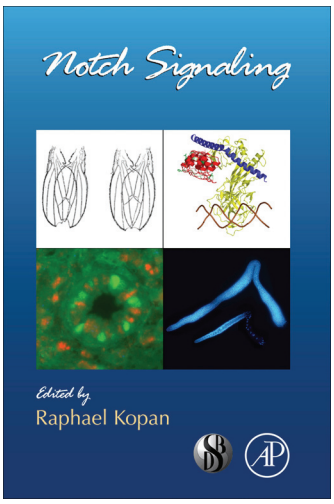

From Pamela Stanley, Roles of Glycosylation in Notch Signaling.
In: Raphael Kopan editor,
Current Topics in Developmental Biology Notch Signaling (Volume -92).
Academic Press 2010, p. 131.
ISBN: 978-0-12-380914-8

CHAPTER FOUR

Roles of Glycosylation in Notch Signaling

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Contents

Abstract

Notch and the DSL Notch ligands Delta and Serrate/Jagged are glycoproteins with a single transmembrane domain. The extracellular domain (ECD) of both Notch receptors and Notch ligands contains numerous epidermal growth factor

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Current Topics in Developmental Biology, Volume 92
ISSN 0070-2153, DOI 10.1016/S0070-2153(10)92004-8

(EGF)-like repeats which are post-translationally modified by a variety of glycans. Inactivation of a subset of genes that encode glycosyltransferases which initiate and elongate these glycans inhibits Notch signaling. In the formation of developmental boundaries in *Drosophila* and mammals, in mouse T-cell and marginal zone B-cell development, and in co-culture Notch signaling assays, the regulation of Notch signaling by glycans is to date a cell-autonomous effect of the Notch-expressing cell. The regulation of Notch signaling by glycans represents a new paradigm of signal transduction. *O*-fucose glycans modulate the strength of Notch binding to DSL Notch ligands, while *O*-glucose glycans facilitate juxtamembrane cleavage of Notch, generating the substrate for intramembrane cleavage and Notch activation. Identifying precisely how the addition of particular sugars at specific locations on Notch modifies Notch signaling is a challenge for the future.

1. Introduction

Notch receptors are covered with a variety of glycans (Fig. 4.1). Mutations that prevent their synthesis cause Notch signaling defects of varying severity (Figs. 4.2 and 4.3). While general populations of glycans might be important in promoting the biologically active conformation, trafficking, and membrane stability of Notch, mutations that affect a single glycan site cause Notch signaling phenotypes. *In vitro* and cell-based assays show that *O*-fucose glycans modulate the degree of binding between Notch and Delta or Serrate/Jagged, but it is not known if Notch ligands bind sugars directly. *O*-glucose on Notch promotes Notch cleavage and activation. The first indication that glycans on Notch may be important for Notch signaling came from hydrophobic cluster analyses of Fringe proteins which led to the proposal that Fringe may encode a glycosyltransferase (Yuan *et al.*, 1997). Fringe was discovered in *Drosophila* in a screen for novel genes that modulate Notch signaling (Irvine and Wieschaus, 1994). It was soon shown to be necessary for Notch signaling at the wing margin (Fleming *et al.*, 1997; Panin *et al.*, 1997) and at other tissue boundaries in *Drosophila* (Irvine, 1999). Mammalian homologues of Fringe (termed Lunatic, Manic, and Radical Fringe) were shown to have conserved functions in *Drosophila* (Johnston *et al.*, 1997). Consistent with a requirement in Notch signaling, inactivation of *Lfng* in the mouse was found to cause defective somitogenesis leading to profound skeletal aberrations (Evrard *et al.*, 1998; Zhang and Gridley, 1998). Meanwhile, several groups were investigating whether Fringe had the sugar transfer ability of a glycosyltransferase. A hint came with the finding that Notch1 in mammals carries two unusual glycans (Moloney *et al.*, 2000b). One began with fucose linked to Ser or Thr located between the second and the third cysteine of an EGF repeat in the consensus $C^2X_{4-5}S/TC^3$, and the other began with glucose linked to

