucleus (13, 14, 18), where it forms a complex with transcriptional co-activators to activate the expression of Notch's downstream target genes (14, 19) (Fig. 1).

C. Glycan Modifications of Notch

The EGF repeats in the NECD can be modified by several types of *O*-linked glycans (20, 21) (Fig. 2A). An EGF repeat has a characteristic structure; it contains six cysteine residues that form three disulfide bonds (1st and 3rd, 2nd and 4th, and 5th and 6th cysteines form disulfide bonds, respectively) in an EGF-like structure (Fig. 2A). Three *O*-linked glycan modifications are known to occur on Notch EGF repeats: *O*-glucose (10, 22, 23), *O*-fucose (10, 11, 24), and *O*-GlcNAc (25–27). These individual modifications have been analyzed to determine their specific functions, leading to potential models describing how glycosylation regulates Notch receptor functions by using model animals.

EGF repeats containing the Cys¹-X-Ser-X-(Pro/Ala)-Cys²

Fig. 1. The Notch signaling activation. Notch is modified by *O*- and *N*-linked glycan in ER and Golgi and cleaved by Furin (S1) during biosynthesis. Notch is then transported to cell surface where it can bind to its ligands. Pulling force generated by ligand endocytosis exposes a protease site for cell-surface ADAM protease (S2), initiating Notch signaling. This is followed by release of the Notch intracellular domain (NICD) by γ -secretase (S3), which moves into the nucleus and activates transcription of target genes.

consensus sequence are typically modified on the Ser with an *O*-glucose glycan modification that is a trisaccharide, EGF-glucose-xylose-xylose (10) (Fig. 2B). The *O*-glucose is attached to the Serine by protein *O*-glucosyltransferase 1 (POGLUT1, *Drosophila* Rumi), and then Glucoside Xylosyltransferase 1/2 (GXYLT1/2, *Drosophila* Shams) can add xylose to this *O*-glucose. The 3rd xylose can then be attached by Xyloside Xylosyltransferase 1 (XXYLT1, not found in *Drosophila*) to generate the Xyl α 1-3Xyl α 1-3Glc α -*O*-Ser trisaccharide.

EGF repeats containing the Cys²-X-X-X-X-(Ser/Thr)-Cys³ consensus sequence are typically modified with an *O*-fucose glycan modification on the Ser/Thr (10) (Fig. 2B). In mammals, the *O*-fucose can be extended to a tetrasaccharide, Sia α 2-3/6Gal β 1-4GlcNAc β 1-3Fuca-*O*-Ser/Thr, but in *Drosophila* only to the disaccharide GlcNAc β 1-3Fuca-*O*-Ser/Thr (28). An unusual *O*-fucose trisaccharide, GlcNAc β 1-3(GlcA β 1-4)Fuc, has been detected in *Drosophila* extracts (29), but not on the Notch receptor (30). The *O*-fucose is attached to the Serine/Threonine by protein *O*-fucosyltransferase 1 (POFUT1) (*Drosophila* *O*-fut1) (31) (Fig.

2B), then a Fringe β 3-*N*-acetylglucosaminyltransferase (*Drosophila* Fringe (Fng), Lunatic, Manic or Radical Fringe (L/M/RFNG) in mammals) adds GlcNAc to the fucose (10, 11) (Fig. 2B). The galactose and sialic acid are added by UDP-Gal: β GlcNAc β 4 galactosyltransferase, polypeptide 1 (β 4GalT1) and α 2,3/6-sialyltransferase (ST3/6Gal1) in mammalian cells, but not in *Drosophila*.

Finally, EGF repeats containing the Cys⁵-X-X-Gly-X-(Ser/Thr)-X-X-Cys⁶ sequence are sometimes modified on the Ser/Thr with an *O*-GlcNAc (25) (Fig. 2B). This *O*-GlcNAc is attached by EGF Domain-Specific *O*-linked *N*-acetylglucosamine Transferase (EOGT) (*Drosophila* Eogt) (26, 27).

