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Fringe Benefits: Functional and structural impacts of *O*-glycosylation on the extracellular domain of Notch receptors

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Abstract

The Notch family of receptors plays essential roles in many phases of development, and dysregulation of Notch activity is increasingly recognized as a player in many diseases. *O*-Glycosylation of the Notch extracellular domain is essential for Notch activity, and tissue-specific alterations in the glycan structures are known to regulate activity. Here we review recent advances in identification and characterization of the enzymes responsible for glycosylating Notch and molecular mechanisms for how these *O*-glycans affect Notch activity.

Introduction

The Notch protein plays an important role as a transmembrane signaling receptor in a wide variety of developmental pathways [1,2]. Notch is conserved across all metazoans, and there are four mammalian homologs (Notch1–4). Loss of individual mouse Notch homologs 1 or 2 results in embryonic lethality, and mutations of Notch or downstream signaling components within the pathway have been implicated in a multitude of disease states in humans, including T-cell Acute Lymphoblastic Leukemia, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), Alagille Syndrome, Spondylocostal Dysostosis, Multiple Sclerosis, several heart defects, and Breast Cancer (reviewed in [1–4]). The Notch locus encodes a large (~300 kDa) single-pass Type I transmembrane receptor, comprised of a large extracellular domain (ECD) with multiple tandem Epidermal Growth Factor-like (EGF) repeats and negative regulatory region (NRR), followed by a transmembrane region and a large intracellular domain (ICD) involved in downstream signaling events (Figure 1A) [1]. The Notch signaling pathway is activated upon binding of the Notch ECD to one of its ligands presented on an apposing cell (Figure 2).

Work in a number of laboratories over the past decade has demonstrated that *O*-fucosylation and *O*-glucosylation of the EGF repeats in the Notch ECD are essential for its function [3–9]. Elimination of the enzyme responsible for addition of *O*-fucose to EGF repeats (Protein *O*-fucosyltransferase 1, Pofut1 in mice, Ofut1 in *Drosophila*) reveals that *O*-fucosylation is universally required for all Notch signaling [10–12]. Elongation at the *O*-fucose by the β 1–

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3*N*-acetylglucosaminyl transferase, Fringe, on EGF repeats modulates Notch activity in a number of tissue specific contexts and serves as a paradigm for how alterations in the glycosylation status of a receptor affect activity [13,14]. Genetic studies on the biological role of *O*-glucosylation have not been as extensive as for *O*-fucosylation, but elimination of the enzyme responsible for addition of *O*-glucose to EGF repeats (Protein *O*-glucosyltransferase, Poglut, gene name *Rumi*) also results in severe Notch-like phenotypes in flies or mice [15,16]. In this review we will focus on advances in our understanding of Notch glycosylation since it was last reviewed in this series [7], with a focus on what we have learned about the biochemistry of Notch *O*-glycosylation and current models for how the glycans affect Notch function.

Biochemistry of Notch *O*-glycosylation

The *O*-fucose and *O*-glucose glycans on Notch occur at specific consensus sequences within the context of EGF repeats, which make up the majority of the Notch ECD (Figure 1). Recently, *O*-GlcNAc modification, a third form of *O*-glycosylation was identified on EGF repeats as well, occurring on hydroxy amino acids between the fifth and sixth conserved Cys of an EGF repeat [17]. This sequence context has been sufficient to identify predicted *O*-GlcNAc sites on other EGF repeat-containing proteins, including the Notch ligand Delta [17]. Although no function for *O*-GlcNAc on Notch has yet been found, it is added to Notch by an enzymatic activity distinct from the well-known nuclear/cytoplasmic *O*-GlcNAc transferase, OGT [8,17,18].

While database searches with the *O*-fucose and *O*-glucose consensus sequences reveal nearly 100 mammalian proteins are predicted to be *O*-fucosylated [4], and over 40 proteins are potentially *O*-glucosylated [16], the Notch family of receptors contain the highest incidence of each consensus sequence and are predicted to be the most widely modified (Figure 1) [4,16]. Recent site-mapping work in our laboratory has revealed that *O*-glucose also exists on EGF 9 of mouse Notch1 at an unconventional sequence: C¹A_SAAC², suggesting that Ala can replace Pro N-terminal to Cys 2 [19]. Database searches with the revised *O*-glucose consensus sequence (C¹-x-S-x-A/P-C²) reveal additional new predicted sites on mouse Notch1 and 3, as well as sites on several novel proteins [19].

