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Multiple Roles for O-Glycans in Notch Signalling

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Abstract

Notch signalling regulates a plethora of developmental processes and is also essential for the maintenance of tissue homeostasis in adults. Therefore, fine-tuning of Notch signalling strength needs to be tightly regulated. Of key importance for the regulation of Notch signaling are O-fucose, O-GlcNAc and O-glucose glycans attached to the extracellular domain of Notch receptors. The EGF repeats of the Notch receptor extracellular domain harbor consensus sites for addition of the different types of O-glycan to Ser or Thr, which takes place in the endoplasmic reticulum. Studies from *Drosophila* to mammals have demonstrated the multifaceted roles of O-glycosylation in regulating Notch signalling. O-glycosylation modulates different aspects of Notch signalling including recognition by Notch ligands, the strength of ligand binding, Notch receptor trafficking, stability and activation at the cell surface. Defects in O-glycosylation of Notch receptors give rise to pathologies in humans. This Review summarizes the nature of the O-glycans on Notch receptors and their differential effects on Notch signalling.

Keywords

Notch signalling; Glycosylation; Glycosyltransferases; O-Glucose; O-Fucose; O-GlcNAc

Introduction:

Notch signalling is a well-characterized, evolutionarily-conserved pathway that plays multiple roles in the regulation of embryonic development, and in the maintenance of tissue homeostasis. Defective Notch signalling leads to numerous pathologies in development, and to different adult diseases [1]. In mammals, there are four different Notch receptors (NOTCH1 to NOTCH4), whereas *Drosophila* has only one homologue which is most similar to NOTCH1. Notch receptors are single transmembrane glycoproteins, comprising an extracellular domain (NECD), a transmembrane region, and an intracellular domain (NICD) (reviewed in [2–4]). The NECD comprises 29–36 epidermal growth factor-like (EGF) repeats, which include Notch ligand-binding domains. The EGF repeats are followed by the negative regulatory region (NRR), which is composed of three cysteine-rich Lin12 Notch repeats and a heterodimerization domain (HD). NECD is non-covalently linked at the HD to the N-terminal 12 amino acids of NICD that are external to the transmembrane domain

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

(termed NEXT for Notch extracellular truncation; [5]). NICD and NECD are generated by furin cleavage at the S1 site during passage through the Golgi. NICD is characterized by an RBP-J κ -associated module (RAM) and ankyrin (ANK) repeats, both of which are required for interactions with the DNA-binding complex CBF1–Suppressor of Hairless–LAG1 (CSL). Near the C-terminus of Notch receptors is a PEST domain, which regulates NICD degradation by the proteasome. Between the ANK repeats and the PEST domain, NICD also contains several nuclear localization signals, and a domain that confers transactivation of transcriptional repressors (TAD).

Notch signalling is mediated by short-range, cell-cell interactions between signal-sending cells expressing Notch ligands, and signal-receiving cells expressing Notch receptors. Signalling strength is fine-tuned by numerous factors, including the expression of Notch ligands that cause cis-inhibition of Notch receptors in signal-receiving cells, molecules involved in secretory pathway trafficking, and the O-glycans attached to NECD. The canonical Notch signalling pathway involves Notch ligands Delta or Serrate (in *Drosophila*) and Delta-like or Jagged (in mammals), binding to NECD of Notch receptors and initiating two, sequential proteolytic cleavages. The first is caused by a disintegrin and metalloprotease (ADAM) and occurs at the S2 site adjacent to the Notch transmembrane domain [6, 7]. This generates soluble NECD bound to Notch ligand that is endocytosed into the signal-sending, ligand-expressing cell [8], and the membrane-bound NEXT fragment described above. The second cleavage occurs within the transmembrane domain of Notch receptors at the S3 site, and is catalyzed by a complex that includes presenilins and has γ -secretase activity [9]. Released NICD complexes with CSL/RBP-J κ , recruits the co-activator Mastermind (MAML) and other factors, and the complex activates Notch target genes [10–12]. Ligand-induced Notch receptor cleavage (activation) alters the expression of many Notch target genes which regulate diverse signalling outcomes, ranging from cell proliferation to cell fate determination, and cell death. Aberrant changes in Notch signalling cause disorders of development and adult diseases. Therefore, precise temporal and spatial regulation of Notch signalling at appropriate levels is critical for optimal Notch signalling [13].

The EGF repeats in NECD are post-translationally modified by distinct O-glycans. Glycosyltransferases catalyze the addition of O-glycans to Notch EGF repeats by transferring fucose from GDP-fucose, glucose from UDP-glucose or N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a Ser or Thr residue in a specific consensus sequence. Each sugar may subsequently be extended by the addition of 1–3 sugar residues added sequentially (Fig. 1). The different O-glycans on NECD can regulate similar or different aspects of Notch signalling (reviewed in [14–18]). Roles for O-glycosylation of Notch have been performed *in vivo* in different tissues, and in culture using cell-based assays with various cell lines. More recently, with the advent of exome sequencing, several human pathologies have been associated with defects in the O-glycosylation of Notch receptors (Fig. 2). This Review will describe the O-glycans of NECD and the different functions of each type of O-glycan in Notch signalling in cells and organisms. It must be noted that Notch ligands also contain EGF repeats that may be O-glycosylated, and a limited subset of other secretory pathway proteins contain EGF repeats with appropriate consensus sequence(s) for modification with O-fucose, O-glucose or O-GlcNAc glycans [19–21].

Functions of O-glycans attributed to Notch signalling arising from mutations in glycosyltransferase genes are therefore based on evidence of Notch signalling dependence using Notch signalling reporter constructs, gamma-secretase inhibitors (GSI) that prevent S3 cleavage, effects on the expression of Notch pathway genes, and also the similarity of observed phenotypes to mutants with defects in other Notch pathway members. Mutation of O-glycan attachment sites (Ser/Thr) to investigate functions of a specific O-glycan is also an important strategy. However, such experiments require probing of several amino acid changes that abolish O-glycosylation, as well as the exchange of Ser to Thr or Thr to Ser respectively, to establish that it is the missing O-glycan, and not the altered amino acid, that is required at a given attachment site [22].

