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Role of Unusual O-Glycans in Intercellular Signaling

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Abstract

In the last two decades, our knowledge of the role of glycans in development and signal transduction has expanded enormously. While most work has focused on the importance of *N*-linked or mucin-type *O*-linked glycosylation, recent work has highlighted the importance of several more unusual forms of glycosylation that are the focus of this review. In particular, the ability of *O*-fucose glycans on the Epidermal Growth Factor-like (EGF) repeats of Notch to modulate signaling places glycosylation alongside phosphorylation as a means to modulate protein-protein interactions and their resultant downstream signals. The recent discovery that *O*-glucose modification of Notch EGF repeats is also required for Notch function has further expanded the range of glycosylation events capable of modulating Notch signaling. The prominent role of Notch during development and in later cell-fate decisions underscores the importance of these modifications in human biology. The role of glycans in intercellular signaling events is only beginning to be understood and appears ready to expand into new areas with the discovery that Thrombospondin type 1 repeats are also modified with *O*-fucose glycans. Finally, a rare form of glycosylation called *C*-mannosylation modifies tryptophans in some signaling competent molecules and may be a further layer of complexity in the field. We will review each of these areas focusing on the glycan structures produced, the consequence of their presence, and the enzymes responsible.

Keywords

Notch; Fringe; *O*-fucose; *O*-glucose; Rumi; EGF repeat; TSR

Introduction

Protein Glycosylation

Glycosylation of proteins has been observed in all organisms from bacteria to man (Wacker et al, 2002, Young et al., 2002). Many functions for glycans have been proposed (for a review see (Varki, 1993)), including stabilization of proteins (Imperiali and Rickert, 1995) antigenic masking (Wyatt et al., 1998), cellular recognition (Lowe, 2003), direct functional consequences on ligand binding (Bruckner et al, 2000, Moloney et al., 2000a), and protein folding and quality control (Moremen and Molinari, 2006, Ricketts et al, 2007, Wang et al., 2007). In the last 15 years, our knowledge of the biological relevance and the impact of protein glycosylation on

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human disease has ballooned. The dramatic and continuing increase in the number of congenital disorders of glycosylation (CDG), all of which result directly from altered glycan structures, is one example (reviewed by (Freeze and Aebi, 2005)). Further support comes from the observation that genetic ablation of many glycosyltransferases results in severe developmental phenotypes (Lowe and Marth, 2003). Examples include protein *O*-fucosyltransferase 1 (Pofut1) in mice (Shi and Stanley, 2003) and flies (Okajima and Irvine, 2002, Sasamura et al., 2003), protein *O*-fucosyltransferase 2 (Pofut2) in mice (Haltiwanger and Holdener, manuscript in preparation), protein *O*-mannosyltransferase 1 (POMT1) in mice (Willer et al., 2004), N-acetylglucosaminyltransferase 1 (GnT1) in mice (Campbell et al., 1995, Ioffe and Stanley, 1994), core β 1,3 galactosyltransferase in mice (Xia et al., 2004) and Rumi, a protein *O*-glucosyltransferase (Poglut) in flies (Acar et al., 2008). Structurally, there are two main classes of protein glycosylation depending on whether carbohydrate is linked to protein through the amide nitrogen of an asparagine (*N*-linked), or through the oxygen of a hydroxyl group on a serine, threonine, or hydroxy-lysine (*O*-linked) (Varki et al, 1999). This review will focus mainly on several fairly rare forms of *O*-linked glycans on cysteine-rich protein motifs, and the effects they exert on proteins involved in intracellular signaling. We will also touch on the potential effects on signaling of a rare form of glycosylation resulting in carbon-carbon bond formation between a sugar and a tryptophan, known as *C*-mannosylation.

Glycosylation of EGF Repeats—Epidermal Growth Factor-like (EGF) repeats are small (~40 amino acid) cysteine-rich motifs with six conserved cysteines forming three conserved disulfide bonds (Appella et al., 1988, Campbell and Bork, 1993, Davis, 1990). EGF repeats containing the appropriate consensus sequence (C^2 -X₍₄₋₅₎-[S/T]-C³ where C² and C³ are the second and third conserved cysteine) are modified by fucose (Fuc) in α -linkage to the serine or threonine by protein *O*-fucosyltransferase 1 (Pofut1 in mammals, OFut1 in *Drosophila*) (Figure 1A) (Harris and Spellman, 1993, Luo and Haltiwanger, 2005, Shao et al, 2003, Wang et al, 2001). Both OFut1 and Pofut1 are localized to the ER and add fucose to properly folded EGF repeats (Luo and Haltiwanger, 2005, Okajima et al, 2005). The fucose can be further elongated in the Golgi by one of the Fringe β 1,3N-acetylglucosaminyltransferases to form the GlcNAc- β 1,3-Fuc disaccharide (Bruckner et al., 2000, Moloney et al., 2000a). While there does not appear to be elongation past the disaccharide in *Drosophila* (at least in *Drosophila* S2 cells) (Xu et al., 2007), the disaccharide is further elongated in mammals by addition of a galactose (Gal) and a sialic acid (Sia) to form the mature tetrasaccharide, Sia- α 2,3/6-Gal- β 1,4-GlcNAc- β 1,3-Fuc (Chen et al., 2001, Moloney et al., 2000b).

In addition to the presence of *O*-fucose glycans, a glucose is added in β -linkage to a serine by protein *O*-glucosyltransferase (Poglut) when the appropriate consensus sequence (C¹-X-S-X-P-C²) is present between the first and second cysteine of the EGF repeat (Figure 1A) (Acar et al., 2008, Moloney et al, 2000b). The *rumi* gene was recently shown to encode a Poglut (Acar et al., 2008, Moloney et al, 2000b). Like Pofut1, Poglut appears to be localized in the ER where it *O*-glucosylates properly folded EGF repeats (Acar et al, 2008, Shao et al, 2002). The *O*-glucose glycan can be further elongated by consecutive addition of two xylose (Xyl) sugars to form a mature trisaccharide, Xyl- α 1,3-Xyl- α 1,3-Glc (Hase et al, 1990). Although activities of the enzymes responsible for addition of these xyloses have been detected, the xylosyltransferases have not yet been identified (Minamida et al., 1996, Omichi et al, 1997). The intracellular localization of xylosylation is likewise unclear at this point, and no function for these modifications has as yet been identified. These modifications may be analogous to the elongation of *O*-fucose initiated by the Fringe enzymes. If so, they may have similar effects on protein-protein interactions (as described below).

Glycosylation of Thrombospondin Type 1 Repeats—Similar to the EGF repeat, a second type of cysteine-rich motif known as a thrombospondin type 1 repeat (TSR) contains six conserved cysteines and three disulfide bonds (Adams and Tucker, 2000). Unlike the EGF