The fully extended forms of *O*-fucose and *O*-glucose glycans are shown diagrammatically in Figure 1B. *O*-Fucose on EGF repeats (including those from Notch [13,20–22]) can be elongated to a tetrasaccharide in mammals: Sia α 2–3/6Gal β 1–4GlcNAc β 1–3Fuc (Figure 1B) [23,24], whereas elongation beyond the disaccharide GlcNAc β 1–3Fuc has not been detected on *Drosophila* Notch protein isolated from S2 cells [25]. Recently, Aoki *et al.* carried out studies to define the *O*-glycome of *Drosophila melanogaster* by mass spectrometry, using β -elimination to release the *O*-linked sugar modifications from total protein extracts of fly embryos [26]. They discovered a novel glucuronic acid-containing *O*-linked fucose trisaccharide: GlcA β 1–4(GlcNAc β 1–3)Fucitol. Given the similarity to the GlcNAc β 1–3Fuc on the EGF repeats of Notch, the trisaccharide was proposed to be derived from Notch, although further work needs to be done to determine whether this trisaccharide exists on Notch or other proteins *in vivo*. *O*-Glucose is typically found elongated to a trisaccharide on EGF repeats (Xyl α 1–3Xyl α 1–3Glc, Figure 1B) [23], including on mouse Notch1 [19,22,27]. In their *O*-glycome analysis of *Drosophila*, Aoki and co-workers also detected *O*-glucose mono- (Glucitol) and disaccharide (Xyl α 1–3Glucitol), but no trisaccharide among the reductively released saccharides [26]. Consistent with these results, mass spectral *O*-glucose site mapping on *Drosophila* Notch has confirmed the presence of mono- and disaccharide forms of *O*-glucose at specific sites ([15], Rana *et al.*, unpublished).

Nearly all of the enzymes responsible for addition of *O*-fucose and *O*-glucose glycans to EGF repeats have been identified (Figure 1B). Pofut1 is a soluble, ER-localized enzyme that adds fucose to Ser or Thr in the C²-x-x-x-x-(S/T)-C³ consensus sequence [28,29]. Fringe is a Golgi-localized β 1-3N-acetylglucosaminyltransferase that extends *O*-fucose to the disaccharide [13,14,30]. While three Fringe homologs exist in mammals (Lunatic, Manic, and Radical Fringe), a single Fringe exists in flies [31]. The structure GlcNAc β 1-3Fuc α 1-*O*-Ser/Thr can be further elongated in mammals to the tetrasaccharide by sequential action of a β 1-4galactosyltransferase and an α 2-3 or α 2-6sialyltransferase.

Rumi was recently identified as the gene encoding Poglut, a soluble, ER-localized enzyme responsible for addition of glucose to Ser in the C1-x-S-x-A/P-C2 consensus sequence [15,16,32]. Two human glucoside α 1-3xylosyltransferases (GXYLT1 and GXYLT2) have been recently identified that extend *O*-glucose to a disaccharide [33]. Both are predicted to be Type II membrane glycoproteins, similar to most Golgi-localized glycosyltransferases [34]. The only enzyme required for *O*-glucose trisaccharide biosynthesis not yet identified is the xyloside α 1-3xylosyltransferase (XXYLT) that adds a second xylose to generate the Xyl α 1-3Xyl α 1-3Glc β 1-*O*-Ser trisaccharide found in mammals. Recent glycoproteomic studies on *O*-glucose site occupancy show *O*-glucose trisaccharide occurs at high stoichiometries at most sites, but some site and cell-specific underglucosylation has been observed on mouse Notch1 and 2 (Figure 1A, [16,19,35]). These results suggest the extent of modification at some sites may be more sensitive to expression levels of *Rumi* than others. Interestingly, recent results suggest that Notch signaling is sensitive to the levels of *Rumi* expressed in a tissue [16].

Models for how *O*-glycans affect Notch function

O-Fucose and Fringe modification affect ligand binding

Elimination of *Pofut1* in mice has a profound effect on ligand binding in both embryonic stem cells [36] and lymphoid cells [37]. A chaperone-like activity has been reported for *Drosophila* Ofut1 that is required for cell-surface expression of Notch in flies [38], but this chaperone activity has not been clearly seen in the mouse system, as cells lacking *Pofut1* have Notch proteins on their surfaces [36,37]. This discrepancy may be explained by differences in species, or in cell-dependent expression of other chaperones. A small decrease in cell surface expression of Notch proteins is seen in embryonic stem cells lacking *Pofut1* [36] and in somites from mice with a hypomorphic allele of *Pofut1*, *cax* [39]. More detailed analysis of the chaperone activity of *Pofut1*/Ofut1 has been reviewed elsewhere [3,5,8].