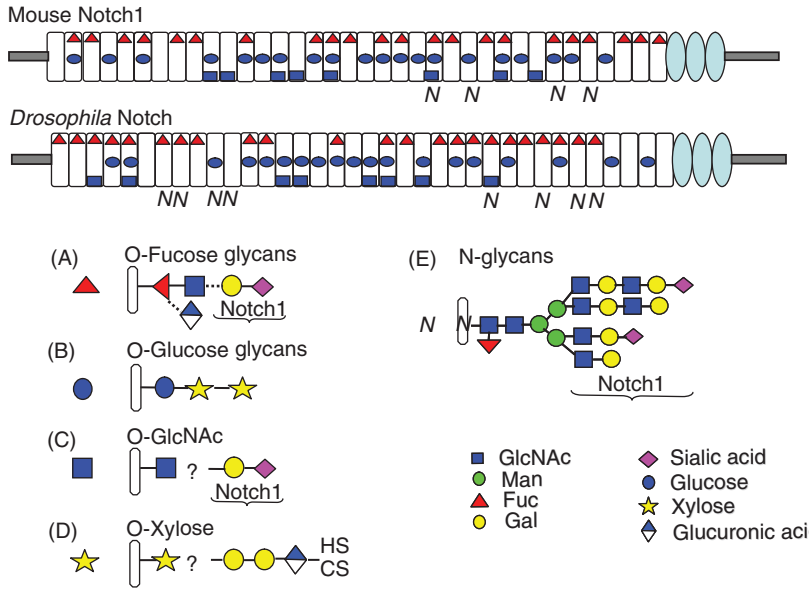

Figure 4.1 Glycans on Notch. A diagram representing the ECDs of mouse Notch1 and *Drosophila* Notch which contain 36 EGF repeats (white ovals) and 3 Lin repeats (blue ovals). Symbols in the EGF repeats identify consensus motifs for *O*-fucose (A), *O*-glucose (B), *O*-GlcNAc (C), *O*-xylose (D), and *N*-glycans (E) that have the potential to contain the sugars shown in the structures below the diagram. *O*-fucose glycans of *Drosophila* Notch may contain a glucuronic acid (Aoki *et al*., 2008) and Notch1 *O*-fucose glycans may contain Gal and SA (Moloney *et al*., 2000b) as noted. *N*-glycans in *Drosophila* are mainly oligomannosyl and rarely contain Gal and SA (Aoki *et al*., 2007; Koles *et al*., 2007), whereas Notch1 probably has complex *N*-glycans (Moloney *et al*., 2000b) as noted. Several of the glycosylation sites in *Drosophila* Notch and mammalian Notch1 are conserved, for example, in EGF12 in the DSL Notch ligand-binding domain. Each sugar of the *O*-fucose (A), *O*-glucose (B), and *O*-GlcNAc (C) glycans is transferred by a specific glycosyltransferase described in the text. *N*-glycans (E) and GAGs (D) are synthesized by the concerted action of many glycosyltransferases and other glycosylation activities (Stanley *et al*., 2009; Esko *et al*., 2009). (See Color Insert.)

Ser or Thr between the first and the second cysteine of an EGF repeat with the consensus C^1XSXPC^2 (Moloney *et al*., 2000b; Panin *et al*., 2002). Consensus sites for *O*-fucose glycans (Fig. 4.1A) and *O*-glucose glycans (Fig. 4.1B) present in mouse Notch1 and *Drosophila* Notch are shown in Fig. 4.1.

The structural observations suggested substrates for *in vitro* assays which led to the discovery that Fringe is a glycosyltransferase which transfers *N*-acetylglucosamine (GlcNAc) to fucose (Fuc) on Notch EGF repeats to generate GlcNAcβ1,3Fuc-*O*-EGF (Bruckner *et al*., 2000; Moloney *et al*., 2000a). EGF repeats with an *O*-fucose consensus site occur in a number of

proteins, including the DSL Notch ligands (Rampal *et al.*, 2007). Nevertheless, the cell-autonomous and phenotypic consequences of blocking these glycosylation pathways as described below indicate that the modification of Notch receptors by sugars is a key factor in regulating Notch signaling *in vivo*. Since publication of the glycosyltransferase activity of Fringe in 2000, there have been a host of investigations into the roles of *O*-fucose, *O*-glucose, and other glycans in Notch signaling. Most studies to date have been performed in *Drosophila* or mammals, although the zebra fish, Xenopus, and *Caenorhabditis elegans* genomes encode protein *O*-fucosyltransferase (Ofut1/Pofut1) homologues. Protein *O*-fucosyltransferases