O-Fucose Glycans

• Discovery and cell-based assays

In a *Drosophila* screen for modifiers of Notch signalling, a gene named Fringe was shown to be required for Notch signalling at the dorsal/ventral boundary of the wing disc in third instar larvae [23, 24]. Fringe was subsequently shown to be a glycosyltransferase that transfers GlcNAc in β 1,3-linkage to O-fucose on certain Notch EGF repeats [25, 26]. These findings established a new paradigm of glycan regulation of a cell fate-determining signalling pathway. Amongst potential substrates of Fringe, Notch receptors contain a high number of putative consensus sites for the O-fucose modification [27], and all are indeed modified [28, 29]. Based on *in silico* and structural studies, the consensus site for the addition of O-fucose is C^2xxxxS/TC^3 (where Ser (S) or Thr (T) accepts the fucose, and C^2 and C^3 are the second and third cysteines of the EGF repeat; x is any amino acid) [19, 21]. The enzyme responsible for the addition of O-fucose to appropriate EGF repeats, protein O-fucosyltransferase 1 (POFUT1), is encoded by *Ofut1* in *Drosophila* and *Pofut1* in mammals [30]. *Pofut2* encodes a distinct protein O-fucosyltransferase that does not act on EGF repeats, but instead transfers O-fucose to thrombospondin type 1 repeats [31].

POFUT1 activity was first identified in Chinese hamster ovary (CHO) cells [32], and cloning revealed high sequence conservation from *C. elegans* to mammals [33]. The OFUT1/POFUT1 enzyme is a resident of the endoplasmic reticulum (ER) with an ER-retention signal at the C-terminus. OFUT1/POFUT1 catalyzes transfer of fucose most efficiently to properly-folded EGF repeats [25, 34, 35]. Loss of *Ofut1* in *Drosophila* S2 cells results in the loss of Notch ligand binding [36, 37]. CHO cells deficient in fucosylation exhibit reduced Notch signalling stimulated by Jagged 1 (JAG1) [25, 38]. Initial studies indicated that knockdown or loss of *Ofut1* did not alter Notch receptor expression at the cell surface [37, 39]. However, subsequent investigations suggested a more complex picture. One group provided evidence that *Drosophila* OFUT1 is a chaperone for Notch required for its trafficking out of the endoplasmic reticulum (ER) [40], while others proposed that OFUT1 was required later in the secretory pathway to maintain Notch stability at the cell surface [41]. To distinguish roles for the O-fucose on Notch EGF repeats versus O-fucosyltransferase activity in Notch receptor trafficking, experiments were performed with a mutant *Ofut1*^{R245A} which has little or no O-fucosyltransferase activity. *Ofut1*-null *Drosophila* expressing *Ofut1*^{R245A} were partially rescued in development, and Notch cell

surface expression was increased in embryos, consistent with enzyme-dead OFUT1 acting as a Notch chaperone [42]. Further analysis in other *Drosophila* mutants with intact *Ofut1*, but lacking the ability to synthesize GDP-fucose, showed that O-fucose has a role in Notch signalling that is not merely to provide a substrate for Fringe [43]. In *Pofut1*-null mouse embryonic stem (ES) cells [44], hematopoietic stem cells (HSC) [45] and CHO cells [46], Notch receptor cell surface expression is essentially unaltered or somewhat reduced [47], but Notch-ligand binding and ligand-induced Notch signalling are greatly reduced. Although *Pofut1*^{R245A} partially rescues Notch signalling in *Pofut1*-null ES cells, so does an enzyme-dead, ER glucosidase [44], suggesting that up-regulation of general chaperones may be responsible. Interestingly, overexpression of the human equivalent *POFUT1R240A*, in *POFUT1*-null human osteosarcoma (U2OS) cells partially rescues Notch ligand binding and Notch signalling [48]. However, mouse embryos that are homozygous for *Pofut1*^{R245A} die at mid-gestation, with a phenotype indistinguishable from *Pofut1*-null embryos [49]. This is presumably because of the unexpected finding that POFUT1(R245A) is degraded in embryos, making homozygous mutant embryos effectively null. Accumulation of NOTCH1 intracellularly is observed in *Pofut1*-null somites [49, 50]. By contrast, *Pofut1*-null inner ear cells [51], endocardium [46], and HSC [52] exhibit substantially reduced Notch signalling, but were shown in the latter two cases to express NOTCH1 at the cell surface equivalently to wild type.

Several groups have addressed the requirement for O-fucose versus POFUT1 by examining mutants unable to synthesize GDP-fucose (GDP-Fuc), the nucleotide sugar substrate of POFUT1. In the mouse, homozygous embryos that cannot make GDP-fucose are rescued by maternal GDP-Fuc to varying degrees [53, 54]. Homozygous mutants that are born have greatly reduced myeloid and lymphoid cells showing that POFUT1, which is present at normal levels, cannot rescue Notch signalling by chaperone activity. In Lec13 CHO cells which have very low levels of GDP-fucose, Notch receptors are well expressed at the cell surface, but Notch ligand binding and ligand-induced Notch signalling are markedly reduced, while POFUT1 levels are unaltered [44]. In *Drosophila*, embryos unable to synthesize GDP-Fuc do not show typical Notch neurogenic defects as observed if *Ofut1* is mutated, but die as first instar larvae, suggesting that O-fucose is required as a substrate of Fringe [42]. However, loss of GDP-Fuc synthesis in large areas of the wing reveal Fringe-independent Notch signalling defects [55]. Similar experiments reveal a temperature-sensitive loss of Notch signaling during neurogenesis in *Drosophila* mutants that cannot make GDP-Fuc [43]. At 30°C, Notch lacking O-fucose is unable to signal whereas signaling is normal at 25°C. Loss of GDP-Fuc from mouse HSC leads to a reduction in self renewal, and an altered ability to occupy the bone marrow niche [56]. Thus, it is apparent that OFUT1/POFUT1 present at normal levels is unable to rescue Notch signalling in several *in vivo* contexts in which Notch does not carry O-fucose glycans, but is nevertheless expressed at the cell surface. Therefore, broad generalizations about OFUT1/POFUT1 as a Notch chaperone required for cell surface expression of Notch cannot be made, since cellular context is clearly of utmost importance in determining effects on Notch receptor trafficking when OFUT1/POFUT1 is enzymatically inactive or absent.