repeat, however, there is some flexibility in the disulfide-bonding pattern in the TSR resulting in two structural groups termed class 1 and 2 (Tan et al., 2002, Tossavainen et al., 2006, Tucker, 2004). The difference in disulfide bonding pattern results from one cysteine being present on separate, neighboring strands in the two classes, although the overall fold is very similar between the two (Tossavainen et al., 2006). Two unusual forms of glycosylation are known to occur on TSRs. A homologue of Pofut1 known as protein *O*-fucosyltransferase 2 (Pofut2) (Luo et al., 2002, Luo et al., 2006a, Luo et al., 2006b) adds a fucose in α -linkage to a serine or threonine within TSRs containing the consensus sequence: C-X-X-[S/T]-C-X-X-G (between the first and second cysteines for class 1 TSRs, second and third for class 2) (Hofsteenge et al., 2001, Ricketts et al., 2007, Wang et al., 2007) (Figure 1D). This fucosylation event also appears to occur in the ER despite the lack of an obvious ER localization sequence in the Pofut2 sequence (Luo et al., 2006a). The *O*-fucose can be elongated by a β 1,3-glucosyltransferase (β 3GlcT) to the disaccharide, Glc- β 1,3-Fuc (Kozma et al., 2006, Sato et al., 2006). In addition to *O*-fucose glycans, tryptophans in the consensus sequence W-X-X-W in a TSR can be *C*-mannosylated (Figure 1D) (Hofsteenge et al., 2001). This glycosylation is performed by an enzyme in the ER membrane using dolichol-P-mannose as a donor substrate (Doucey et al., 1998, Hofsteenge et al., 1994). *C*-mannosylation is an unusual form of glycosylation that involves a carbon-carbon bond formed between the tryptophan and the mannose (Man). The W-X-X-W motif does not need to be in the context of a TSR to be *C*-mannosylated (Krieg et al., 1997, Krieg et al., 1998, Löffler et al., 1996), although this sequence is commonly found in TSRs. The gene encoding the enzyme(s) responsible for addition of *C*-mannose has not yet been identified. While little is yet known about the effects of *C*-mannosylation on modified proteins, there are reports that suggest effects on protein folding and trafficking (Hilton et al., 1996) and on signaling by an unknown mechanism (Muroi et al., 2007).

Importance of Epidermal Growth Factor-like Repeat Glycosylation in Notch Signaling

Notch Signaling

Notch is a membrane anchored signaling receptor important for development of many tissues in all metazoans, with four homologues in mammals (Bray, 2006, Wharton et al., 1985). Disruption of Notch signaling interrupts cell fate decisions during neuronal development in flies, leading to a “neurogenic” phenotype, where neurons develop at the expense of epidermal cells (reviewed by (Louvi and Artavanis-Tsakonas, 2006)). Notch plays similar roles in development of many tissues. Not surprisingly, aberrant Notch signaling contributes to many diseases (reviewed by (Rampal et al., 2007)).

Notch becomes activated upon interaction with membrane-anchored ligands of the Delta/Serrate/LAG-2 (DSL) family on adjacent cells. The DSL ligands are subdivided into Delta (Delta-like1, 3 and 4 in mammals) and Serrate (Jagged1 and 2 in mammals) subfamilies (reviewed by (Bray, 2006)). The nascent Notch receptor is subject to cleavage by a furin-like protease in the Golgi. This creates a Notch heterodimer with the extracellular domain (ECD) tethered to a transmembrane-intracellular domain (Figure 2B). The ECD is composed mainly of tandem EGF repeats, with three Notch/Lin12 repeats at the C-terminus forming a negative regulatory region (see Figure 2A). Ligand binding induces a proteolytic event where the majority of the extracellular portion of Notch is released from the cell surface (Figure 2C). This cleavage can be catalyzed by *ADisintegrin* And *Metalloprotease* (ADAM) 10 (Hartmann et al., 2002) in mammals, while Kuzbanian performs the cleavage in *Drosophila* (Mumm et al., 2000). Recent structural studies suggest that the cleavage site is masked by the Notch/Lin12 domains in the resting state (Figure 2B). Thus, ligand binding must induce a conformational rearrangement in the extracellular domain of Notch to expose the ADAM cleavage site (Gordon et al., 2007) (see Figure 2C). The ECD is then endocytosed with the ligand by the signal-sending

cell (Parks et al., 2000, Nichols et al., 2007). After release of the Notch ECD, the presenilin/ γ -secretase complex cleaves the remaining Notch fragment just inside the inner leaflet of the membrane, releasing the intracellular domain from the membrane (De Strooper et al, 1999) (Figure 2D). The ICD then translocates to the nucleus where it binds to the CSL (for CBF1/SuH/LAG-1) transcriptional regulators, converting them from transcriptional repressors to transcriptional activators, resulting in expression of a number of downstream targets (Bray, 2006). Notch signaling is regulated at a variety of levels by both intracellular and extracellular modulators. Intracellular modulators include E3 ubiquitin ligases, E3 ligase inhibitors, coactivators, corepressors, DNA binding proteins, and signaling inhibitors (reviewed by (Bray, 2006, Stanley, 2007)). Notch activity is also regulated by glycosylation of its ECD, as described below.

Fringe Modulates Notch by Elongating *O*-fucose on EGF repeats

The importance of *O*-fucosylation for Notch function was first realized when the Fringe family of genes were demonstrated to catalyze addition of N-acetylglucosamine (GlcNAc) to *O*-fucose on the EGF repeats of the Notch ECD (Figure 3A) (Bruckner et al., 2000, Moloney et al., 2000a). Fringe was originally described in *Drosophila* for its role in boundary formation during wing development (Irvine and Wieschaus, 1994). Although Notch and its ligands are expressed throughout the wing imaginal disc, Notch is only activated at the dorsal/ventral boundary. Elongation of *O*-fucose by Fringe inhibits Notch activation from Serrate while potentiating activation from Delta (Panin et al, 1997) (Figure 3). In this fashion, Fringe functions to position Notch activation at the dorsal/ventral boundary (Panin et al., 1997). Fringe performs a similar positioning of Notch activation during development of limbs and in the eye in flies (reviewed by (Irvine, 1999)). This provides a clear example of how a signaling event can be regulated by changing the glycosylation state of a receptor.

Vertebrates have a family of three Fringe enzymes named Lunatic, Manic and Radical fringe. Knocking out Lunatic fringe in mice produces segmentation and somitogenesis defects (Evrard et al., 1998, Zhang and Gridley, 1998, Zhang et al., 2002). Lunatic fringe homozygous mutants have severely disrupted skeletal patterning, with shortened, missing, fused, and bifurcated ribs, shortened tails, and disrupted vertebral patterning due to effects on the Notch dependent process of somitogenesis (Zhang and Gridley, 1998). Radical fringe knockout did not show any obvious phenotype (Zhang et al., 2002). Lunatic and Radical fringe double knockouts were produced with no obvious differences between the double mutant and the Lunatic fringe homozygous mutant mice (Zhang et al., 2002). There have been no reports of a Manic fringe knockout.

The fact that the Radical fringe knockout shows no obvious phenotype raises the question of functional redundancy for the Fringe enzymes. As for their catalytic activity, all three mammalian Fringe enzymes catalyze the transfer of GlcNAc to *O*-fucose on EGF repeats in in vitro assays, although with differing efficiency (Rampal et al., 2005b). Lunatic and Radical fringe both modified *O*-fucose on a Factor IX EGF repeat with similar efficiency, while Manic fringe was three-fold less efficient with this substrate (Rampal et al., 2005b). In the case of an *O*-fucosylated EGF repeat from mouse Notch1, Lunatic fringe activity was more than six-fold greater than Radical fringe and more than one hundred and fifty fold greater than Manic fringe (Rampal et al., 2005b). Comparison of the sequences of the EGF repeats from human Factor IX (a good substrate for Lunatic fringe) with Factor VII (a poor substrate for Lunatic fringe) revealed two amino acids which are necessary for efficient Lunatic fringe recognition (Rampal et al., 2005b). These results reveal that the Fringe enzymes preferentially modify *O*-fucose on some EGF repeats over others, and that the Fringe enzymes recognize specific amino acids within EGF repeats in addition to the *O*-fucose (Shao et al., 2003). These patterns indicate that while the Fringe enzymes all catalyze the same reaction, their specificity for any given EGF repeat is different. The three enzymes may have evolved optimum specificities for different

substrates and may be promiscuous to varying degrees with sub-optimal substrates, suggesting the Fringe enzymes are not functionally redundant. More detailed analyses of the knockout mice, and evaluation of a Manic fringe knockout, are necessary to resolve these issues.