D. The Function of the *O*-Glucose Modifications

The gene *rumi* is widely expressed in *Drosophila* (22), and *rumi* mutants show a similar phenotype to that of Notch signaling mutants at 28°C, but not at 18°C (22). Thus, the monosaccharide *O*-glucose glycan appears to be essential for Notch signaling at 28°C. In *rumi* mutant cells, Notch accumulates in both the ER and the apical cell membrane at 28°C (22). However, the binding affinity

Fig. 2. O-glycan modifications of Notch EGF-like repeats. A. The schematic of full length *Drosophila* Notch. EGF repeats containing consensus sequences for O-fucose are marked with red triangles, O-glucose with blue circles, and O-GlcNAc with blue squares. A schematic of a single EGF repeat with O-fucose, O-glucose, and O-GlcNAc consensus sequences is shown below. B. Enzymes responsible for synthesis of O-glucose, O-fucose, and O-GlcNAc glycans.

between Notch and Delta is normal at 28°C in *Drosophila rumi* mutant cells (32). These observations suggest that the monosaccharide O-glucose is not required for ligand binding but affects Notch localization and is essential for the S2 cleavage of Notch at 28°C (22). The temperature sensitivity suggests that Notch is destabilized in the absence of O-glucose glycans on the receptor.

The expression of *shams* has not been studied in detail; however, in *shams*-mutant cells in wing imaginal discs, Notch accumulates at the apical cell membrane and the Notch signaling is hyper-activated (23), while the ectopic expression of *Drosophila shams* and human *GXYLT1/2* in wing disc cells reduces the Notch signaling activation (23). Generally, the Notch hyper-activation phenotype is caused by the acceleration of *trans*-activation, interaction of Notch with ligand on the neighboring cell, or reduction of *cis*-

inhibition, interaction of Notch and ligand on the same cell. Recent results show that the terminal di-xylose of the O-glucose-linked saccharides of Notch selectively impedes the binding of Notch with *trans*-Delta without affecting its binding with *cis*-ligands (33).

E. The Functions of O-Fucose Modifications

The *POFUT1* gene is widely expressed and essential for the Notch signaling activation, given that its null mutant phenotypes are similar to those of *Notch*-null mutants (24, 34, 35). Although the analyses of *O-fut1* mutants in *Drosophila* and *Pofut1* mutants in mice revealed that POFUT1 is essential for Notch signaling in these species, it was uncertain whether the enzymatic activity of POFUT1 is essential for Notch signaling *in vivo*. In *Drosophila O-fut1*-null cells, Notch accumulates in the ER, with less Notch cell

surface expression (36, 37). To test whether this was due to a loss of the fucosyltransferase activity, an *O-fut1* mutant with an amino acid substitution in its GDP-fucose-binding site, *O-fut1*^{R245A}, was generated (36). The 245th arginine of *O-Fut1* is essential for its binding to GDP-fucose, and the *O-fut1*^{R245A}-encoded protein largely lacks *O*-fucosyltransferase activity *in vitro* (36). However, over expression of *O-fut1*^{R245A} in *O-fut1*-null mutant cells rescued the Notch accumulation phenotype in wing imaginal discs, suggesting that Notch-specific chaperone activity was distinct from the fucosyltransferase activity (36). Different results have been obtained in mice depending on cell type. For instance, endogenous mouse NOTCH1 is not expressed on the cell surface but accumulates in the ER in *Pofut1*-null presomitic mesoderm (38), while Notch1, 2 and 3 are expressed on the surface of *Pofut1*-null mouse embryonic stem cells (39). Recently, mouse *POFUT1*^{R245A} (40) and *Drosophila O-fut1*^{R245A} (41) knock-in mutants were generated in mice and flies, respectively. *Drosophila O-fut1*^{R245A} knock-in mutants show the neurogenic phenotype, typical phenotype of loss of function of *Notch*, and accumulates Notch in the ER, at 30°C but not at 25°C (40). Thus, the monosaccharide *O*-fucose is essential for proper Notch localization at 30°C but not at 25°C in *Drosophila* (40). This temperature sensitive Notch phenotype is also observed in *Drosophila GDP-mannose 4,6-dehydratase (Gmd)* mutant, which should prevent *O*-fucosylation as well as fucosylation of *N*-glycans (40). These data suggest that the monosaccharide *O*-fucose modifications are essential for the Notch trafficking and signaling at 30°C but not 25°C in *Drosophila*. In mouse *POFUT1*^{R245A} knock-in mutants, Notch accumulates in the ER and is rarely observed on the cell surface, and this phenotype is also observed in *POFUT1* null mice (41). In both *Drosophila* and mouse studies, however, the R245A mutation destabilizes the *O-Fut1* and *POFUT1* protein, reducing *O-Fut1*^{R245A} and *POFUT1*^{R245A} protein levels *in vivo* (41), raising concerns about conclusions drawn from experiments using these mutations.