While ligand-induced Notch signalling assays reveal that O-fucose glycans regulate the strength of Notch signalling, and flow cytometry and surface plasmon resonance assays

reveal effects on the binding of different Notch ligands, such assays do not address whether O-fucose glycans on Notch receptors physically interact with Notch ligands. However, recent X-ray studies have begun to elucidate mechanistic roles for O-fucose in Notch receptor-ligand binding. Thus, co-crystals of NOTCH1 EGF11–13 with a Delta-like 4 (DLL4) N-terminal ECD fragment identified precisely how the O-fucose on EGF12 of NOTCH1 interacts directly with the module at the N-terminus of Notch ligands (C2 domain) of DLL4 [57]. The authors propose that the fucose functions as a twenty-first amino acid. Subsequently, they solved co-crystals of a larger NOTCH1 fragment (EGF8-EGF12) and a JAG1 N-terminal ECD fragment [58]. In this case, they observed not only interactions with the O-fucose in NOTCH1 EGF12 with the C2 domain of JAG1, but also between EGF3 of JAG1 and the O-Fuc in EGF8 of NOTCH1, thereby extending the ligand binding domain of NOTCH1. This finding was consistent with the effects of a mutation in *Drosophila* Notch EGF8 that affects Serrate-induced, but not Delta-induced, Notch signalling [59]. Co-culture assays using engineered forms of Notch receptors and ligands have further defined mechanisms by which DLL1, DLL4 and JAG1 regulate Notch signalling [60, 61]. Single cell reporter assays showed that DLL1 stimulates Notch signalling in a pulsatile fashion, while DLL4 stimulates in a sustained manner [61]. These differences lead to different cell fate outcomes – DLL1-induced Notch signalling promotes myogenesis, whereas DLL4-induced signalling inhibits myogenesis [61, 62]. Such assays elegantly dissect the Notch signalling pathway and reveal the critical necessity of optimal regulation of Notch signalling strength. Too much, or too little Notch signalling may each have deleterious consequences.

The O-fucose modification on Notch may be elongated to form a disaccharide, trisaccharide or tetrasaccharide by the sequential addition of GlcNAc, galactose (Gal) and N-acetylneuraminic acid (NeuAc), respectively [25, 27]. Fringe adds GlcNAc to O-fucose on properly-folded EGF repeats at highest efficiency [25, 63]. *Drosophila* has a single Fringe, but there are three homologues in mammals - Lunatic (LFNG), Manic (MFNG) and Radical (RFNG) [64]. *In vitro* assays of recombinant enzymes show mouse LFNG to be the most active [65]. Co-culture Notch reporter assays and ligand binding assays revealed the important role of GlcNAc addition to O-fucose in promoting Delta binding to Notch receptors and Delta-induced Notch signalling, while simultaneously inhibiting Jagged binding to Notch and reducing Jagged-induced Notch signalling [25, 36, 38, 44, 66–69]. Mosaic experiments in *Drosophila* showed that removal of Fringe from Notch ligand-expressing cells of the wing disc did not alter ligand ability to induce Notch signalling [24]. Co-culture assays with CHO cells expressing a Notch reporter with or without LFNG, MFNG or RFNG, or inducible DLL1 or JAG1, showed that Fringe modification in *cis* (i.e. in the Notch expressing cell) promotes *cis* inhibition of Notch signalling by DLL1, but weakens *cis* inhibition by JAG1 in the cases of LFNG and MFNG, whereas RFNG promotes *cis* inhibition by DLL1 and JAG1 alike [60]. These effects are similar to the effects of Fringe on Notch signalling in *trans*. It will be most interesting to see co-crystals of NOTCH1 and Notch ligand fragments with O-fucose plus GlcNAc to gain molecular insights into the different effects of Fringe on Delta versus Jagged binding. Modeling GlcNAc into the DLL4/NOTCH1 fragment crystal structure predicts specific amino acid interactions [57]. Binding to both DLL1 and JAG1 is increased when the O-fucose in EGF12 of the NOTCH1 EGF11–13 fragment is elongated by Fringe [70]. A Fringe code has been proposed based on

the differential modification of NOTCH1 EGF repeats by LFNG versus MFNG or RFNG, and the consequences for Notch ligand binding and Notch signalling in human embryonic kidney (HEK)-293T cells [29]. However, these results come from overexpressed transfected Fringe genes in cultured cells. It will be interesting to determine whether a Fringe code leads to functional consequences *in vivo* in mice expressing only a single Fringe [71]. Elongation of O-Fuc-GlcNAc by Gal was shown to be necessary for optimal Notch signalling in CHO cell reporter assays, whereas the further elongation of Gal by NeuAc appears to be dispensable for Notch signalling [38, 72].