Similar to *O*-fucosylation, the major effect of Fringe-mediated *O*-fucose elongation appears to be modulation of Notch-ligand binding, whereby Delta activation of Notch is potentiated, while signaling via Serrate is inhibited. In flies, data indicate that GlcNAc is the terminal sugar added to *O*-fucose residues on *Drosophila* Notch, and that the disaccharide is sufficient for observing a Fringe effect. *In vitro* extension to trisaccharide causes no change in *in vitro* ligand binding as assessed *in vitro* [25]. While the mammalian system is more complicated (4 receptors, 5 ligands, and 3 Fringes), the majority of data suggest mammalian Fringe modification also alters ligand binding [3,5,8,9]. Elongation beyond GlcNAc to the trisaccharide (Gal β 1-4GlcNAc β 1-3-Fucose α 1-*O*-Ser/Thr) is necessary to see a Fringe effect in a mammalian cell system [40], an important departure from requirements in the fly system. Consistent with these findings, β 4-galactosyltransferase-1 knockout mice exhibit a minor Notch phenotype [41].

O-Glucose affects a step after ligand binding but prior to proteolysis

To examine what roles *Rumi* and *O*-glucose play in proper Notch function in *Drosophila*, Acar *et al.* examined steps of the canonical signaling cascade where *Rumi* may be exerting

regulation [15]. Notch is present at the cell surface in cells lacking Rumi, indicating that *O*-glucosylation is not required for cell surface presentation of the receptor, and that Rumi does not have any chaperone-like activity. Defects in ligand binding were ruled out as the cause for the *rumi* phenotype, as Notch generated in *rumi* knockdown cells binds Delta as strongly as protein generated from control cells. Interestingly, analysis of protein extracts from various tissues of *rumi* mutant flies showed significantly decreased Notch proteolysis (S2 or S3 cleavage, Figure 2). As proteolysis requires conformational changes in the ECD (Figure 2), this suggests *O*-glucosylation may affect the structure of the ECD. Similar results have been obtained in mammalian cells where *rumi* has been knocked down using RNAi [16]. The temperature sensitivity of the phenotype in flies is consistent with destabilization of the Notch ECD due to the absence of *O*-glucose [15]. This lack of stability could in turn interfere with the ability of the receptor to couple ligand binding to requisite conformational changes necessary for proteolysis (Figure 2).

O-Glycosylation sites important for Notch activity

Several studies have examined whether specific *O*-fucose or *O*-glucose sites on Notch are important for activity. Eliminating any of three highly conserved *O*-fucose sites at EGF 12, 26, or 27 within mouse Notch1 alters activity in cell-based Notch signaling assays [21]. EGF 12 is part of the ligand-binding region of Notch (Figure 1A). Ge and Stanley generated a mouse line carrying a point mutation in the *O*-fucosylation site of EGF 12 in endogenous Notch1 [42]. Loss of this site resulted in a mild Notch phenotype with defects in T cell development. This result shows that *O*-fucosylation of EGF 12 plays an important role in Notch1 function, but suggests that additional *O*-fucose sites, such as those on EGF 26 or 27, are likely contributing to Notch activity. Interestingly, EGF 26 and 27 are in the *Abruptex* region of the Notch receptor (Figure 1A), named for a group of mutations in *Drosophila* Notch that result in hyperactivated Notch that is refractory to Fringe [43].

We have also performed cell-based signaling assays to examine the role of each *O*-glucosylation site in mouse Notch1. Only elimination of *O*-glucose at EGF 28 results in significant decreases in Delta-like1-mediated Notch1 signaling [19]. This is a surprising result, as there are 17 sites of *O*-glucosylation on Notch1, and suggests that individual mutations may not explain the major effects of Rumi. Of interest is the fact that like EGF repeats 26 and 27, EGF 28 also maps to the *Abruptex* region of the receptor (Figure 1A).