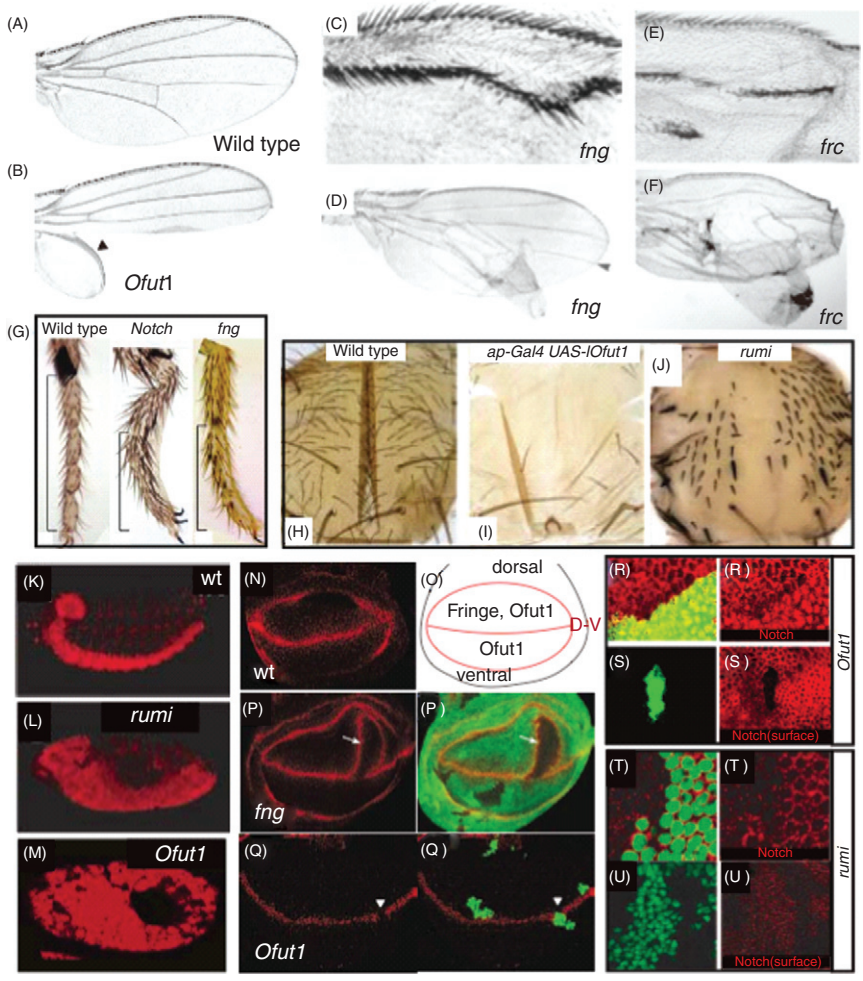

Figure 4.2 *(Continued)*

transfer fucose directly to Ser or Thr in an EGF-like domain with the appropriate consensus (Wang *et al.*, 2001). The chick (Sakamoto *et al.*, 1997), zebra fish (Qiu *et al.*, 2004), and Xenopus (Wu *et al.*, 1996) express up to three Fringe genes, but Fringe does not appear to be present in *C. elegans* based on phylogenetic comparisons (Haines and Irvine, 2003).