Role of Lunatic Fringe During Somitogenesis

The formation of vertebrate somites is regulated by waves of gene expression (including Notch pathway components hairy enhancer of split (Hes)7, Lunatic fringe, and Dll3) termed the somitogenesis clock (for recent reviews see (Andrade et al., 2007, Kageyama et al., 2007)). These unidirectional waves of expression originate in the caudal presomitic mesoderm and travel to the rostral presomitic mesoderm where they eventually terminate when encountering mesoderm posterior 2 (Mesp2) expression (Morimoto et al., 2005). This sets the boundary for the developing somite. Lunatic fringe turns Notch on and off in this context, and its expression is regulated in concert with periodic somite formation. Thus, Lunatic fringe is now recognized as an integral component of the somitogenesis clock. In computer simulations, Zhu and Dhar showed that transient blockage of Notch signaling involving Notch1, Lunatic fringe, and Hes7, a downstream target of Notch, could maintain a unidirectional wave of signaling (Zhu and Dhar, 2006). Hes7 negatively regulates its own expression and that of Lunatic fringe (Chen et al., 2005). Thus, when Hes7 is expressed, Hes7 and Lunatic fringe transcription is inhibited, resulting in waves of expression. Disruption of the clock by elimination or misexpression of Lunatic fringe results in somitogenesis defects. Recently, Shifley and Cole have reported that the N-terminus of the Fringe family of enzymes contain sequences affecting proteolysis by subtilisin-like proprotein convertases and that these cleavage events decrease the intracellular half-life of Lunatic fringe (Shifley and Cole, 2008). Substitution of a Radical fringe N-terminus for the wild type Lunatic fringe sequence significantly increased the intracellular half-life of Lunatic fringe. This strongly suggests that the different N-terminus of Fringe enzymes could regulate how they function, and that proteolysis by subtilisin-like proprotein convertases could play a role in the cycling of Lunatic fringe function in somitogenesis. Additionally, Cole and coworkers have reported that disruption of Lunatic fringe cycling in somitogenesis has substantially greater effects on the patterning of the anterior skeleton compared with the posterior (Shifley et al., 2008). Lewis and coworkers, using computer simulations and experiments in zebrafish, conclude that Notch signaling in fish is required only for synchronization of the segmentation clock and is unlikely to play a role in initiation of the clock or boundary formation in the somites (Özbudak and Lewis, 2008). Whether or not this is true in all organisms is an unanswered question. Together, these results suggest that the clock in somitogenesis could ultimately be found to be composed of multiple components with disruption of any one component being insufficient to completely disrupt somite patterning.

Lunatic Fringe knockout mice share a striking resemblance to mice lacking Delta-like3, suggesting that disruption of these genes has similar effects in vivo (Kusumi et al., 1998). The Delta-like3 ligand has significant sequence divergence from the other Notch ligands. Delta-like3 has recently been shown to negatively regulate Notch signaling in a cell autonomous manner, while being incapable of activating the Notch signaling pathway in trans like the other Notch ligands (Ladi et al., 2005). The genetic disease spondylocostal dysostosis in humans can be caused by mutations in Delta-like3 (Bulman et al., 2000), Lunatic fringe (Sparrow et al., 2006), Mesp2 (Whitlock et al., 2004), or Hes7 (Sparrow et al., 2008), presumably due to the effects these Notch pathway components have on somitogenesis during development (for a review see (Sparrow et al., 2007)).

Mechanisms For the Effects of Fringe on Notch Function

The majority of data suggests that Fringe-mediated elongation of *O*-fucose on Notch results in a change in the binding between Notch and its ligands (Bruckner et al., 2000, Hicks et al., 2000, Okajima et al., 2003, Shimizu et al., 2001, Stahl et al., 2008, Xu et al., 2007, Yang et

al., 2005). This is clearly the case in the *Drosophila* system. The in vivo effects of Fringe can be recapitulated using purified components in vitro (Xu et al., 2007). Addition of GlcNAc to *O*-fucose using Fringe causes an increase in Delta binding and a decrease in Serrate binding (Table 1) (Xu et al., 2007). Although the data using mammalian components also suggests that Fringe modulates the binding between Notch and its ligands, the results are complicated by the increased number of receptors, ligands, and Fringe enzymes. Weinmaster and coworkers found that with Notch1, Lunatic fringe increased signaling from Delta-like1 and inhibited Jagged1 mediated signaling using either NIH3T3 or C2C12 myoblast cells (see Table 1) (Hicks et al., 2000, Yang et al., 2005). This mirrors the situation known to occur in *Drosophila* where Fringe increases signaling from Delta and inhibits signaling from Serrate (Fleming et al., 1997, Panin et al., 1997). The Weinmaster group has shown that Manic fringe mirrors the effects of Lunatic fringe on Notch1, although Manic fringe appears to be less efficient (Hicks et al., 2000). Importantly, although Lunatic fringe causes an increase in binding of Delta-like1 to Notch1, it does not appear to significantly alter the binding of Jagged1 to Notch1 (see Table 1) (Hicks et al., 2000; Yang et al., 2005). Thus, unlike in *Drosophila* where Fringe decreases Serrate binding, there is no immediately obvious mechanism for Lunatic fringe mediated inhibition of Jagged1 signaling. The Weinmaster group proposed that Lunatic fringe reduces the strength of the interaction between Notch1 and Jagged1, preventing activation. Weinmaster and coworkers also found that the effects of Radical fringe on signaling through Notch1 are different from those of Lunatic and Manic. Radical fringe increased signaling with both Delta-like1 and Jagged1 (see Table 1) (Yang et al., 2005). In the case of Jagged1 and Notch, Moloney et al. had identical results to the Weinmaster group (see Table 1) using Chinese hamster ovary (CHO) cells. Both Lunatic and Manic fringe decreased Jagged1 mediated Notch signaling in a cell based assay, although these cells express more than one Notch (Notch1 is indicated in Table 1 as it appears to be the major form of Notch in CHO cells) (Chen et al., 2001, Moloney et al., 2000a). Recent results demonstrate that Lunatic fringe decreases binding of Jagged1 and increases binding of Delta-like1 to endogenous Notch in CHO cells (Stahl et al., 2008).

The effect of Fringe on Notch2 shows different effects. The Hirai group reported that both Lunatic and Manic fringe cause a decrease in Notch2 signaling from Jagged1, with the level of decrease significantly higher from Manic fringe (see Table 1) (Shimizu et al., 2001). They saw no effect of Lunatic or Manic fringe on the ability of Delta-like1 or Jagged2 to activate Notch2 using CHO cells. The effect of Lunatic or Manic fringe on Notch2-ligand binding was consistent with the signaling data (Shimizu et al., 2001). In contrast, the Weinmaster group reported (using C2C12 myoblasts) that Lunatic fringe causes increased activation of Notch2 with either the Delta-like1 or Jagged1 ligands (see Table 1) (Hicks et al., 2000). Clearly, the results for Lunatic fringe modulation of Notch2 signaling will require further investigation. These differences may reflect further layers of complexity caused by the presence or absence of various Notch modulators such as Delta-like3 that may be present in some cells but not others. The data in Table 1 shows that the effect of Fringe on activation of Notch by Delta-family ligands appears to be at the level of binding, while the effect on the activation of Notch by the Serrate/Jagged-family of ligands may be more complicated.