Structural analysis of Notch signaling

Structural studies of the Notch family of receptors are required to fully understand the molecular mechanisms by which *O*-fucose and *O*-glucose glycans affect Notch function. The structural implications of *O*-glycosylation on Notch-ligand interactions have yet to be defined, but recent structural studies provide insights into Notch ECD global structure, and the possible impact of *O*-glycosylation on altering that structure. Several studies have focused on the structure of EGF 11–12, the ligand-binding region of Notch [44,45]. Recently, the structures of unmodified, *O*-fucosylated, and Fringe elongated mouse Notch1 EGF 12 were compared by NMR [46]. Interestingly, the fucose moiety appears to contribute to stability of the anti-parallel β -sheet in the EGF repeat, and Fringe elongation to the GlcNAc- β 1,3-Fucose causes a significant conformational shift of several residues within the *O*-fucose consensus region. This may provide a mechanism for how Fringe modification indirectly exerts its effects on Notch activity at EGF 12.

Although the Notch ECD is traditionally schematically represented as a linear and rigid structure (Figure 1A), several recent studies indicate that this is unlikely to be an accurate depiction of the global structure of the receptor. Some EGF repeats are capable of coordinating calcium via conserved acidic/polar residues at the N-terminal linker region

between two adjoining EGFs (calcium binding EGF, or cbEGF). Using EGF 11–13 of human Notch1, Hambleton and co-workers observed that in the presence of calcium, the calcium-binding motifs in the linker between two adjacent EGF repeats impart rigidity to this region [47]. The flexibility of non-cbEGF linkers, which do not coordinate calcium, is supported by the observation that consecutive non-cbEGF repeats from *Plasmodium falciparum* merozoite surface protein-1 assume a U-shaped structure in which the non-cbEGF domains share a hydrophobic interface. Based on these results, Hambleton and co-workers proposed that the Notch ECD consists of both rigid and flexible regions dependent on the presence or absence of cbEGFs. Of special interest is the fact that the organization of calcium-binding sites within the Notch ECD is highly conserved [45], suggesting these regions of structural rigidity and flexibility are essential for Notch ECD function.

Support for this model of regions of flexibility and rigidity comes from electron microscopy (EM) studies by Kelly *et al.* [48]. Using the ECD of both *Drosophila* and human Notch1, their images depict structures that appear to have globular structures where the ECD is folded up on itself, consistent with the flexible regions predicted to exist in both proteins based on positioning of cbEGFs in these receptors. Further work remains to be performed to address whether these structures are biologically active, or whether *O*-fucose or *O*-glucose glycans affect this structural conformation.

Conclusions

Just over 10 years ago, Notch activity was shown to be regulated by Fringe-mediated elongation of *O*-fucose on its ECD [13,14]. Since then most of the enzymes responsible for addition of *O*-fucose and *O*-glucose glycans have been identified, and their importance for Notch activity has been confirmed. We now know that the predicted consensus sites are modified, usually at high stoichiometries, at least in Notch protein overproduced in tissue culture systems. Future studies need to examine the site-specific modification of Notch proteins isolated from physiologically relevant *in vivo* sources. We have also learned through mutagenesis of the *O*-fucose site in the ligand-binding domain (EGF 12) and individual *O*-fucose or *O*-glucose sites in the *Abruptex* region (EGF 26, 27, and 28), that *O*-glycosylation of these sites plays important roles in Notch1 function. Elimination of the *O*-fucose site at EGF 12 decreases Notch1 activity *in vivo* [42]. Future studies need to examine the *in vivo* importance of other sites (*e.g.* *O*-fucose on EGF 26 or 27, *O*-glucose on EGF 28). Finally, structural studies on the Notch ECD need to be performed on protein with different modifications (*e.g.* with or without Fringe or GXYLT elongation), to better understand how these glycans modulate Notch function.