• ***In vivo* consequences of defective O-fucose glycan synthesis or loss of O-fucose sites**

In *Drosophila*, expression of *Ofut1* is regulated during embryonic development and *Ofut1* is differentially expressed in adult tissues. Loss or suppression of *Ofut1* in *Drosophila* results in phenotypes similar to those of Notch pathway mutants such as lateral inhibition in the nervous system, and cell lineage decisions in sensory organ precursor cells [37, 39]. Expression of *Ofut1* with no or low fucosyltransferase activity (*Ofut1*^{R245A}) rescues fringe-dependent neurogenesis in *Drosophila* embryos [42]. Loss of *Pofut1* in mice is embryonic lethal [50, 73], as are *Pofut1*^{R245A} homozygotes due to degradation of the mutant enzyme [49]. These mice show the characteristic phenotype associated with defective Notch signalling, including growth retardation, due in part to disrupted somitogenesis, vascularization defects, and defects in neural tube formation. *Pofut1* null embryos share phenotypes not only with Notch receptor null mice, but also with Notch downstream effector deficient mice. Therefore, only conditional deletion of *Pofut1* can be used to study requirements for O-fucose glycans in Notch signalling in different cell types. Alternatively, mutation of O-fucose sites in the EGF repeats of Notch receptors or Notch ligands has been used.

Conditional deletion of *Pofut1* in bone marrow cells and stroma with *Mx1*-Cre causes cell-fate defects in lymphoid and myeloid cell differentiation. Cells lacking POFUT1 exhibit no Notch ligand binding but only a slight decrease in cell surface expression of NOTCH1 and NOTCH2 receptors [45]. Residual Notch signalling occurs in these bone marrow cells, since deletion of the Notch downstream effector RBP-J κ via the same method gave a more severe phenotype [52, 74]. Similarly, deletion of *Pofut1* in the endocardium via *Nfatc1*-Cre is less severe than deletion of *Notch1* by the same strategy [46]. Residual DLL4-induced Notch signalling in this case allowed the identification of angiogenic precursor cells involved in coronary arteriogenesis. Yet another example in which conditional deletion of *Pofut1* gives a milder phenotype than deletion of *Notch1* is deletion via *Pax2*-Cre in the inner ear [51]. These examples were unexpected given that the *Pofut1*-null embryonic phenotype is similar to that of a *Notch1*-null. Deletion of *Pofut1* in intestinal epithelium by *Villin*-Cre [75] or in bone marrow by *Mx1*-Cre [45] has milder consequences than deleting RBP-J κ in the same manners [52, 76]. By contrast, deletion of *Pofut1* in lung [77] or skin [78] gives severe Notch signalling-defective phenotypes.

In humans, several heterozygous autosomal dominant mutations, and a homozygous recessive mutation in the *POFUT1* gene, have been associated with disease (Fig. 2). Pigmentation defects are characteristic of the autosomal dominant mutations which give rise

to a syndrome termed Dowling-Degos Disease 4 (DDD4) [79–81]. A recently identified heterozygous mutation in *POFUT1*, associated with DDD4 is also accompanied by Hidradenitis Suppurativa (HS) which is marked by recurrent painful nodules and abscesses [82]. A homozygous recessive mutation in *POFUT1* ablates a N-glycan site and is correlated with more severe developmental defects [83]. However, the loss of the N-glycan is not the basis of the reduced activity of POFUT1. Rather, it seems that Ser162 in the Asn-Lys-Ser N-glycan sequon cannot be replaced by Leu, though it can be replaced by Gln, a change that apparently enhances POFUT1 activity. Enhanced POFUT1 activity has been associated with tumor progression and increased Notch signalling in liver cancer [84].

Since POFUT1 may modify ~100 different proteins that contain appropriate EGF repeat(s), it is also important to determine functions of O-fucose by mutating O-fucose sites in POFUT1 substrates. Removal of the O-fucose site in EGF12 of *Drosophila* Notch (N-EGF12f) revealed that the O-fucose glycan is important for inhibiting Serrate-induced Notch functions in the wing disc, and binding of Delta and Serrate to N-EGF12f was enhanced [85]. This is surprising considering the crystal structures showing the key role of NOTCH1 EGF12 O-fucose in mammalian DLL4 and JAG1 binding [57, 58]. The *Notch1*[12f] mutation in mouse causes reduced ligand binding to thymocytes, but no apparent effects on viability or fertility [86]. However, Notch signalling is compromised in *Notch1*[12f/12f] mice, as reflected by the cell-autonomous reduction in T cell development. Point mutations have also been introduced into Notch ligands DLL1 [87] and DLL3 [88]. Only in the case of DLL3 did elimination of two O-fucose sites in EGF2 and EGF5 have a functional effect, in that a mutant transgene could not rescue somitogenesis in *Dll3* null embryos. It will be important to perform this experiment by mutating the endogenous *Dll3* gene and replacing the O-fucose Ser/Thr with the alternative (Thr/Ser) that could receive a fucose, as well as with amino acids other than Ala that cannot be O-fucosylated. DLL1 has four EGF repeats that receive O-fucose but DLL1 expressed in *Pofut1*-null presomitic mesoderm or mouse embryo fibroblasts was localized to the cell surface and stimulated Notch signalling [87]. By contrast, experiments with intestinal cells showed that Paneth cells have slightly reduced cell surface expression of DLL1 and DLL4 when RFNG is absent, and cells from cultured intestinal organoids have reduced DLL1 on the cell surface after knockdown of *Lfng* [89]. *Lfng* knockout mice have reduced DLL1 and DLL4 on the surface of goblet cells, whose numbers are increased due to a reduction in Notch signalling in the absence of *Lfng* [89]. Thus, depending on cellular context, cell surface expression of Notch ligands may be promoted by Fringe modification.