The molecular details for how elongation of *O*-fucose by Fringe can change the binding between Notch and its ligands is not at all clear. Fringe modifies *O*-fucose on many but not all of the EGF repeats in the ECD of Notch (Rampal et al., 2005a, Shao et al., 2003, Xu et al., 2007). EGF repeats 11 and 12 are believed to be essential for ligand binding, and mutation of the *O*-fucose site in EGF repeat 12 (known to be modified by Fringe) decreases Notch1 function both in cell-based assays (Rampal et al., 2005b) and in vivo in mice (Ge and Stanley, 2008). Elimination of the same site in *Drosophila* Notch reduces the ability of Fringe to inhibit Serrate-induced Notch activation (Lei et al., 2003). Recent structural studies provide evidence for interaction between specific amino acids in the DSL domain of Jagged1 and EGF repeats 11 and 12 of Notch1 (Cordle et al., 2008b). Interestingly, the *O*-fucose on EGF repeat 12 lies on

the opposite face of the EGF repeat from that predicted to interact with Jagged1. This suggests that the effects of the *O*-fucose (and Fringe elongation) on EGF repeat 12 may be indirect. In addition, the fact that other EGF repeats in Notch not directly implicated in ligand binding can dramatically affect function suggests that glycosylation of other EGF repeats may also be important (Perez et al., 2005, Rampal et al., 2005a). For instance the *Abruptex* mutations cluster in EGF repeats 24–29 outside the ligand binding domain (Perez et al., 2005). These mutations result in hyperactivated Notch that is refractory to Fringe (De Celis and Bray, 2000). Several EGF repeats in the *Abruptex* region are modified by Fringe, and mutation of the *O*-fucose sites in EGF repeat 26 or 27 in mouse Notch1 alters Notch signaling in cell-based assays (Rampal et al., 2005a). These data suggest that Fringe may mediate its effects on Notch activity not just at EGF repeat 12, but at numerous sites along the ECD.

One possible explanation for how modification by Fringe at numerous sites scattered across the ECD could modulate activity would be effects on the conformation. Structural studies on a short region of human Notch1 including the ligand binding region around EGF repeat 12 revealed that the presence of calcium-binding EGF repeats results in a fairly rigid structure, while the absence of calcium binding allows flexibility (Cordle et al., 2008a, Cordle et al., 2008b, Hambleton et al., 2004). Calcium binding is conferred by the presence of certain amino acids in the short linker between two EGF repeats (NNxNC¹, where N can be D/E/Q/N, x any amino acid, and C¹ is the first conserved C of the EGF) and can be predicted based on sequence (Figure 2A shows the location of predicted calcium-binding sequences in human Notch1). The pattern of calcium-binding EGF repeats in various Notch proteins is highly conserved (Xu et al., 2005), and as such, may play a crucial role in the function of the protein. This suggests a model whereby the regions containing calcium-binding EGF repeats are rigid, with more flexible “hinge” regions between them, allowing the rigid regions to stack against each other. The elongation of *O*-fucose by Fringe could inhibit interactions between neighboring rigid sections of Notch, or conversely, interactions between elongated glycans could facilitate a new interaction between the neighboring rigid regions. The Delta class of ligands would prefer the conformation produced by Fringe elongated glycans in the Notch ECD, and the Serrate/Jagged class would prefer the Notch ECD conformation in the absence of Fringe elongation. Recently, Pei and Baker showed that the EGF repeats from the *Abruptex* region of Notch can bind to EGF repeats containing the ligand-binding domain (EGF repeats 11 and 12). This interaction appears to compete with Delta in binding assays. These data, coupled with knowledge that *Abruptex* mutations activate Notch signaling, suggests that the *Abruptex* and ligand binding regions may interact in vivo (Pei and Baker, 2008). A conformational change disengaging this interaction would allow Delta ligand binding. Fringe elongated *O*-fucose glycans may function to modulate the interaction between the *Abruptex* region and ligand-binding region of Notch.

O-fucose on Notch EGF Repeats

While elongation of *O*-fucose by Fringe modulates Notch function, the addition of *O*-fucose appears to be essential for Notch function through contributions distinct from its role as a substrate for Fringe modification. Elimination of OFut1 in *Drosophila* results in severe Notch-like phenotypes. An *Ofut1* mutant called *neurotic* results in a classic neurogenic phenotype where there is an overabundance of neurons due to failure of Notch-dependent lateral inhibition (Sasamura et al., 2003). Knockdown of *Ofut1* using RNAi also results in lateral inhibition defects in sensory organ precursor cells, as well as in wing formation, both of which require Notch function (Okajima and Irvine, 2002). Similarly, elimination of Pofut1 in mice produces a severe embryonic lethal phenotype that is more dramatic than single Notch receptor knockouts in mice. This is presumably due to the wholesale destruction of signaling through all four of the Notch receptors present in mammals. The mice die in mid-gestation with defects in neurogenesis, somitogenesis, vasculogenesis, and cardiogenesis due to disrupted Notch signaling (Shi and Stanley, 2003).

Although the importance of Pofut1 (or OFut1) for Notch function in both flies and mice is clear, the mechanism by which it affects Notch is not. Irvine and coworkers have provided evidence that OFut1 functions as a chaperone for Notch in flies (Okajima et al., 2005). Their data show that loss of OFut1 causes a loss of cell-surface expression of Notch. Interestingly, an enzymatically inactive form of OFut1 (OFut1^{R245A}) can rescue the secretion defect in cell culture (Okajima et al., 2005) as well as rescue the *Ofut1* null Notch-like neurogenic phenotype (Okajima et al., 2008). These results suggest that some property of OFut1 other than the fucosyltransferase activity is essential for cell surface expression of Notch. Because Notch was accumulating in the ER, they proposed that OFut1 has a chaperone activity (assisting in the proper folding of Notch) that is distinct from the *O*-fucosyltransferase activity. Consistent with this hypothesis, flies null for GDP-mannose 4,6 dehydratase (Gmd), which is required for the production of GDP- β -L-fucose, do not show a neurogenic phenotype as would have been expected with non-functional Notch (Okajima et al., 2008). Since GDP-fucose is the donor substrate for OFut1, this result suggests that fucosylation is not required for Notch function during neurogenesis. The major phenotype observed in clones of cells expressing OFut1^{R245A} is similar to that of Fringe mutants, consistent with the loss of the substrate for Fringe. These results suggest that in flies, *O*-fucose is essential as a substrate for Fringe, but not for other Notch activities (Okajima et al., 2008).

The situation in mice may be different. Stanley and coworkers have shown convincingly that embryonic stem cells lacking Pofut1 have wild type levels of Notch receptors on their cell surfaces (Stahl et al., 2008). Notch activity (either ligand binding or Notch activation) is severely compromised in these cells, suggesting that Notch requires the addition of *O*-fucose for full activity. Overexpression of an enzymatically inactive Pofut1 (equivalent to the R245A mutant in OFut1) in these cells partially restores Notch activity. However, overexpression of another ER protein has the same effect, suggesting the “chaperone” activity of Pofut1 may be non-specific in mammalian cells. Notch activity (both ligand binding and Notch activation) in CHO cells lacking GDP-fucose is also compromised, again suggesting the importance of *O*-fucose for Notch function in mice (Chen et al., 2001, Moloney et al., 2000a, Stahl et al., 2008). In contrast, Okamura and Saga recently reported that Notch1 fails to properly localize to the cell surface in the presomitic mesoderm of *Pofut1* knockout mice (Okamura and Saga, 2008). They present data suggesting partial co-localization of Notch1 with ER markers in *Pofut1*^{-/-} mice and suggest that this mis-localization contributes to the mutant phenotype. Further work will need to be done to carefully examine the effects of eliminating Pofut1 on Notch localization in vivo.