Highlights

- Notch is modified at multiple sites with *O*-fucose and *O*-glucose glycans
- Elongation of *O*-fucose glycans by the glycosyltransferase Fringe regulates Notch-ligand binding
- *Rumi* encodes the protein *O*-glucosyltransferase responsible for adding *O*-glucose to Notch
- *O*-Glucose may stabilize Notch to allow proper receptor activation
- Multiple *O*-glycan sites contribute to Notch ligand-binding and activity
- *O*-Glycosylation may alter the flexibility of the Notch extracellular domain

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Abbreviations

ICD	Intracellular Domain
ECD	Extracellular Domain
Poglut	Protein <i>O</i> -Glucosyltransferase
Pofut1/Ofut1	Protein <i>O</i> -Fucosyltransferase 1/ <i>O</i> -fucosyltransferase 1
EGF repeat	Epidermal Growth Factor-like Repeat
cbEGF	Calcium-binding EGF repeat
OGT	<i>O</i> -GlcNAc Transferase
LNR	Lin-12/Notch Repeat
RAM	Regulation of Amino Acid Metabolism

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expressing cells, suggesting it may be present on Notch. These same studies showed the presence of *O*-glucose monosaccharide (Glucitol) and disaccharide (Xyl α 1-3Glucitol), but no *O*-glucose trisaccharide (Xyl α 1-3Xyl α 1-3Glucitol) nor *O*-fucose monosaccharide (Fucitol) or disaccharide (GlcNAc β 1-3Fucitol).

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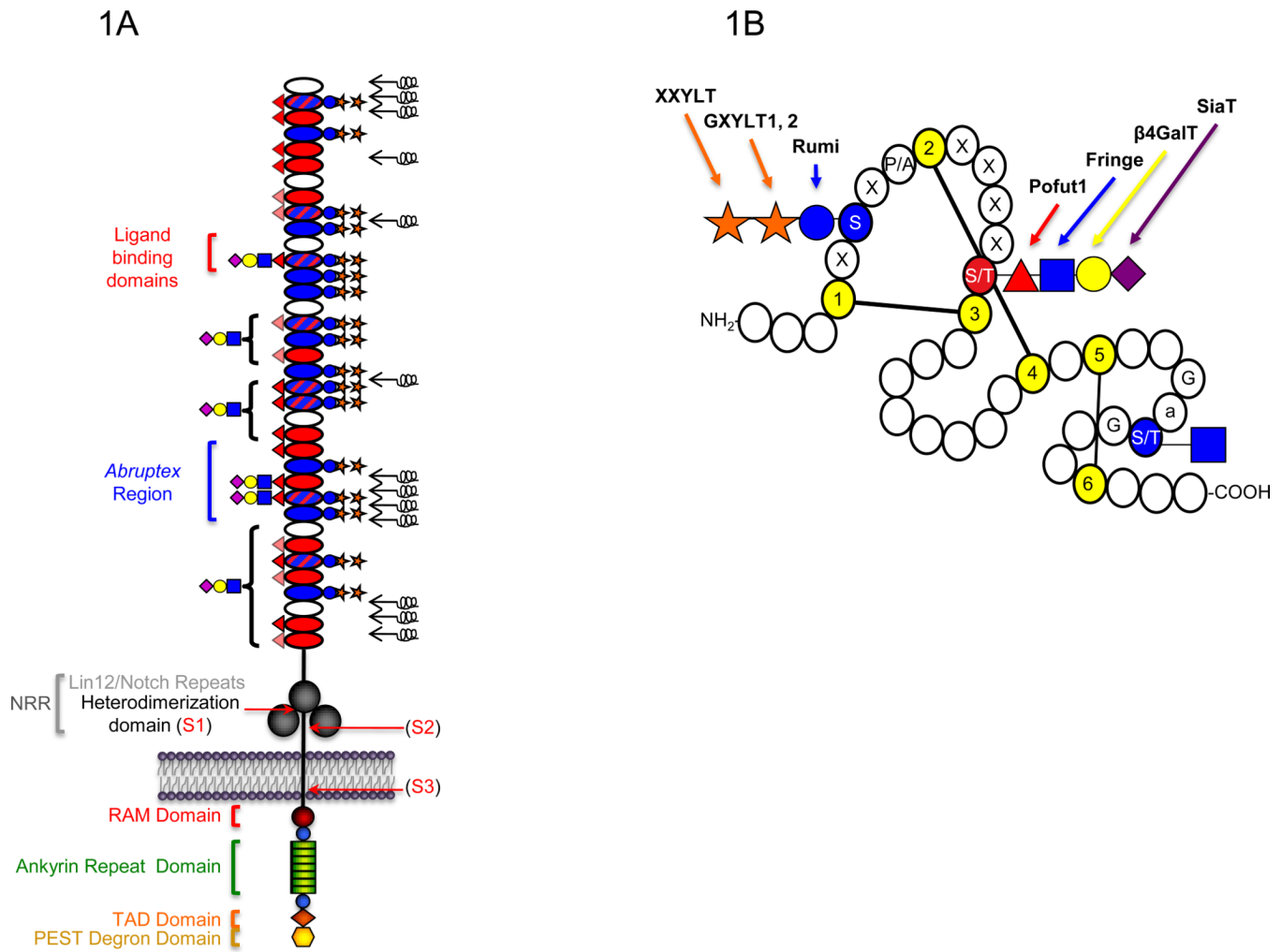