Demonstration that addition of *O*-fucose to Notch is directly involved in Notch-ligand interactions has come from studies examining the function of *O*-fucose on specific EGF repeats. Elimination of *O*-fucose sites on Notch1 EGF repeats 8 or 12, both part of the ligand-binding domain, reduce Notch1 binding to both Delta-like and Jagged-ligands as well reduce Notch1 activation by these ligands in cell-based assays (42). *In vitro* studies with purified proteins showed that the monosaccharide *O*-fucose modification on EGF12 enhances binding of Notch1 (EGF11-13) to DLL1, DLL4 and JAG1 (43). The recent Notch1-DLL4 co-crystal structure provides a molecular explanation for this enhancement by showing that the *O*-fucose on EGF12 (Thr466) is in direct contact with DLL4's module at the N-terminus of Notch ligands (MNNL)

(44). The more recent Notch1-Jagged1 co-crystal structure shows direct contact of *O*-fucose on both EGF8 and EGF12 with Jagged1 (45). Finally, the importance of *O*-fucose on EGF12 has also been demonstrated *in vivo*. Elimination of the *O*-fucose site on EGF12 in both flies and mice alters, but does not eliminate, Notch activity (46, 47).

The gene *fringe* is expressed in a region-specific manner in flies, and *fringe* mutants have the reduced Notch signaling activation in a subset of contexts (11, 48, 49). Notch modified with monosaccharide *O*-fucose binds to the Delta- and Serrate-type ligands equally well; however, *O*-fucose-GlcNAc disaccharide modification of Notch enhances the binding with the Delta-type ligands but not Serrate-type ligands (28, 49) (Fig. 3A). Modeling the GlcNAc onto *O*-fucose on EGF12 in the Notch1-DLL4 structure predicts enhanced interaction with the DLL4 MNNL domain (44). This may provide a molecular explanation for how the Fringe modification increase the affinity of Notch-Delta binding (Fig. 3B).

The mammalian genes encoding *Lunatic*, *Manic*, and *Radical fringe* are expressed in developmentally regulated and tissue specific patterns (50). LFNG, MFNG, RFNG is encoded by *Lunatic*, *Manic*, and *Radical fringe*, respectively (50). Knockout of individual *fringe* genes in mice shows distinct phenotypes suggesting differential functions (51–54). Interestingly, while LFNG and MFNG have similar effects on Notch1 function in cell-based assays (enhancing signaling from Delta ligands but inhibiting signaling from Jagged), RFNG activates signaling from both (42, 49). Recent results showed that LFNG and MFNG modify *O*-fucose on similar Notch1 EGF repeats, consistent with their effects on signaling (42). In contrast, RFNG modifies a subset of the same EGF repeats. Elimination of single *O*-fucose sites by mutagenesis revealed that Fringe modifications on EGF8 and 12 (modified by all three Fringes) enhance the ability of Delta ligands to activate Notch1, while modifications at EGF6 and 36 (modified by LFNG and MFNG but not by RFNG) inhibit Notch1 activation by Jagged1 in a dominant fashion (42). Thus, Fringes “mark” regions of the Notch ECD for either activation or inhibition of Notch function, allowing Notch to discriminate between ligands.

F. The Function of *O*-GlcNAc Modification

Eogt is mainly expressed in vascular cells in mice and *Drosophila* (27, 55). *Drosophila Eogt* mutants are larval stage lethal and *Eogt* mutant larva have dumpy phenotype in epithelial tissue (27). Dumpy is a protein which contains about 200 of EGF-like repeats, and *Eogt* mutants lack the Dumpy-dependent epithelial cell–matrix interactions; however *Eogt* mutants do not display the phenotype caused by defects in Notch signaling in *Drosophila* (27). Mouse *EOGT* mutants show a retinal angiogenesis pheno-

Fig. 3. Model of Fringe function. Notch binds to Delta type and Serrate type ligands when Notch has monosaccharide *O*-fucose modification (A: left). Fringe elongation enhances the binding between Notch and Delta (A: right), because Fringe elongation gives additional interaction to Notch EGF12-Delta MNLL (B) (44).

type: more blood vessels are observed in *EOGT* mutants compare with wild-type (55). *Eogt* has genetic interaction with *NOTCH1*, so reduced Notch signaling augments the blood vessel phenotype in *Eogt* mutants in mice *in vivo* (55). Adams–Oliver syndrome (AOS) is characterized by aplasia cutis congenita of the scalp and terminal transverse limb defects. Recessive forms of AOS are caused by mutations in several genes in the Notch pathway including *EOGT*, providing evidence that *O*-GlcNAc modification of EGF repeats plays an important role in Notch function in humans (56).