Elongation of O-fucose with a GlcNAc transferred by Fringe is critical for development in *Drosophila* and in mammals [90]. While there are three Fringe homologues in mammals, numerous studies reveal a dominant role for *Lfng* [91–93]. Mice lacking *Lfng* display severe defects in somitogenesis [91, 94, 95], reproduction [96, 97], and T cell and B cell development [71, 98, 99]. However, genetic background affects survival and longevity of *Lfng*-null mice [71, 91]. The dominance of *Lfng* is clearly observed in retinal angiogenesis, in which all three Fringe homologues are expressed by tip cells of the growing angiogenic front [100]. Deletion of *Lfng* causes excess vessel sprouting, despite the continued expression of *Mfng* and *Rfng*. *Lfng* is proposed to promote DLL4-induced Notch signalling in tip cells, and to inhibit JAG1-induced Notch signalling in stalk cells, thereby promoting

selection of tip cells [101]. An additive role of *Lfng* and *Mfng* occurs in marginal zone B cell development and in T cell development [71, 102, 103]. In fact, each Fringe gene expressed in the absence of the other two can rescue altered T and B cell development compared to triple Fringe knockout mice [71]. Fringe regulates Notch signalling based on differential interactions between Notch modified by Fringe with Delta-like versus Jagged Notch ligands. Fringe may also promote the cell surface expression of Delta-like ligands [89]. A role for the addition of Gal to Fringe-modified Notch receptors was observed in *B4galt1* null embryos which exhibit reduced expression of several Notch target genes during somitogenesis [104]. A patient with a mutation in B4GALT1 had severe neurological defects and other pathologies consistent with reduced Notch signalling [105].

Human mutations in *LFNG* give rise to spondylocostal dysostosis [106, 107], but no mutations in *MFNG* or *RFNG* have yet been associated with any human pathology. However, upregulation of *MFNG* has been correlated with tumor progression in claudin-low breast cancer, due to increased Notch signalling and the induction of *PI3KCG* [108]. By contrast, loss of *Lfng* which suppresses JAG1-induced NOTCH1 signalling in mammary epithelium, in cooperation with MET/CAVEOLIN gene amplification, promotes basal-like breast cancer [109]. *LFNG* also functions as a tumor suppressor in melanoma metastasis [110], and in mouse models of pancreatic [111] and prostate cancer [112]. These results reveal fundamental roles of Fringe in regulating Notch signalling. Further studies will help better elucidate different functions of the three fringe homologues in mammals.

O-Glucose glycans

• Discovery and cell-based assays

Bovine blood coagulation factors VII and IX, were the first proteins identified with an O-glucose modification on EGF repeats [113]. O-glucose was subsequently identified on NOTCH1 in CHO cells [27]. O-glucose was also found extended by xylose in α 1,3-linkage, or by two xylose moieties to form a trisaccharide [114]. In *Drosophila*, only one enzyme has been identified as a glucoside xylosyltransferase (GXYLT) termed *Shams* [115], whereas in mammals, the addition of the first xylose is mediated by GXYLT1 or GXYLT2, and the second xylose is added in α 1,3-linkage by xylose xylosyltransferase 1 (XXYLT1) [114, 116]. A *Drosophila* Xxytl1 was recently identified and shown to repress Delta-Notch signaling [117]. Thus, loss of *Drosophila* Xxytl1 was found to promote Delta-Notch signaling in an appropriately sensitized genetic background. The consensus site for O-glucose addition to most EGF repeats of Notch receptors and ligands is $C^1xSxAPC^2$, where C^1 and C^2 are the first and second cysteines of the EGF repeat, **S** is the Ser that accepts glucose, **x** is any amino acid, **P** is Pro and **A** is Ala [118, 119] (Fig. 1). Mass spectrometric analysis of *Drosophila* NOTCH1 ECD from S2 cells and NOTCH isolated from *Drosophila* embryos demonstrated the presence of O-glucose on all predicted 18 sites. However, O-glucose-xylose disaccharide was found only on EGF13–20 and EGF25. Similarly, the trisaccharide was found on a restricted subset of the EGF repeats with O-glucose [28]. Both the addition of O-glucose and its elongation by xylose is dependent on the amino acids in the consensus site and the proper folding of the EGF repeat [120]. The O-glucosyltransferase is encoded by *Rumi* in *Drosophila* [121] and the protein O-

glucosyltransferase 1 gene *Poglut1* in mammals [122]. POGLUT1 is an ER resident enzyme [121]. *In vitro* knockdown of *Rumi* in *Drosophila* S2 cells and mammalian cell lines reduces Notch signalling due to defects in Notch receptor cleavage upon ligand binding. However, loss of O-glucose glycans does not reduce Notch ligand binding. Thus, O-glucose glycans appear to promote a conformational change in Notch receptors after ligand interaction, and are required for S2 cleavage by ADAM proteases [123, 124]. Further, crystal structures of a NOTCH1 ligand-binding fragment bound to a N-terminal fragment of DLL4 demonstrate that O-glucose on EGF12 and EGF13 are located away from the DLL4 binding face, and cover hydrophobic residues Pro and Phe in these EGF repeats [57], hence supporting the notion that O-glucose is not required for Notch-ligand interactions. The presence of a novel, O-linked hexose attached to Ser in EGF11 at a site which does not match the consensus of an O-glucose site was revealed in crystal structures [57, 125]. This modification is conserved in other Notch receptors, except NOTCH2, at the consensus site C³xNTxGSFxC⁴. The hexose was recently identified as a glucose residue which is added by POGLUT2 or POGLUT3, homologues of POGLUT1 [126]. Mutations of single O-glucose sites in NECD do not affect the cell surface expression of Notch, nor impair Notch activation, except for mutation in EGF28 [15, 127]. Though, the O-glucose consensus site in EGF28 is present in mammalian NOTCH1, and not present in *Drosophila* Notch or other mammalian Notch receptors, mutation of Ser to Ala in EGF28 of NOTCH1 causes a decrease of Delta-induced Notch signalling, but does not affect signalling mediated by Jagged ligands. Deletion of *Poglut1* in HEK-293T cells causes a mild reduction in cell surface expression of NOTCH1, and enhanced secretion of soluble NECD, suggesting that O-glucose glycans contribute to trafficking or stability of Notch receptors [47]. Mutation of the O-glucose site in EGF11 to Ala has no effects on cell surface expression of NOTCH1, DLL1 binding or DLL1-induced NOTCH1 signalling [126]. However, the combined mutation of EGF11 and the O-fucose site in EGF8 or EGF12 of NOTCH1 has somewhat greater effects than the single O-fucose mutations. It would be interesting to see if cells lacking both POGLUT2 and POGLUT3, but retaining Ser in EGF11, give the same results with NOTCH1 carrying the EGF8 or EGF12 O-fucose mutations.