Figure 1. Schematic representation of the domain structure of the Notch receptor and an Epidermal Growth Factor-like (EGF) repeat

A. The extracellular domain (ECD) of mouse Notch1 consists of 36 tandem EGF repeats (ovals) followed C-terminally by three Lin2/Notch repeats (LNRs) (large grey circles) and a heterodimerization domain (black line) [1]. EGF repeats containing the consensus sequence for *O*-glucosylation, *O*-fucosylation, or both, are filled with blue, red, or blue and red hatched lines, respectively. Mapped *O*-fucose and *O*-glucose glycans are indicated on each EGF repeat based on published data [19–21] (lightly shaded red triangles indicate *O*-fucose sites not yet confirmed). Results suggest that all *O*-glucose sites are modified with *O*-glucose trisaccharide, although some *O*-glucose monosaccharide form also exists at lower levels (not shown) [19]. Elongation of the saccharide beyond the *O*-fucose is based on work from [20,21]. Brackets indicate that elongation occurs on some or all of the *O*-fucose sites in that region. Symbols are based on Consortium for Functional Glycomics guidelines: glucose, blue circle; xylose, orange star; fucose, red triangle; GlcNAc, blue square; Galactose, yellow circle; Sialic Acid, purple diamond. Non-calcium-binding EGF repeats are unable to coordinate calcium ions to their N-terminal linker region, and are predicted to be flexible regions of the ECD (black springs) [47]. Ligands are known to physically interact with EGF11–12 [44,45]. The *Abruptex* region is defined by a series of mutations in EGF 24–29 of *Drosophila* Notch known to hyperactivate Notch and be refractory to Fringe [43]. The LNRs and heterodimerization domain together form the Negative Regulatory Region

(NRR). Recent structural studies suggest that in the resting state, the LNRs mask the heterodimerization domain, preventing proteolysis [49,50]. Ligand binding is believed to result in a conformational change exposing the S2 protease site (see Figure 2). Sites of receptor cleavage events (S1-S3) are indicated with red arrows. The intracellular domain (ICD) is rich in several regulatory motifs as indicated, including a RAM (Regulation of Amino Acid Metabolism) domain, immediately followed by a Nuclear Localization Signal (NLS) (blue circles) and seven tandem ankyrin repeats (green rectangles). Following these motifs are a second NLS (blue circle), a Transcriptional Activation Domain (TAD) (orange diamond), and a PEST (Pro, Glu, Ser, Thr-rich) degron sequence involved in turnover of the ICD at the C-terminus (yellow hexagon). **B.** Short 40 amino acid motifs known as EGF repeats are defined by the presence of six conserved Cys (yellow) that form three disulfide bridges. Potential sites of *O*-glucosylation (C^1 -x-S-x-P/A-C²), *O*-fucosylation (C^2 -x-x-x-S/T-C³), and *O*-GlcNAc modification (no consensus sequence defined, but observed between Cys 5 and Cys 6 on Ser or Thr following Gly and an aromatic residue (a), and preceding Gly) are indicated in red or blue. Glycosyltransferases that carry out addition of each sugar are indicated with arrows.