G. Collaboration between Two or More Glycan Modifications is Essential for Full Notch Function

Notch EGF11 and EGF12 are the classically defined ligand binding domain, and EGF12 has an *O*-fucose modification which is evolutionally conserved (57). As noted above, mouse Notch1 with EGF12 *O*-fucose site mutation (*mNOTCH1*^{12f}) significantly reduces Notch1 activity in cell-based assays (58). Nonetheless, *mNotch*^{12f} knock-in mutants do not display typical the *Notch* loss of function phenotypes *in vivo*, but show a *Notch* haploinsufficient phenotype (47). In contrast, *POFUT1* null mutant mice show the strong *Notch* loss of function phenotypes (58). These data suggest that not only Notch1 EGF12 *O*-fucose but also *O*-fucose on other Notch EGF repeats are important for Notch activity. The function of individual

O-fucose modifications were tested by eliminating of *O*-fucose sites on mouse Notch1 and testing effects using cell-based assays (42). Mouse *Notch1* EGF8 (*EGF8*^{T→V}) or EGF12 (*EGF12*^{T→V}) mutations result in diminished Notch signaling in cell-based assays, but *EGF8*^{T→V} and *EGF12*^{T→V} double mutation displayed a nearly complete loss of signaling *in vitro* (42). This data suggest that the collaboration between EGF8 and EGF12 *O*-fucose is essential for the Notch signaling activation.

Not only do the same glycan on different sites collaborate, but different glycans also collaborate in Notch trafficking and signaling. *O*-Fucose and *O*-glucose glycans are simultaneously found on many of the EGF-like repeats of Notch. In addition, *Drosophila* *O*-*fut1*^{R245A} and *rumi* mutants show a similar temperature-sensitive neurogenic phenotype (22, 40). These findings suggest that the monosaccharide *O*-fucose and *O*-glucose glycans have similar roles in Notch function in *Drosophila*. While single mutants of *O*-*fut1*^{R245A} or *rumi* do not show a neurogenic phenotype at 25°C, the double mutant of *O*-*fut1*^{R245A} and *rumi* exhibit the sensory organ precursors (SOPs) neurogenic phenotype at 25°C in *Drosophila* wing imaginal discs. These observations indicate that the monosaccharide *O*-fucose and *O*-glucose glycans on Notch have collaborative roles in the Notch signaling activation in *Drosophila* (40, 59). Notch is completely accumulated in ER in double mutant of *O*-*fu*-

Fig. 4. The collaborative functions of monosaccharide *O*-fucose and *O*-glucose glycan in Notch trafficking. After Notch is synthesized and modified by glycan modifications in ER and Golgi, it is transported to the plasma membrane first, and then relocated to the adherence junction (AJ) where Notch bind to its ligand via transcytosis in *Drosophila* wing imaginal discs (A) (60). The schematic of Notch localization in wild-type, *rumi*, *shams*, *O-fut1^{R245A}*, *rumi* and *O-fut1^{R245A}* double, and *shams* and *O-fut1^{R245A}* double mutant in *Drosophila* wing imaginal discs (B) (58).

t1^{R245A} and *rumi* in 25°C, although single mutants of *O-fut1^{R245A}* or *rumi* do not show the Notch trafficking defect (Fig. 4A, 4B). This suggests *O*-fucose and *O*-glucose collaborate in Notch trafficking from ER to cell membrane (Fig. 4A).