Experiments utilizing *O*-fucose site mutants in Notch reveal that *O*-fucose glycans are important for optimal Notch function in both flies and mice, but they do not resolve whether the *O*-fucose has a role distinct from being a substrate for Fringe. Expression of Notch lacking fucose in EGF repeat 12 of the ligand binding region (N^{12f}) in flies showed a reduction in the ability to respond to Fringe (Lei et al., 2003). Expression of the same mutant (N^{12f}) of mouse Notch1 in place of endogenous Notch1 produced mice that were viable and fertile, indicating that this site is not essential for Notch function (Ge and Stanley, 2008). Nonetheless, the mice showed growth defects and T cell abnormalities (T cell development is Notch 1 dependent), suggesting subtle changes in Notch activity. While *O*-fucose on EGF repeat 12 can be modified by Fringe in some contexts (Shao et al., 2003; Xu et al., 2007), it is not known whether the effects observed in the N^{12f}/N^{12f} mice are due to loss of *O*-fucose or to the lack of ability of Fringe to modify the *O*-fucose at that site.

Other studies support additional roles for OFut1 in flies. Sasamura et al. proposed that extracellular OFut1 is necessary for proper cycling of cell surface Notch through endosomes and on to lysosomes in a fucose-dependent manner (Sasamura et al., 2007). Sasaki et al. provide evidence that Notch is localized to the sub-apical complex/adherens junction in *Drosophila*

epithelial cells, and that Notch localization in this context is dynamin dependent and thus likely dependent on transcytosis (Sasaki et al., 2007). In both papers, the authors suggest a role for OFut1 and fucosylation in these processes. These are intriguing ideas, but some concerns about the results have been raised. For instance, it is not clear whether OFut1, which is retained in the ER by virtue of a C-terminal KDEL-like sequence, is actually found at significant levels in extracellular spaces in vivo. These results are discussed in more detail elsewhere (Vodovar and Schweisguth, 2008). Obviously, further work will need to be done to work out the details of how OFut1 affects Notch activity in flies.

All of the experiments performed in the absence of OFut1/POFUT1, as well as those suggesting a non-enzymatic function for OFut1, suffer from the lack of any analysis of the glycosylation state of Notch. The “enzymatically inactive” mutants of OFut1 (e.g. R245A) could retain small amounts of activity allowing partial *O*-fucosylation of Notch. In the absence of GDP-fucose, it is possible that other nucleotide-sugars could substitute as a donor substrate for the enzyme. This might explain why Notch without *O*-fucose could maintain at least some of its Fringe-independent functions.

There are several potential explanations for the apparent divergence between the role of *O*-fucose in the *Drosophila* and mouse models. First, flies and mice may simply be different. Perhaps to function in flies, Notch does not require *O*-fucose, while in higher organisms it does. It is known that mammals and more primitive organisms use Notch signaling at different stages in development (Ge et al., 2008, Good et al., 2004, Peterson and McClay, 2005, Sherwood and McClay, 1999, Sherwood and McClay, 2001, Shi and Stanley, 2006). Most dramatically, mammals do not use Notch for early stages in development such as germ layer formation whereas *C. elegans* and sea urchins do. Shi and Stanley have suggested that adoption of Notch signaling for earlier processes in development may be a later adaptation, rather than the ancestral function (Shi and Stanley, 2006). A second possibility is that the robustness of developmental processes in the two model systems may differ. Perhaps *Drosophila* development provides less strenuous demands upon Notch signaling than mice, and as such, despite Notch signaling at a less than peak efficiency in *Ofut1* null flies, it is sufficient. This may be testable if there are differences in the mutant flies with regard to the number of larvae that hatch, and/or the time it takes them to reach this stage. Additionally, placing the developing larvae under a stress such as higher temperature may reveal a phenotype not apparent under normal developmental conditions. Indeed, if OFut1 is functioning as a classical chaperone, it is exactly this type of stress that the OFut1^{R245A} mutant enzyme should be able to rescue. Thirdly, the differences between mice and flies may not involve differences in the Notch signaling pathway *per se*, but differences in the robustness of the protein expression machinery in the two species. Perhaps even an inefficient rescue might be enough to achieve a Notch signaling threshold to permit fly development to continue. Differences in the complement of chaperones in different cells could also help to explain the differences observed.

Elongation of *O*-fucose Glycans Beyond the Disaccharide

Recent results show that there is no elongation past the GlcNAc- β 1,3-Fuc disaccharide on *Drosophila* Notch produced in S2 cells (Xu et al., 2007). The effect of Fringe on Notch-ligand binding can be recapitulated using purified components in vitro, suggesting that no further elongation is required for Fringe to modulate Notch activity (Xu et al., 2007). A recent report suggests that a branched *O*-fucose trisaccharide exists in total fly extracts (GlcNAc- β 1,3-(GlcA- β 1,4)-Fuc) (Aoki et al., 2008). Further work needs to be done to determine whether this trisaccharide exists on Notch, and whether the addition of the glucuronic acid has effects on Notch activity. Nonetheless, further elongation of the glycan in mammals is significant. Stanley and coworkers showed that Lunatic fringe could not inhibit Jagged1-mediated Notch activation in CHO cells incapable of adding Gal to the GlcNAc- β 1,3-Fuc disaccharide (Chen et al.,

2001). Detailed analysis of mice lacking the β 1,4-galactosyltransferase 1 (β 4GalT1) enzyme responsible for addition of this Gal revealed a mild segmentation defect, suggesting some involvement of the Gal in the ability of Lunatic fringe to modulate Notch function in vivo (Chen et al., 2006). In contrast, Stanley and coworkers have shown that the presence or absence of Sia does not affect Fringe modulation of Notch signaling from Jagged1 (Chen et al., 2001). The effects of the Gal on Notch function may represent an additional level of fine tuning acquired through evolution.

O-Glucose on Notch EGF-like Repeats

While the importance of *O*-fucose modifications in Notch biology has been clear for several years, the importance of *O*-glucose glycans has only recently been realized. Acar et al. have shown that the *rumi* gene encodes the protein *O*-glucosyltransferase (Poglut) (Acar et al., 2008). Mutations in *rumi* exhibit a temperature sensitive Notch phenotype in flies (Acar et al., 2008). The authors reported that loss of Rumi results in cell-autonomous defects affecting the extracellular domain of Notch. They also observed accumulation of Notch at the cell surface, but no apparent ER-related folding defects for Notch in the *rumi* mutants (Acar et al., 2008). Thus, Rumi does not appear to be a chaperone analogous to OFut1. RNAi-mediated knockdown of *rumi* in S2 cells does not affect Delta binding to Notch (Acar et al., 2008) suggesting that loss of Rumi affects a step between ligand binding and the S2 cleavage (Figure 2C). Temperature sensitivity is often associated with effects on protein conformation. Thus, these data suggest that *O*-glucose holds the extracellular domain of Notch in a functionally active conformation. This supports the conclusion that *O*-glucose glycans are necessary for the function of Notch in flies, and that the *rumi* temperature-sensitive phenotype is due to impaired *O*-glucosylation of the Notch receptor.

Although *O*-glucose can be elongated by two xylose residues (Figure 1), little is known about the biological function of the xyloses. Enzymatic activities for the two xylosyltransferases have been identified (Minamida et al., 1996, Omichi et al., 1997), and the β -glucose α 1,3-xylosyltransferase has been partially purified (Omichi et al., 1997, Ishimizu et al., 2007). The biochemical data suggests that each Xyl is added by a separate enzyme. Determination of whether it is *O*-glucose alone, or the elongated glycan that is required for Notch function will require the identification of the genes encoding these enzymes.