2. Glycans of Notch Receptors and DSL Notch Ligands

A variety of glycans can be added to the portion of Notch that transits the secretory pathway—the Notch ECD—and the intracellular domain of Notch is potentially modified by *O*-GlcNAc which is found on many cytoplasmic and nuclear proteins (Butkinaree *et al.*, 2010). The ECD of Notch and the DSL Notch ligands in *Drosophila* and mammals have

Figure 4.2 Notch signaling phenotypes of *Drosophila ofut1, fng, frc*, and *rumi* mutants. (A) A wild-type adult wing. (B) A wing-bearing clones of *Ofut1* mutant cells shows wing nicking and vein thickening (arrowhead), indicating defective Notch signaling. (C and D) Wings bearing *Fringe* mutant clones show duplications of wing margins (C) and an additional wing outgrowth from the wing blade (D). (E and F) *frc* mutant clones in the wings exhibit similar phenotypes to *Fringe* mutant clones. (G) Adult legs with *Notch* or *Fringe* clones show shortened legs and fused joints. A wild-type leg is shown *left*. Tarsal segments 2–5 are indicated by brackets. (H) A wild-type notum. (I) RNAi-mediated suppression of *Ofut1* in notum (*ap-Gal4 UAS-iOfut1*) results in loss of bristles, indicating defective Notch signaling. (J) Loss of bristles was also observed in a notum bearing *rumi* clones. (K) A wild-type embryo stained with a neuronal marker, ELAV. (L) *rumi* embryos lacking zygotic expression show a neurogenic phenotype at 28°C, in which ectodermal cells are replaced by excess neural cells. (M) *Ofut1* embryos lacking maternal and zygotic expression also exhibit the neurogenic phenotype. (N) A wild-type third instar wing disc stained for Wingless (WG) expression (red). (O) Schematic drawing. WG expression is indicated in red. D–V indicates dorsal–ventral boundary. *Ofut1* is expressed in both compartments whereas *Fringe* is expressed only dorsally. WG expression at D–V depends on Notch signaling. (P) *Fringe* mutant clones, marked by absence of GFP (green). Ectopic WG is indicated (arrow). (Q) *Ofut1* mutant clones, marked by presence of GFP (green). Loss of WG is indicated (arrowhead). (R) A wing disc with *ofut1* mutant clones (green), stained with antibodies against the Notch ECD (NECD; red) after detergent treatment. Increased and mislocalized Notch protein is observed within *ofut1* mutant cells. (S) A wing disc stained without detergent treatment. An *ofut1* mutant clone (green) is devoid of cell surface Notch (red). (T) *rumi* clones marked by GFP (green) also showed an accumulation of Notch (red). (U) A wing disc with *rumi* clones (green) stained with Notch (red) antibody in the absence of detergent. Notch expression is elevated at the apical cell surface. Panels A and B are adapted from (Sasamura *et al.*, 2003); C and D are from (Irvine and Wieschaus, 1994); E and F are from (Selva *et al.*, 2001); G is from (Rauskolb and Irvine, 1999); H and I are from (Okajima and Irvine, 2002); J, K, L, T, and U are from (Acar *et al.*, 2008); M, N, O, P, Q, R, and S are from (Okajima *et al.*, 2008) with permission of the publishers. (See Color Insert.)