• ***In vivo* consequences of defects in O-glucose glycan synthesis or loss of O-glucose sites**

Loss of *Rumi* from *Drosophila* results in temperature-sensitive Notch signalling defects [121]. *Rumi*-null flies are viable at 18°C but exhibit a slight Delta wing vein phenotype. However, at 28°C lethality occurs at the larval stage, and reduced Notch signalling is observed in all contexts studied. O-glucose on Notch in flies is critical for S2 cleavage of Notch but ligand binding remains unaffected. Further, *rumi* mutant G189E lacking transferase activity did not rescue defective Notch signalling in *rumi* null flies, suggesting the importance of O-glucose glycans to Notch receptor trafficking and stability at the cell surface. Multiple mutations in O-glucosylation sites on NECD were essential for temperature-sensitive Notch signalling defects to arise, whereas single site mutations did not affect Notch signalling [127]. *Poglut1*^{-/-} mice are embryonic lethal and die before E9.5 with severe defects in neural tube development, cardiogenesis and somitogenesis [122]. *Poglut1* null mutants die earlier than Notch pathway null mutants, apparently due to the loss of O-glucose from CRUMBS2 [122]. Haploinsufficiency of *Poglut1* on a *Jag1* heterozygous background results in decreased O-glucosylation of NOTCH1 and severe defects in bile duct

morphogenesis, suggesting a genetic interaction between *Poglut1* and members of the Notch signalling pathway [123]. Additionally, mutations in human *POGLUT1* cause an autosomal dominant form of Dowling-Degos Disease termed DDD2 [128–131], or a recessive limb-girdle muscular dystrophy [132].

O-GlcNAc glycans

• Discovery and cell-based assays

The presence of O-GlcNAc on EGF repeats of Notch receptors was first identified in S2 cells on a *Drosophila* NECD fragment containing EGF20 [133]. O-GlcNAc is added to Ser/Thr between the fifth and sixth cysteines of an EGF repeat with the consensus site C⁵xxG(Y/F)(T/S)Gx_{2–3}C⁶ [20, 133–135]. Of the 36 EGF mouse NOTCH1 repeats, 17 have a consensus site for O-GlcNAc and *Drosophila* Notch has 18 consensus sites. However, mass spectrometry on Notch purified from S2 cells and *Drosophila* larvae identified O-GlcNAc on only 5 sites [28]. In mammalian cells, O-GlcNAc can be further elongated by Gal [134] and probably sialic acid. The enzyme responsible for the addition of O-GlcNAc on EGF repeats was identified in *Drosophila* as EGF-domain specific O-GlcNAc-transferase (EOGT). EOGT is conserved across species and is localized to the ER by a signal peptide at the N terminus and a C-terminal KDEL sequence [134]. Studies using knockdown and knockout of *Eogt* in mammalian cell lines suggest that O-GlcNAc on Notch receptors promotes Delta-mediated Notch signalling, but does not significantly affect JAG1-induced Notch signalling [136]. Loss of EOGT inhibits binding of Delta-like ligands but not JAG1, suggesting that O-GlcNAc on Notch plays specific roles in Notch ligand binding and Notch signalling [136].

• In vivo consequences of defects in O-GlcNAc glycans and loss of O-GlcNAc sites

Loss of *eogt* in flies results in lethality, mostly during second instar larval development, with a few survivors at the early third-instar stage [134, 135]. However, larvae lacking *eogt* do not show a phenotype similar to flies with Notch-deficient signalling. Knockdown of *eogt* in the fly wing results in blistering that may arise from roles for O-GlcNAc on *Dumpy*, an extracellular matrix protein with a large number of EGF repeats. Ligand-induced Notch signaling promotes blistering because the phenotype is partially rescued with the loss of one allele of *Notch* or Notch pathway members such as Delta or Serrate that reduces Notch signalling, suppressor of hairless (Su(H) or *maml* [135]. Such genetic interaction studies provided the first link between Notch signalling and *Eogt* which has now been validated in mammalian cells and mice. In the mouse, *Eogt* expression is enhanced in the presomitic mesoderm at E9.5, and limited to the digits of developing limbs by E12.5 [137]. *Eogt* null mice are viable, fertile and do not show a typical Notch phenotype [136]. Using retinal angiogenesis as a sensitive assay for ligand-induced Notch signalling [100], defective angiogenesis with leaky blood vessels is observed in mice lacking *Eogt* [136]. Loss of *Eogt* results in increased blood vessel branching and increased tip cell numbers, which is characteristic of disrupted DLL4-NOTCH1 signalling. Thus, in retinal angiogenesis, loss of *Eogt* recapitulates results in cell lines and reveals the importance of O-GlcNAc in promoting optimal Delta-induced Notch signalling. In humans, mutations in *EOGT* cause a rare, congenital disorder termed Adams-Oliver Syndrome 4 (AOS4) [137–139]. Symptoms

include cutis aplasia of the scalp, defects in the development of digits, vascular defects and, in some cases, cardiac defects. Autosomal dominant mutations in *NOTCH1*, *DLL4* and *RBPJ* genes have also been identified in patients diagnosed with AOS [140–143].