Biological Roles for O-fucosylation of Thrombospondin Type I Repeats

O-Fucose on TSRs

Little is known about the function of *O*-fucosylation of TSRs, although database searches using the consensus sequence for modification suggest more than 40 proteins may be modified in mammals, including thrombospondins 1 and 2, the ADAMTS family of metalloproteases, brain-specific angiogenesis inhibitors, properdin, members of the spondin family, and others (Luo et al., 2006a, Luo et al., 2006b). TSRs are known to interact with fibronectin, heparin, glycosaminoglycans (including heparan sulfate), and CD36 (reviewed by (Adams and Lawler, 2004, Adams and Tucker, 2000). Thrombospondins 1 and 2 have potent anti-angiogenic activity that is mediated at least in part by interaction between their TSRs and CD36 (Iruela-Arispe et al, 1999, Lee et al., 2006, Tolsma et al, 1993), and this interaction is reported to encompass the peptide that is *O*-fucosylated (Iruela-Arispe et al, 1999, Silverstein and Febbraio, 2007). Whether the presence of *O*-fucose on the TSR has an effect on this anti-angiogenic activity is as yet unknown. Studies investigating the efficacy of peptides derived from TSRs to combat cancer are under way. Another potential role for *O*-fucosylation of TSRs is neurite outgrowth. Meiniel and coworkers have shown that a peptide encompassing the *O*-fucose site of a TSR from (SCO)-spondin can promote neurite outgrowth in culture (Meiniel et al., 2003). The neurite outgrowth is stimulated through α 1/ β 1 integrin, although it remains

to be determined whether the TSR peptide is binding directly to the integrin or is associated with some other integrin activating factor (Meiniel et al, 2003). As with the other peptide studies mentioned, the effect of glycosylation on their function has yet to be determined.

In addition to these potential direct effects on TSR function, recent studies suggest that *O*-fucosylation of TSRs may play a role in quality control. Both ADAMTS13 and ADAMTS-like1 are modified by *O*-fucose on multiple TSRs (Ricketts et al., 2007, Wang et al, 2007). Seven of the eight TSRs in ADAMTS13 contain a consensus sequence for *O*-fucosylation, all of which are modified with either the monosaccharide fucose or disaccharide Glc- β 1,3-Fuc. Similarly, all four of the TSRs in ADAMTS-like1 contain consensus sequences, three of which are modified (the fourth also appears to be modified, but the data is inconclusive) (Wang et al., 2007). In all cases the stoichiometry of modification at individual sites is very high. Interestingly, elimination of *O*-fucosylation reduced secretion of both proteins. A variety of approaches were used to eliminate *O*-fucosylation. Mutation of individual glycosylation sites reduced secretion, and the effect was more pronounced when multiple sites were mutated (Ricketts et al., 2007, Wang et al, 2007). Lec13 CHO cells have a defect in GDP-fucose biosynthesis and do not fucosylate any protein (Ripka et al, 1986). The presence of a salvage pathway for GDP-fucose biosynthesis allows rescue of the fucosylation defect by supplementation of the medium with fucose. Secretion of both ADAMTS13 (Ricketts et al, 2007) and ADAMTS-like 1 (Wang et al., 2007) is significantly impaired in Lec13 cells, but fucose supplementation rescues secretion, suggesting that *O*-fucosylation is required for proper secretion. Finally, knockdown of Pofut2 using siRNA also caused a decrease in secretion of ADAMTS13 (Ricketts et al., 2007). Together, these results suggest that *O*-fucosylation of TSRs is required for optimal secretion of both ADAMTS13 and ADAMTS-like1.

As an initial step towards understanding the biological function of TSR *O*-fucosylation, we have generated a mouse lacking Pofut2. Preliminary analysis suggests that homozygous mutants die during early embryogenesis (Du et al, 2007). Although the specific defect in these mice is not known, the data suggests that *O*-fucosylation of one or more of the target proteins is essential for early embryogenesis.

Elongation of *O*-fucose on TSRs with β 1,3-linked Glucose

Mutations in the β 3GlcT that modifies *O*-fucose on TSRs results in a human genetic deficiency known as Peter's Plus syndrome (Lesnik Oberstein et al., 2006). Patients with Peters Plus syndrome display developmental delay, cleft lip or palate, defects in the anterior eye chamber, and short stature (for a review see (Maillette de Buy Wenniger-Prick and Hennekam, 2002)). Recently, the Hofsteenge group has shown by mass spectrometry that the TSR containing protein properdin from the serum of Peter's Plus patients does not contain glucosylated *O*-fucose glycans, confirming that the genetic deficiency results in loss of the β 1,3linked glucose on *O*-fucosylated TSRs (Hess et al, 2008). Properdin is present at slightly lower levels in the serum of Peter's Plus patients compared to controls, suggesting that the addition of the glucose is not essential for secretion, but may have a minor effect. The molecular basis for the symptoms displayed in these patients is not understood, but the data suggest that loss of glucose from the TSR of one or more of the target proteins is the cause of these severe developmental defects.

C-Mannosylation

C-Mannosylated tryptophans have been found in proteins from both mammals and insects (Doucey et al, 1999, Hofsteenge et al, 2001, Munte et al, 2008). The W-X-X-W consensus sequence is frequently, but not exclusively, found in the amino terminus of TSRs (Doucey et al., 1998). Several proteins without TSRs are also C-mannosylated, including RNase (Krieg et al, 1997, Krieg et al., 1998, Löffler et al, 1996) and the erythropoietin receptor (Furmanek et al., 2003). The absence of C-mannosylation in erythropoietin receptor has been implicated

in functional defects for the receptor, apparently through effects on folding and trafficking of the protein (Hilton et al, 1996). Little is known about how C-mannosylation affects protein folding, but the addition of a hydrophilic mannose will undoubtedly have significant effects on the ability of a tryptophan to be buried deep within the core of a protein. The fact that the mannose is added to proteins in the ER, a folding and quality control compartment of the cell, adds weight to this idea.

Several recent studies suggest that C-mannosylation has the potential to affect cell signaling. Ihara and coworkers demonstrated that C-mannosylated peptides from TSRs enhance the production of tumor necrosis factor alpha (TNF- α) by lipopolysaccharide in mouse macrophage like RAW264.7 cells (Muroi et al, 2007). In contrast, production of TNF- α was not enhanced by the unglycosylated peptides, suggesting that C-mannosylation of TSRs has the potential to affect cell signaling. TNF- α production was enhanced partly through C-jun N-terminal kinase (JNK) activation by transforming growth factor beta (TGF- β)-activated kinase 1 (TAK1) (Muroi et al., 2007). Nuclear factor kappa B (NF κ B) was also reported to play a smaller role in the lipopolysaccharide induced TNF- α increase. Peptides from TSRs have also been shown to possess anti-angiogenic effects, including a peptide comprised of the tryptophan containing the C-mannosylation consensus sequence (Iruela-Arispe et al, 1999). What effect the presence or absence of C-mannosylation might have on the activity of these peptides is as yet unknown. The SCO-spondin TSR peptide used by Meiniel and coworkers to promote neurite outgrowth in culture contained both the C-mannosylation and O-fucose consensus sequences (Meiniel et al., 2003). As such, the relative contribution of either consensus sequence, with or without glycosylation awaits further investigation.

Does Glycosylation Play a Role in Folding of TSRs or EGF Repeats?