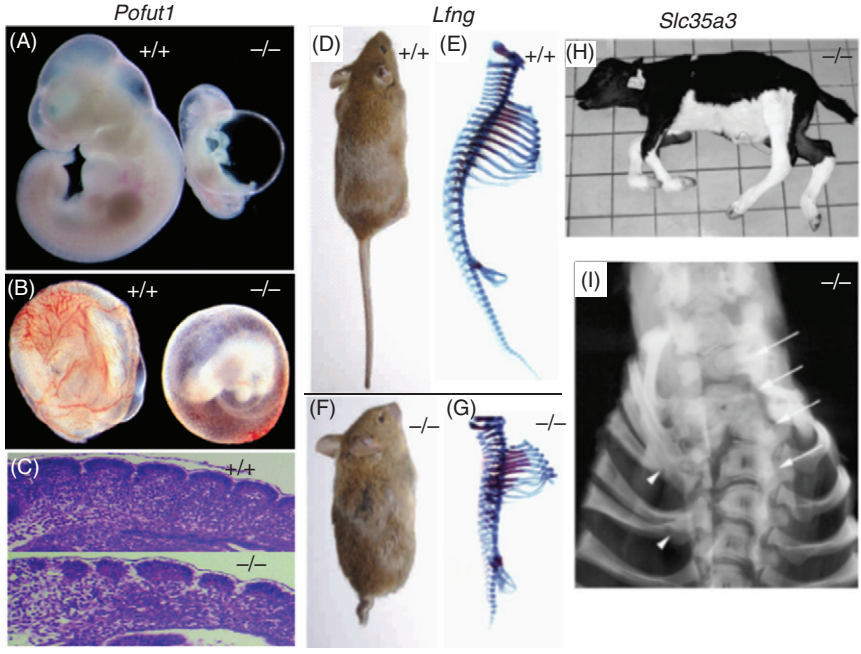

Figure 4.3 Notch signaling phenotypes of *Pofut1, Lfng*, and *Slc35a3* mutants. Inactivation of the *Pofut1* gene is embryonic lethal in mice with embryos showing defective development of the heart (A), defective vasculogenesis (B), and defective somitogenesis (A and C). Deletion of the *Lfng* gene causes marked skeletal defects apparent in *Lfng* mutant mice that lack a tail (D, wild-type $Lfng^{+/+}$; F, affected $Lfng^{-/-}$) and in skeletal preparations (E, wild-type $Lfng^{+/+}$; G, affected $Lfng^{-/-}$). A calf homozygous for a mutant *Slc35a3* allele is moribund due to skeletal defects (H), highlighted by arrows in an X-ray of the skeleton (I). Panels A, B, C are adapted from (Shi and Stanley, 2003); panels D, E, F, G are modified from (Serth *et al.*, 2003); and panels H and I are adapted from (Thomsen *et al.*, 2006) with permission of the publishers. (See Color Insert.)

consensus sites for the addition of *N*-glycans (at N-X-S/T or N-X-C where X is not proline), as well as *O*-Fuc, *O*-Glc, and *O*-GlcNAc glycans at Ser or Thr residues (Fig. 4.1). *O*-glycosylations of *Drosophila* Notch have been extensively studied using one of the common *Drosophila* cell lines, Schneider-2 (S2), while Chinese hamster ovary (CHO), NIH-3T3, and COS-7 cells have been used to investigate *O*-glycosylation in mammals. Cell lines are noted because glycosyltransferase gene expression and other factors affecting glycosylation may vary between cell lines. Proof of occupancy of individual EGF repeats by *O*-glycans is available for certain *O*-fucose (Fig. 4.1A), *O*-glucose (Fig. 4.1B), and *O*-GlcNAc (Fig. 4.1C) sites based on either radioactive labeling or western analysis of Notch EGF fragments or mass spectroscopy of Notch EGF fragments produced in cultured cells (Table 4.1), as discussed below. While this is important

Table 4.1 Sites of O-glycosylation on *Drosophila* Notch, Delta, and Serrate

	Fuc-*O*-	**GlcNAcβ1, 3Fuc-*O*-**	**Peptide**	**References**
Serrate	Yes	Yes	ND	(Panin *et al.*, 2002)
Serrate 4M	Yes	Yes	ND	(Panin *et al.*, 2002)
Serrate 8M	No	No		(Panin *et al.*, 2002)
Delta	Yes	Yes	ND	(Panin *et al.*, 2002)
Notch				
EGF3	Yes	Yes	CLNGGTC	(Xu *et al.*, 2007)
EGF5	Yes	Yes	CKYGGTC	(Xu *et al.*, 2007)
EGF7	Yes	Yes	CQNGGTC	(Xu *et al.*, 2007)
EGF12	Yes	Yes	CQNEGSC	(Lei *et al.*, 2003)
EGF17	Yes	Yes	CNNGATC	(Xu *et al.*, 2007)
EGF20	Yes	Yes	CQHGGTC	(Matsuura *et al.*, 2008)(Xu *et al.*, 2007)
EGF23	Yes	Yes	CRNGASC	(Xu *et al.*, 2007)
EGF25	Yes	Yes	CQNGATC	(Panin *et al.*, 2002)
	Glc-*O*-	**Xylα1, 3Glc-*O*-**		
EGF14	Yes	ND	CQSQPC	(Acar *et al.*, 2008)
EGF16	Yes	ND	CESNPC	(Acar *et al.*, 2008)
EGF17	Yes	ND	CHSNPC	(Acar *et al.*, 2008)
EGF19	Yes	ND	CASNPC	(Acar *et al.*, 2008)
EGF20	Yes	YES	CSSNPC	(Matsuura *et al.*, 2008)
EGF35	Yes	ND	CDSNPC	(Acar *et al.*, 2008)
	GlcNAc-*O*			
EGF20	Yes		CMPGYTG	(Matsuura *et al.*, 2008)
EGF1-10	Yes		ND	(Matsuura *et al.*, 2008)
EGF22-32	Yes		ND	(Matsuura *et al.*, 2008)
Delta	Yes		ND	(Matsuura *et al.*, 2008)