The *O*-glucose glycan on *Drosophila* Notch *in vivo* exists as mono-, di- or trisaccharide depending on the site (30). EGF14-EGF20 of *Drosophila* Notch are modified by di- or trisaccharide, and the other EGF repeats are modified by monosaccharide *O*-glucose (30). *O*-Fucose monosaccharide and *O*-glucose trisaccharide have a collaborative role in Notch signaling (40, 59). To address which part of the *O*-glucose glycan functions collaboratively with the monosaccharide *O*-fucose, *O-fut1^{R245A}* and *shams*

double mutants were generated in *Drosophila*. The double mutant of *Drosophila O-fut1^{R245A}* and *shams* exhibited a very weak SOPs neurogenic phenotype compare with *O-fut1^{R245A}* and *rumi* double mutant at 25°C (59). The double mutant of *O-fut1^{R245A}* and *shams* cells showed a distinct Notch trafficking defect at 25°C, Notch was accumulated on the cell surface and diminished from the adherence junction (AJ), but not accumulated in the ER, in the double mutant of *O-fut1^{R245A}* and *shams* cells in the wing imaginal discs of *Drosophila* (59) (Fig. 4A, 4B). Notch is transported in order of the ER, the cell membrane, and AJ in wing imaginal discs of *Drosophila* (60). This suggests *O*-fucose and dixylose modification of *O*-glucose collaborate in Notch trafficking from cell membrane to AJ. And those results also suggest that the “monosaccharide *O*-fucose and *O*-glucose glycans on Notch” and “monosaccharide *O*-fucose and di-xylose modification of *O*-glucose glycan modification on Notch” on Notch have distinct collaborative functions in Notch trafficking and signaling, respectively.

In mammalian cells, *O*-fucose and *O*-glucose modifications also appear to collaborate, although slightly differently than in flies. Notch1 levels are reduced about 50% on the cell surface of *POGLUT1* or *POFUT1*-null HEK293T cells, but are completely eliminated in *POGLUT1/POFUT1* double mutant cells (61). The protein appears to accumulate in the ER in the double mutant cells (61). These results suggest that *POGLUT1* and *POFUT1* function collaboratively in Notch trafficking from ER to the cell surface in HEK293T cells (61). Each cell type has a unique chaperone protein expression pattern and the chaperone protein expression is changed by stress (ex. heat) condition of cells. All *Drosophila* experiments were performed at 18–28 degree, while mammalian cell experiments were performed at 37 degree. Thus, some differences in result may be caused by differences in cell-specific chaperone expression.

H. Finally ...what Factors Regulate the Glycan Modifications of Notch?

It is clear from these studies that the Notch signaling is regulated by its glycan modifications. Glycan modification of Notch may be one of the major switches controlling Notch signaling activation in several contexts. Clearly, developmental or tissue-specific genetic regulation of the enzymes responsible for the addition of the glycans is a major factor in determining their structure and subsequent effects on Notch activity. In addition, some recent results suggest that Notch glycosylation can also be influenced by environmental factors. For instance, Fringe modification of Notch appears to be regulated by insulin signaling in *Drosophila* ovary. *FOXO*, a transcriptional factor that is suppressed by the insulin signaling, regulates *fringe* expression in the *Drosophila* ovarian stem

cells. So *fringe* expression is regulated by diet conditions, resulting in changes in Notch-Delta and/or Notch-Serrate signaling (62). Also in human cells, Notch glycan modifications can be regulated by nutrient in some contexts. For instance, *O*-GlcNAc modification of Notch1 (Fig. 2) is limited by the amount of donor substrate *in vitro*. Addition of glucose, GlcNAc, or glucosamine to the media results in the more *O*-GlcNAc modifications of Notch1 (63). *O*-

glucose modifications of Notch may also be affected by alterations in glucose levels in a system.

The Notch signaling activation is regulated by two or more glycan modifications. Further studies are essential; however, changes in nutrients may regulate Notch signaling activation mediated by altering its glycan modifications simultaneously.

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Robert S. Haltiwanger, Ph.D.: Dr. Haltiwanger received his B.S. in Biology (1980) and Ph.D. in Biochemistry (1986) from Duke University. He went on to do postdoctoral work at Johns Hopkins University School of Medicine, and took his first independent position as an Assistant Professor in the Department of Biochemistry and Cell Biology at Stony Brook University (1991). He rose through the ranks to full Professor and served as Chair of that Department for 8 years. He moved to the CCRC in 2015 as the Georgia Research Alliance Eminent Scholar in Biomedical Glycosciences. He has served as President of the Society for Glycobiology, Chair of the Glycobiology Gordon Conference, and currently serves as Editor-in-Chief of the journal *Glycobiology*.