Synergistic and redundant roles for O-fucose and O-glucose glycans

While the majority of studies to date have focused on understanding the functions of each type of O-glycan independently, a few studies have investigated roles for O-glucose and O-fucose glycans together. In *Drosophila*, loss of O-fucose or O-glucose separately causes temperature-sensitive Notch signalling defects that manifest at 30°C [43]. However, at 25°C each mutant behaves essentially like wild type. When, however, both O-fucose and O-glucose glycans are not transferred to Notch, Notch signalling is lost at 25°C. This correlates with accumulation of Notch in the ER, whereas loss of O-fucose or O-glucose alone allowed exit of Notch from the ER. Thus O-glucose and O-fucose glycans function synergistically to support Notch trafficking out of the ER in *Drosophila*. Consistent with this, in mammalian cells in which both POFUT1 and POGLUT1 were deleted to give NOTCH1 lacking both O-fucose and O-glucose glycans, NOTCH1 was not expressed well at the cell surface, whereas loss of either O-glycan alone allowed cell surface expression of NOTCH1 [47]. In another study, the presence of xylose in O-glucose glycans was also found to contribute to Notch trafficking and Notch signalling in *Drosophila* but only in the context of a double mutant [144]. Thus, Notch lacking both O-fucose and the dixylose on O-glucose was mislocalized from the apical plasma membrane to adherens junctions, and had reduced Notch signalling [144]. Notch lacking O-glucose (including dixylose) and O-fucose was not exported from the endoplasmic reticulum. Thus, some functions of sugars (e. g. dixylose) may only be observed in the absence of a compensatory sugar (e. g. O-fucose), and this may in turn depend on cellular context.

Conclusions

It is apparent from studies published over the past 18 years that distinct O-glycans on Notch receptors are essential for regulating and optimizing different aspects of Notch signalling. O-glucose glycans on Notch positively regulate the cleavage of Notch receptors upon ligand binding, and promote receptor trafficking to the cell surface, but do not directly mediate interactions with Notch ligands. However, extension of O-glucose by xylose negatively regulates Notch signalling, in a context dependent manner. O-fucose glycans influence Notch signalling by differentially regulating Notch ligand binding to Delta and Jagged ligands. In *Drosophila* and certain numerous mammalian cell types, POFUT1 is important for promoting Notch receptor trafficking to the cell surface. However, in mammalian cells lacking POFUT1 NOTCH1 and other Notch receptors are well expressed at the cell surface but do not bind Notch ligands or exhibit ligand-induced Notch signalling. The addition of GlcNAc to O-fucose by Fringe differentially modulates Notch receptor interactions with the various ligands. LFNG and MFNG generally promote Delta ligand binding and inhibit Jagged ligand binding, whereas RFNG promotes both Delta and Jagged ligand binding. The more recently identified O-GlcNAc modification on Notch appears to mediate Notch signalling via Delta but not Jagged ligands based on both cell-based and *in vivo* studies. Mutations in several of the glycosyltransferases that synthesize the O-glycans on Notch

receptors cause a variety of defects in Notch signalling, establishing the biological importance of O-glycans in regulating and optimizing the strength of Notch signalling. Human mutations in several glycosyltransferase are associated with different pathologies, which are also associated with mutations in Notch and Notch pathway members. The ExAc browser [145] describes exon sequencing data from 60,706 unrelated individuals, not including people with known congenital mutations, and reports single nucleotide mutations including missense and nonsense mutations in the different glycosyltransferase genes described above. A few healthy people with homozygous missense mutations have been reported, indicating that these mutations are not important for the activity of the relevant glycosyltransferase. While the majority of studies to date have focused on understanding the roles of each type of glycan independently, a few studies have revealed synergistic and redundant roles of O-glucose and O-fucose glycans. Future efforts should continue along this line of inquiry to reveal how all the O-glycans on Notch receptors and ligands work separately and together to optimize Notch signalling. These studies will reveal synergistic, redundant and non-overlapping functions of the glycans which will further help to elucidate how O-glycans on Notch regulate diverse cell fate decisions. Insights from these studies will help to design potential targets for therapeutic purposes.

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Abbreviations

NECD	Notch extracellular domain
NICD	Notch intracellular domain
EGF	Epidermal growth factor-like
NRR	Negative regulatory region
HD	Heterodimerization domain
NEXT	Notch extracellular truncation
RAM	RBP-J κ -associated module
ANK	Ankyrin repeats
TAD	transcriptional activation domain
CSL	CBF-1 Suppressor of Hairless-LAG1
ADAM	A disintegrin and metalloprotease
MAML	Mastermind-like
GlcNAc	N-acetylglucosamine
GSI	gamma-secretase inhibitor

POFUT1	Protein O-fucosyltransferase 1
CHO	Chinese hamster ovary
ER	Endoplasmic reticulum
ES	Embryonic stem cells
HSC	Hematopoietic stem cells
U2OS	Human osteosarcoma cell line
GDP-Fuc	GDP-Fucose
Gal	Galactose
NeuAc	N-acetylneuraminic acid
LFNG	Lunatic fringe
MFNG	Manic fringe
RFNG	Radical fringe
HEK	Human embryonic kidney
DLL	Delta-like
JAG1	Jagged 1
C2 domain	module at the N-terminus of Notch ligands
POGLUT1	Protein O-glucosyltransferase 1
EOGT	EGF-domain specific O-GlcNAc
Su(H)	Suppressor of hairless
AOS4	Adams-Oliver Syndrome 4
DDD	Dowling-Degos Disease
SCDO3	Spondylocostal dysostosis 3
LGMD2Z	Limb-girdle muscular dystrophy type 2Z
HS-DDD4	Hidradenitis Suppurativa-Dowling-Degos Disease 4
GXYLT1	Glucoside xylosyltransferase 1
GXYLT2	Glucoside xylosyltransferase 2
XXYLT1	Xylose xylosyltransferase 1