and represents the current state-of-the-art, identifying sites in Notch that are occupied by glycans *in vivo,* under conditions of endogenous and regulated expression of enzymes and their substrates, in a particluar cell type at a specific time in development, is the goal. Clearly the latter is

needed to eventually understand how and why glycans modulate Notch signaling.

2.1. *N*-glycans and *O*-GalNAc glycans

Drosophila Notch was inferred to be a glycoprotein based on the ability of Notch ECD to bind to a lentil lectin affinity column and be eluted with α-methylmannoside (Johansen *et al.*, 1989). These properties are consistent with modification by oligomannosyl or simple complex *N*-glycans (Fig. 4.1E) found in *Drosophila* (Aoki *et al.*, 2007). Mammalian Notch1 was shown to carry *N*-glycans based on its sensitivity to peptide *N*-glycosidase F (*N*-glycanase) (Shao *et al.*, 2003) which cleaves *N*-glycans from Asn, thereby generating Asp. It is not known if Notch carries *O*-GalNAc or mucin *O*-glycans (Brockhausen *et al.*, 2009), although predictions of the NetOGlyc 3.1 database (Julenius *et al.*, 2005) suggest that neither *Drosophila* Notch nor mammalian Notch1 ECDs have potential sites of *O*-GalNAc glycosylation.

2.2. *O*-fucose glycans

Modification of Notch EGF repeats with ^{3}H-fucose was discovered in Lec1 CHO cells that incorporate very little fucose into *N*-glycans (Moloney *et al.*, 2000b). Previous studies had shown that EGF repeats of tissue plasminogen activator, blood clotting factor VII, and factor IX contain *O*-fucose at a Ser or Thr residue just before the third Cys of the EGF repeat (Harris and Spellman, 1993). Notch1 EGF repeat sequences were examined for Ser or Thr at this position, and a consensus motif for *O*-fucosylation was proposed (Moloney *et al.*, 2000b). This was later modified based on experimental evidence and theoretical considerations to $C^{2}XXX(A/G/S)\mathbf{S/T}C^{3}$ based on the fact that EGF15 in mouse Notch1 ($C^{1}HYG\mathbf{S}C^{2}$) is not modified (Li *et al.*, 2003; Rampal *et al.*, 2005a; Shao *et al.*, 2003). The *O*-fucose in coagulation factors is elongated to a tetrasaccharide by the addition of GlcNAc, Gal, and sialic acid (SA) (Fig. 4.1A). This fact, and the suspicion that Fringe might be a glycosyltransferase, led to *in vitro* assays of sugar transfer using pNP-*O*-fucose as substrate and Fringe on beads. These experiments revealed that Fringe in *Drosophila* and mammals is a GlcNAc transferase which generates GlcNAcβ1,3Fuc-*O*-EGF on Notch (Moloney *et al.*, 2000a). While *Drosophila* has only one Fringe (*Fringe*) and one Notch gene (*N*), mammals have four Notch genes (*Notch1–4*) and three Fringe genes as noted above. *In vitro* comparisons of the mammalian Fringes identify mouse Lfng as the most active followed by Mfng and then Rfng (Rampal *et al.*, 2005b). Although all three mammalian Fringe proteins have a single transmembrane domain and are thought to reside and function in the Golgi, both Lfng and Mfng are secreted from cells (Johnston *et al.*,

1997), perhaps as a way to regulate their activity (Shifley and Cole, 2008), whereas Rfng remains predominantly intracellular. *Drosophila* Fringe was also shown to be secreted (Irvine and Wieschaus, 1994).