All of the modifications discussed in this review (O-fucosylation of EGF repeats and TSRs, O-glucosylation of EGF repeats, C-mannosylation) have been proposed to play some role in folding or quality control of folding. Each of these modifications is believed to occur in the ER, the folding compartment of the secretory pathway. Pofut1, Pofut2, and Poglut (Rumi) are ER localized (Acar et al., 2008, Kozma et al., 2006, Luo and Haltiwanger, 2005, Luo et al., 2006a, Okajima et al., 2005), and although the C-mannosyltransferase has not been identified, the evidence suggests that C-mannosylation occurs co-translationally in the ER (Doucey et al., 1998). In addition, Pofut1, Pofut2, and Poglut only modify properly folded substrates (EGF repeats for Pofut1 and Poglut, TSRs for Pofut2) in vitro (Luo et al., 2006b, Shao et al., 2002, Wang and Spellman, 1998). The fact that these enzymes can differentiate between folded and unfolded structures, and that they are localized to the ER, has led us to propose that they are well suited to play a role in quality control. The observation described above that elimination of O-fucosylation reduces secretion of ADAMTS13 and ADAMTS-like 1 (Ricketts et al., 2007, Wang et al., 2007) is consistent with this idea. A model summarizing these concepts for Pofut2 is shown in Figure 4. Defects in various aspects of the model (e.g. loss of GDP-fucose as in Lec13 cells, loss of Pofut2 by siRNA knockdown, or mutation of O-fucosylation sites) could have effects on ER exit. The fact that Pofut1 and Poglut are also ER-localized and recognize properly folded structures, and that OFut1 is reported to have chaperone activity (Okajima et al., 2005), suggest that they may perform similar functions.

It is not at all clear how the addition of a single fucose, glucose, or mannose could affect folding or quality control. It is possible that the sugars "mark" the properly folded EGF repeat or TSR, and that the glycosylated domains are recognized by lectins specific for the fucose or glucose, targeting them for ER-exit. This is analogous to ERGIC53, which recognizes specific N-glycan structures on properly folded proteins and assists in their exit from the ER (Hebert et al., 2005). Alternatively, the sugar could affect local protein structure, contributing to the folding process itself. O-Glycosylation is known to affect the physical dynamics of peptides and protein

sequences in solution. A nuclear magnetic resonance (NMR) study of *O*-fucosylated and unfucosylated forms of the *Pars intercerebralis* major peptide C (PMP-C) from *Locusta migratoria* showed only small changes on the backbone fold of the protein due to glycosylation, but the thermal stability of the protein was substantially increased (Mer et al., 1996). This included decreases in the rate of deuterium exchange for amide protons remote from the site of fucosylation (Mer et al., 1996). Similarly, the presence of an *O*-fucose on a factor VII EGF repeat showed no significant effect on backbone structure, but showed NMR chemical shift effects in regions distant from the site of glycosylation (Kao et al., 1999). Interestingly, the *O*-fucose glycan produced a modest increase in the affinity of the amino-terminal calcium binding domain for calcium (Kao et al., 1999). Considering that calcium is known to produce rigidity between EGF repeats (Hambleton et al., 2004, Rao et al., 1995), and that this was an NMR study on a single EGF repeat, the effect could be greater in proteins with tandem EGF repeats such as Notch. In the Notch ECD as a whole, *O*-fucosylation might alter the affinity of calcium binding EGF repeats for calcium, making some regions more rigid. Thus, the presence of the *O*-fucose on multiple EGF repeats may affect the folding of the entire Notch ECD. The fact that loss of *rumi* results in a temperature sensitive Notch phenotype suggests *O*-glucose may play a similar role (Acar et al., 2008, Moloney et al., 2000b).

Conclusions

The last two decades has seen a dramatic increase in our knowledge of the role of glycans in signaling and disease. *O*-fucose glycans play key roles in the modulation of Notch signaling, and the potential role of *O*-glucose glycans in Notch signaling is just now beginning to come to light. Recent evidence suggests that *O*-linked glycans may have a role in modulating the conformation of the Notch ECD. Thus, the glycosyltransferases responsible for these modifications may serve as pharmaceutical targets considering the vast array of diseases linked to aberrant Notch signaling. Determination of exactly how Fringe modification of Notch modulates signaling is a crucial step to advancing our understanding of the Notch signaling pathway. This will undoubtedly require new and innovative approaches to characterize the ECD structure of Notch in the presence and absence of elongated glycans. While much has been learned about the nature and role of glycans on Notch to this point, we have clearly entered a period where it will be necessary to directly characterize the glycans on endogenous Notch in various biological contexts. Identification of the array of Notch pathway components present in any given context will likely also be necessary to explain some of the apparent inconsistencies between the *Drosophila* and mouse models. The question of whether the three mammalian Fringe enzymes have different specificities and different target substrates is another outstanding question that needs to be answered. In addition, questions about the potential role of *O*-fucose glycans on the more than one-hundred proteins predicted to be *O*-fucosylated other than Notch (Rampal et al., 2007) remains largely unanswered. For instance, a very recent paper suggests *O*-fucosylation of an EGF repeat in agrin alters its function (Kim et al., 2008).

Our understanding of the other types of glycosylation discussed in this review is less well developed. While *O*-fucosylation of TSRs appears to be biologically essential, less is known about the specific biochemical effects of this modification. The presence of *O*-fucose consensus sequences in the TSRs of signaling competent proteins such as Thrombospondins, and the evidence suggesting peptides encompassing these consensus sequences have anti-angiogenic effects is intriguing. The potential therapeutic effects of these anti-angiogenic peptides toward tumor growth are an exciting area of inquiry. Presumably the effects of glycosylation on TSRs will become clearer once knockout phenotypes are reported for the enzymes responsible for these modifications. Current evidence for the role of the rare *C*-mannose modification in signaling is likewise limited at this point. Investigations in this area are hampered by the absence of any gene identified as a *C*-mannosyltransferase, which is undoubtedly a priority in

this field. Finally, the potential role of all of these modifications in folding and quality control is an area that is wide open for further study. Ten years from now, we will no doubt look back and marvel in a similar fashion at how much more we have learned about these unusual carbohydrate modifications. It is exciting to witness the advent of an age of glycans in biological research.

Abbreviations Used

ADAM, A Disintegrin And Metalloprotease
 ADAMTS, A Disintegrin And Metalloprotease with thrombospondin type 1 repeats
 β 3GlcT, β 1,3-glucosyltransferase
 CDG, congenital disorder of glycosylation
 CSL, CBF1/SuH/LAG-1
 DSL, Delta/Serrate/LAG-2
 ECD, extracellular domain
 EGF, Epidermal Growth Factor-like
 ER, endoplasmic reticulum
 Fuc, fucose
 Gal, galactose
 Glc, glucose
 GlcA, glucuronic acid
 GlcNAc, N-acetylglucosamine
 Man, mannose
 OFut1, protein *O*-fucosyltransferase 1 (*Drosophila*)
 Pofut1, protein *O*-fucosyltransferase 1 (mammalian)
 Pofut2, protein *O*-fucosyltransferase 2
 Poglut, protein *O*-glucosyltransferase
 SCO, subcommissural organ
 Sia, sialic acid
 TSR, thrombospondin type 1 repeat
 Xyl, xylose

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Figure 1. O-Glycan Containing Epidermal Growth Factor-like and Thrombospondin Type 1 Repeats