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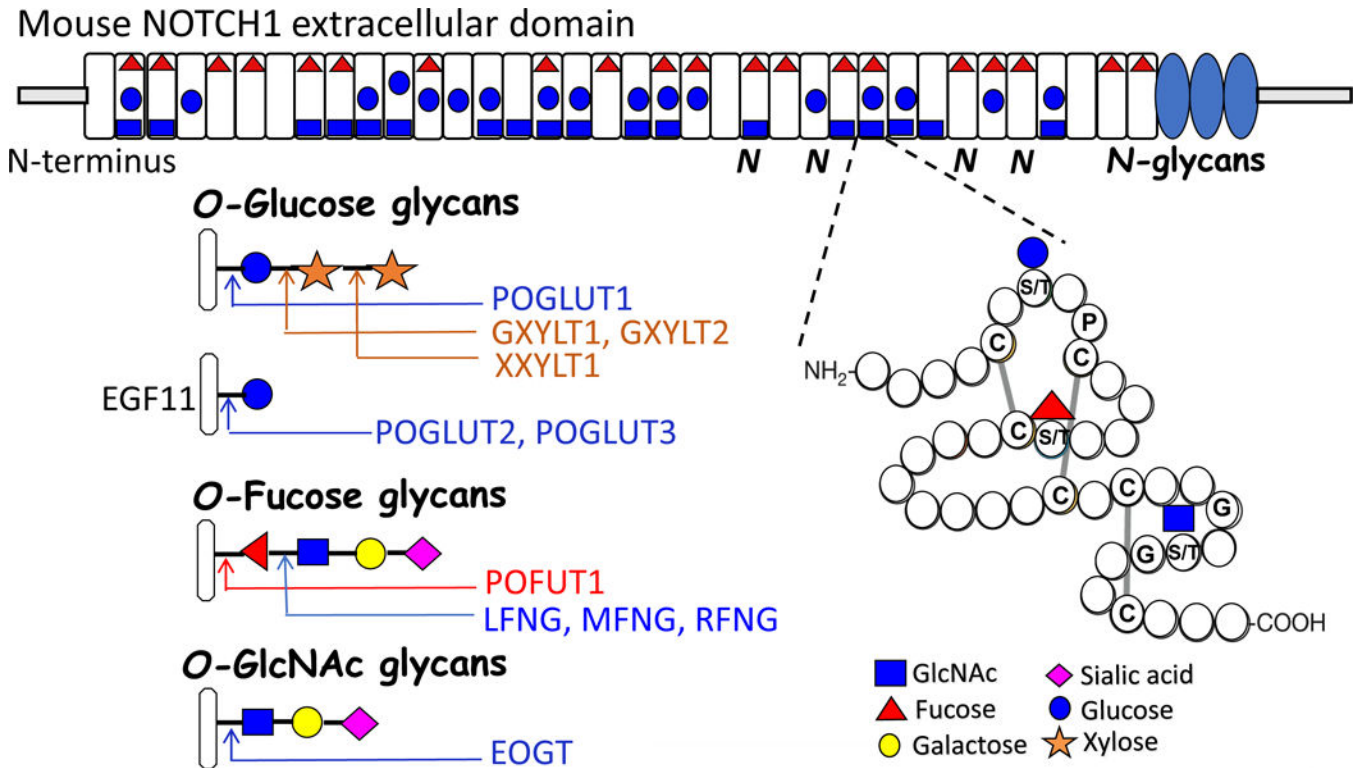


Figure 1. Representation of mouse NOTCH1 extracellular domain depicting EGF repeats with different O-glycan consensus sites that may be modified with the O-glycans shown. One of the EGF domains is magnified to show the consensus site for each type of O-glycan. Different O-glycans, their respective differential extension with sugars (+/-), and the glycosyltransferases responsible for the transfer of each sugar are shown below the diagram. The transfer of O-glucose by POGLUT2 or POGLUT3 occurs only on EGF11 in NOTCH1. The consensus site is between Cys³ and Cys⁴, indicated by the different location of the glucose symbol in EGF11 in the diagram.





	Enzyme	Location	Disease	Refs
POFUT1	 <p>1 26 RDEF 388</p> <p>p.Glu144* c.430G→T p.Lys161Serfs*42 c.482delA; -- c.246+5delG p.Ser162Leu c.485C→T -- c.430-1G→A</p>	ER	DDD4	75-77 79
LFNG	 <p>9 29 379</p> <p>p.Phe188Leu c.564C→A p.Trp195Arg c.583T→C p.Thr281Lys c.842C→A p.ala16Argfs*135 c.44dupG</p>	Golgi	HS-DDD4 SCDO3	78 102,103
POGLUT1	 <p>1 23 KTEL 392</p> <p>p.Trp4* c.11G→A p.Arg69* c.205C→T p.Gly170Glu c.509G →A p.Arg132* c.394C→T p.Ser212* c.635C→G p.Arg218* c.652C→T -- c.798-2A→C p.Arg279Trp c.835C→T p.Arg279Profs*3 c.833_834insC p.Cys286Tyr c.857G → A p.Gly342Glufs*22 c.1023_1025delG p.Asp233Glu c.699T→G</p>	ER	DDD2	123-126
EOGT	 <p>1 18 HDEL 527</p> <p>p.Trp207Ser c.620G→C p.Arg377Gln c.1130G→A p.Gly359Aspfs* c.1047delA</p>	ER	LGMD2Z AOS4	127 134-136

Figure 2.

The human mutations and pathologies associated with glycosyltransferases that modify Notch receptors. The diagram of each glycosyltransferase represents protein size, with the signal peptide or transmembrane domain identified by a grey box and each ER retention sequence given at the C-terminus. The human mutations identified so far are mentioned below each protein diagram, with the corresponding pathologies referred to in the disease column.