All Fringe enzymes transfer GlcNAc to *O*-Fuc on a folded EGF repeat with a ~1000-fold improved efficiency over a denatured EGF repeat (Moloney *et al.*, 2000a) and ~10-fold better than to a simple fucose acceptor (Luther *et al.*, 2009). Fringe glycosyltransferases are glycoproteins (Rampal *et al.*, 2005b) and probably need their *N*-glycans to be active in the cell. Two predominant transcripts of Lfng exist, and a large number of differentially spliced forms of Mfng, as well as numerous splice forms of Rfng, have been isolated as cDNAs from different sources (see AceView at http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/ (Thierry-Mieg and Thierry-Mieg, 2006). Attempts to determine whether the different mammalian Fringes are regulated primarily by transcription or have different substrate specificities with respect to the amino acid sequence of the EGF repeat to which *O*-Fuc is attached have provided interesting insights (Rampal *et al.*, 2005b). The general conclusion from *in vitro* assays is that there is no simple motif for Fringe recognition of a Fuc-*O*-EGF repeat and that differences observed between the Fringes may primarily reflect differences in their catalytic efficiency (Rampal *et al.*, 2005b). However, only a limited number of EGF repeat substrates have been explored in this context, and evidence discussed below for additive effects amongst Fringe genes argues for some degree of variation in the sites modified by different Fringe enzymes.

Once Fringe has acted in a mammalian cell there is a possibility of further elongation of the disaccharide with Gal followed by SA to generate the tetrasaccharide SAα2,3Galβ1,4GlcNAcβ1,3Fuc-*O*-EGF (Fig. 4.1A). This elongation is variable, however, so that any Fuc-*O*-EGF may not be modified further, or may be a disaccharide, a trisaccharide or a tetrasaccharide. The functional significance of this diversity is an important question for the future. In S2 cells, Fringe expression is negligible but *O*-fucose is present on Notch (Okajima and Irvine, 2002). Upon expression of Fringe in S2 cells, GlcNAcβ1,3Fuc disaccharide is synthesized, but unlike mammalian *O*-fucose glycans, no further elongation has been observed. This could be because the *O*-fucose glycan *in vivo* is not faithfully replicated in cultured S2 cells. Interestingly, a novel glucuronyl trisaccharide *O*-fucose glycan, GlcNAcβ1,3(GlcAβ1,4)-Fucitol, was amongst the *O*-glycans released from glycoproteins of *Drosophila* embryos (Aoki *et al.*, 2008). This trisaccharide is enriched in the dorsal compartment of the wing imaginal disc, which is consistent with the dorsal expression of Fringe. However, it is not known whether this unique *O*-fucose glycan is actually attached to Notch *in vivo*. Intriguingly, while it was reduced in amount in embryos lacking Fringe, the trisaccharide was still detected (Aoki *et al.*, 2008), though this may be due to maternal Fringe.

After Fringe was found to elongate Fuc-*O*-EGF on Notch, the search was on for the *O*-fucosyltransferase that transfers the fucose to Notch. This is encoded by the *Ofut1* gene in *Drosophila* and the *Pofut1* gene in mammals (Wang *et al.*, 2001). A second distantly related gene termed *Pofut2* transfers fucose to Ser or Thr on thrombospondin repeats, but not to EGF repeats (Luo *et al.*, 2006; Shi *et al.*, 2007). Ofut1 and Pofut1 have a KDEL-like sequence at their C-terminus and are luminal proteins of the endoplasmic reticulum (ER)/*cis*-Golgi network (Luo and Haltiwanger, 2005). When Ofut1 and Notch are overexpressed in S2 cells, they physically associate (Okajima *et al.*, 2005; Sasamura *et al.*, 2007). This finding, and the fact that Ofut1 aids in the folding of Notch as discussed below, supports the proposal that Ofut1 is a chaperone for Notch in *Drosophila* (Okajima *et al.*, 2005; Sasamura *et al.*, 2007).