A) Cartoon representation of fully glycosylated Factor IX EGF repeat containing both an *O*-fucose tetrasaccharide (Sia- α 2,3/6-Gal- β 1,4-GlcNAc- β 1,3-Fuc) on the right and an *O*-glucose trisaccharide (Xyl- α 1,3-Xyl- α 1,3-Glc) on the left. The *O*-fucose tetrasaccharide containing EGF repeat was described previously (pdb accession 1EDM) (Luo, 2007). The *O*-glucose trisaccharide was modeled by taking a solution structure model from the Sweet website (<http://www.glycosciences.de/modeling/sweet2/doc/index.php>) and adding the modification to the appropriate serine. The serine side-chain was then cycled through known rotamer conformations and the position judged most appropriate by eye was chosen. **B)** Surface

representation of panel A without glycosylation. **C)** Surface representation of panel A. **D)** Cartoon representation of a fully glycosylated TSR containing a Glc- β 1,3-Fuc disaccharide and a mannosylated tryptophan. The disaccharide was modeled by taking a Glc- β 1,3-Fuc solution structure model from the Sweet website, and aligning the fucose from the disaccharide with the fucose of TSR3 from the structure of the Thrombospondin 1 TSRs (pdb accession 1LSL) (Tan et al., 2002). The mannose on the tryptophan was modeled by superimposing the planar ring of 4-nitrophenyl- α -D-mannose with the tryptophan ring, removing the oxygen on C1 of the mannose, and placing the mannose at an approximated carbon bond length from the appropriate indole carbon. **E)** Surface representation of panel D without glycosylation. **F)** Surface representation of panel D. All panels are at the same scale. The carbons of sugar residues are colored according to the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) recommended nomenclature. Fucose is red, glucose and N-acetylglucosamine are blue, galactose is yellow, xylose is white, sialic acid is purple, and mannose is green. Oxygens are colored pink, nitrogen is cyan, carbons of amino acid side-chains involved in disulfide bonding or glycosylation are grey, and the sulfur of cysteines are colored yellow. All other atoms are brown. All pictures produced using PyMol (Delano, 2002).

Figure 2. Model of Notch Receptor Activation

A) Diagram of human Notch1 ECD. EGF repeats are indicated with black ovals, and Notch/Lin12 repeats as green hexagons. The glycans on Notch are indicated with the suggested shapes and colors of the Consortium for Functional Glycomics

(<http://www.functionalglycomics.org>). Calcium ions are shown as orange spheres associated with EGF repeats and the Notch/Lin12 repeats. The glycan modifications and calcium binding EGF repeat pattern is based on human Notch 1. The diagram indicates a maximal level of glycosylation at all predicted sites. Mass spectral analysis suggests that this is not the case (Haltiwanger lab unpublished results, Nita-Lazar and Haltiwanger, 2006a, Nita-Lazar and Haltiwanger, 2006b, Xu et al, 2007), but that it varies depending on species, Notch subtype, and cell type. The in vivo glycosylation pattern could involve monosaccharide *O*-fucose or *O*-glucose on some repeats. Note that consecutive EGF repeats containing calcium binding sites are predicted to be rotated approximately 120° relative to one another (Hambleton et al., 2004). Thus the glycans are drawn pointing up or down (switching sides) on neighboring EGF repeats to reflect this. B–D) Notch activation. B) A simplified Delta ligand is shown on an adjacent cell in brown. The Delta ligand interacts with the Notch receptor on a neighboring cell. Note that the Lin12/Notch repeats interact non-covalently with the transmembrane/intracellular portion of Notch. The transmembrane portion of Notch is in grey, and the intracellular Notch is shown in simplified fashion in blue. C) Subsequent to Delta/Notch

interaction, Delta begins to be endocytosed by the sending cell. This causes a conformational change in the Notch/Lin12 repeats exposing the cleavage site. ADAM10, which catalyzes the cleavage, is shown as a red lightning bolt. **D)** After the extracellular cleavage, the presenilin/ γ -secretase complex (green lightening bolt) catalyzes the intracellular cleavage, releasing the intracellular domain from the membrane. The Notch intracellular domain transits to the nucleus to transactivate downstream targets.

Figure 3. Fringe Modulation of Notch Signaling

A. Diagram of the chemical reaction catalyzed by Fringe. The EGF repeat is drawn as described in Figure 1. The fucose is shown in red sticks, and the GlcNAc is shown in blue sticks. **B.** Notch activity without Fringe. Without Fringe, Notch is modified with *O*-fucose monosaccharide. Under these conditions, Serrate can activate Notch, but Delta cannot. Notch is drawn with a single *O*-fucose monosaccharide (red triangle) for simplicity. **C.** Notch activity with Fringe. With Fringe, Notch is modified with *O*-fucose disaccharide. Under these conditions, Delta can activate Notch, but Serrate cannot. As in panel B, Notch is drawn with a single disaccharide (GlcNAc, blue square; fucose, red triangle) for simplicity. The diagrams in panels A and B are drawn based on what is known about *Drosophila* Notch and Fringe.

Figure 4. Model of the Effects of Pofut2 on TSR Folding in the Endoplasmic Reticulum

As a protein containing TSR sequences is co-translationally translocated into the lumen of the ER, the cysteines emerge unpaired. For simplicity, the unpaired sulfhydryls of six cysteines from a single TSR are shown, although these typically occur in tandem repeats (*e.g.* ADAMTS13 has 8 TSRs (Ricketts et al., 2007)). Soon after entering the lumen, the TSR will enter a folding cycle catalyzed by chaperones and an oxidoreductase such as protein disulfide isomerase (PDI). A number of folding intermediates could result, although only one is properly folded. Since Pofut2 is ER localized and specifically recognizes properly folded TSRs (Luo et al., 2006a, Luo et al, 2006b), as soon as a properly folded TSR is formed, Pofut2 should bind. We have previously presented data that *O*-fucosylation of TSRs and EGF repeats occurs in the

ER, suggesting the presence of GDP-fucose in the ER (Sturla et al., 2003). Thus, the TSR should be *O*-fucosylated at this point. *O*-Fucosylation may occur co-translationally, although definitive evidence for this does not yet exist. The *O*-fucosylated protein would then be ready for ER exit. Defects at any point in this process could affect the efficiency of ER exit. For instance mutation of *O*-fucosylation sites on the TSRs, elimination of Pofut2 by siRNA, or elimination of GDP-fucose in Lec13 cells, would all eliminate or reduce *O*-fucosylation. Each of these appears to affect secretion of ADAMTS13 or ADAMTS-like1 (Ricketts et al., 2007, Wang et al., 2007) which may be a result of reduced ER exit.

Results of Fringe Effects on Various Notch/Ligand Cell-based Signaling and Binding Assays
Lfng: Lunatic fringe, Rfng: Radical fringe, Mfng: Manic fringe, Dll1: Delta-like1, J1: Jagged1, J2: Jagged2, N1: Notch1, N2: Notch2, DEL: Delta, SER: Serrate. Arrows indicate an increase or decrease in signaling through the indicated ligand or changes in measured binding. n.c. indicates no change. The bottom two rows are *Drosophila* experiments while the remainder of the table refers to data from mammalian systems. The table contains data from references where the complement of Notch pathway components is clearly known except for the studies in CHO cells, where endogenous Notch (mainly Notch1) was used (Chen et al., 2001., Stahl et al., 2008). There is considerable data from other references where there is less certainty of the exact complement of components present [‡] (Shimizu et al., 1999, Shimizu et al., 2000b, Shimizu et al., 2000a).

Table 1

		Cell-based Signaling				Cell-based Binding				References	
		N1		N2		N1		N2			
		Dll1	J1	Dll1	J1	J2	Dll1	J1	Dll1		J1
	Lfng			n.c.	↓↓	n.c.			n.c.	↓	(Shimizu et al., 2001)
	Mfng			n.c.	↓	n.c.			n.c.	↓	
	Lfng	↑	↓	↑	↑		↑	n.c.			(Hicks et al., 2000, Yang et al., 2005)
	Mfng	↑(weak)	↓				↑	n.c.			
	Rfng	↑	↑				↑	n.c.			
	Lfng		↓				↑	↓			(Chen et al., 2001, Moloney et al., 2000a, Stahl et al., 2008)
	Mfng		↓								
							DEL	SER			(Xu et al., 2007)
	Eng						↑	↓			

[‡] Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., et al. (1999). Mouse Jagged1 physically interacts with Notch2 and other Notch receptors. Assessment by quantitative methods. *J Biol Chem.*, 274, 32961–9.
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