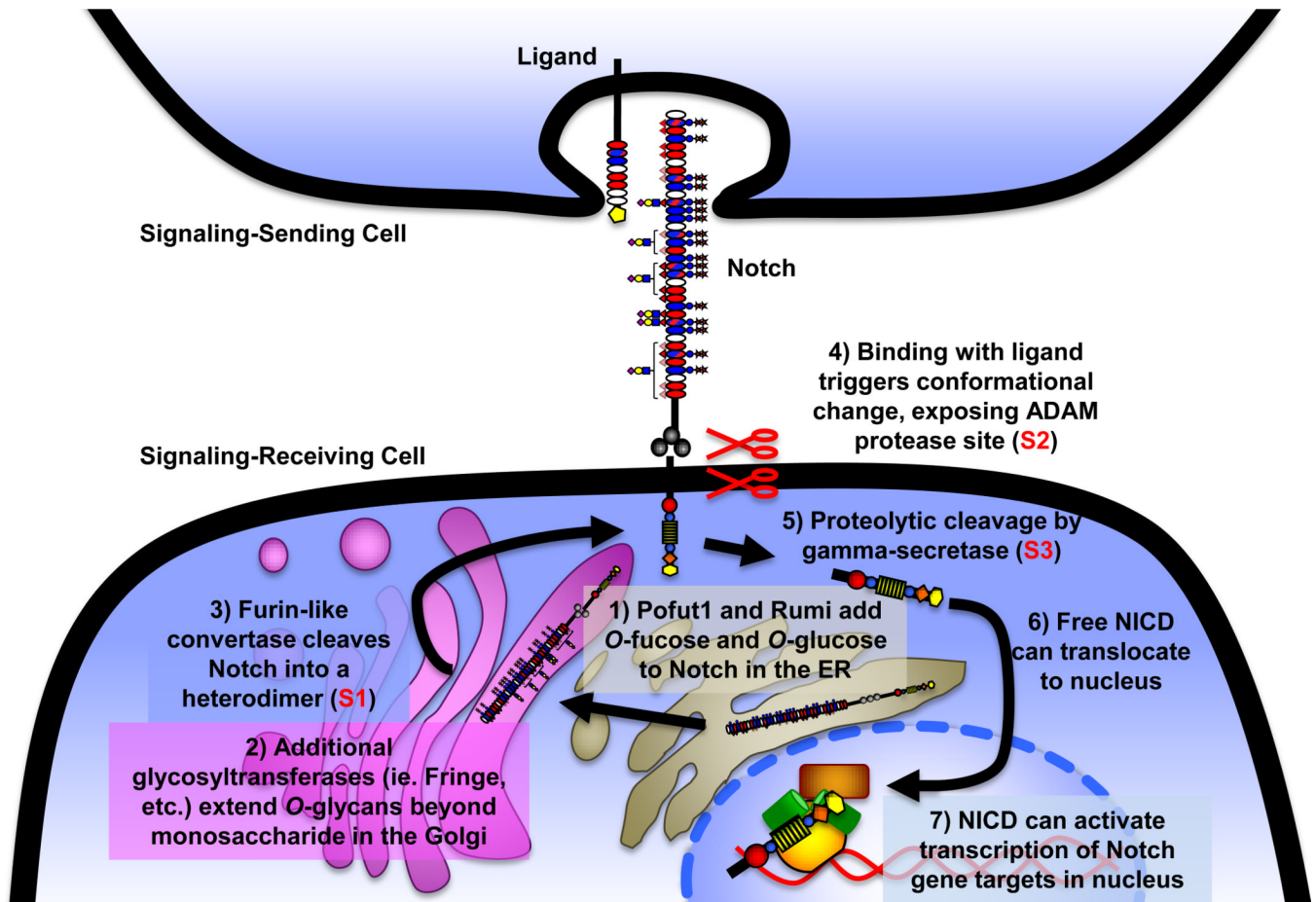


Figure 2. Notch activation pathway

Notch is *O*-fucosylated and *O*-glucosylated in the ER (1), and elongated by additional glycosyltransferases in the Golgi (2). Notch is also cleaved by a furin-like convertase in the Golgi (S1 cleavage, 3), generating the mature heterodimeric receptor that is subsequently expressed at the cell surface. Following interaction with a DSL (Delta, Serrate, Lag2) ligand presented on an apposing cell, Notch ECD undergoes a conformational change exposing the protease site for an ADAM protease (Kuzbanian in *Drosophila*) at the membrane interface (S2 cleavage, 4), and is subsequently cleaved by gamma-secretase just inside the cell membrane (S3 cleavage, 5), causing ICD release. Upon S3 cleavage, the ICD is free to translocate to the nucleus (6), bind to a member of the CSL (CBF/SuH/LAG-1) family of transcription factors *via* its RAM domain, and this binary complex serves as a platform for Mastermind-like (MAML)-family cofactors, which in turn can act as scaffolding for transcriptional complex formation. The assembled transcriptional activation complex can then activate expression of Notch target genes (7), including *Hairy-Enhancer of Split* (Hes) and *Hairy-Enhancer of Split related with YRPW motif* (Hey) family members [1].