While most studies of *O*-fucose glycans have focused on their presence on the Notch ECD, it was shown early on that the DSL Notch ligands Delta and Serrate are also modified by both *O*-fucose and Fringe (Panin *et al.*, 2002). Sequence comparisons indicate that a cohort of about 50 proteins are potential carriers of *O*-fucose glycans (Rampal *et al.*, 2007). However, mechanistic studies described below indicate that it is necessary to determine for each site of modification, whether the presence of an *O*-fucose glycan affects biological activity, and if so, how.

2.3. *O*-glucose glycans

The presence of *O*-glucose glycans on Notch1 was discovered along with *O*-fucose glycans in Lec1 CHO cells (Moloney *et al.*, 2000b). The Glc-*O*-EGF modification of Notch1 is found at a Ser or Thr adjacent to the second Cys in the consensus C^1XSXPC^2. The Glc-*O*-EGF is elongated by the addition of xylose (Moloney *et al.*, 2000b) and was proposed to form Xylα1,3Xylα1,3Glc-*O*-EGF (Fig. 4.1B), as detected on bovine coagulation factors VII and IX (Hase *et al.*, 1988). This structure and the glycosyltransferases that generate it have now been confirmed. The *O*-glucosyltransferase is encoded by the *rumi* gene in *Drosophila* (Acar *et al.*, 2008), and two genes in mammals encode a xylosyltransferase that transfers Xyl to Glc-*O*-EGF (Sethi *et al.*, 2010). The gene encoding the Xyl-to-Xyl xylosyltransferase remains to be identified. While the nature and distribution of *O*-glucose glycans on endogenous Notch is not known, *Drosophila* Notch fragments expressed in S2 cells transfected with Rumi carry Glc- or a Xly-Glc-disaccharide (Acar *et al.*, 2008; Matsuura *et al.*, 2008; Table 4.1). As for *O*-fucose glycans, *O*-glucose glycans are potentially present on any EGF repeat that contains the acceptor motif for the *O*-glucosyltransferase. Most surprisingly, Rumi has also been found to transfer xylose to the same EGF consensus motif and thereby to generate Xyl-*O*-EGF (Fig. 4.1D; R. S. Haltiwanger and H. Takeuchi, personal communication). It is not known if xylose occurs on EGF repeats *in vivo*, whether a second Xyl or other sugars

are subsequently added, or if this Xyl serves as an initiator of proteoglycan synthesis, as discussed below.

2.4. Glycosaminoglycans

The transfer of xylose to Ser or Thr residues that usually occur in a cluster but may be isolated residues initiates glycosaminoglycan (GAG) synthesis (Esko *et al.*, 2009). The subsequent addition of two Gal residues and a glucuronic acid provides the core structure on which long GAG chains are synthesized to generate heparan sulfate or chondroitin sulfate. To date there has been no structural evidence that Notch or its ligands are modified by GAGs. However, this certainly is a possibility since elimination of the GAG-specific sulfotransferase Hst-3b in *Drosophila* affects Notch signaling and trafficking (Kamimura *et al.*, 2004).

2.5. A novel *O*-GlcNAc modification

The presence of *O*-GlcNAc at Ser or Thr in cytoplasmic and nuclear proteins is now well established (Butkinaree *et al.*, 2010), and Notch ICD may carry *O*-GlcNAc, which could regulate the expression of Notch target genes. However, it was a big surprise to find *O*-GlcNAc as a modification of the ECD of *Drosophila* Notch (Matsuura *et al.*, 2008). Based on galactosyltransferase labeling, β-*N*-acetylhexosaminidase digestion and immunoblotting with *O*-GlcNAc-specific antibody (CTD110.6), the modification was determined to be GlcNAc-β-*O*-EGF (Fig. 4.1C). Intracellular *O*-GlcNAc transfer is catalyzed by a single *O*-GlcNAc transferase (OGT) (Kreppel *et al.*, 1997). However, this OGT is not responsible for the O-GlcNAc glycosylation of Notch ECD, since O-glycosylation of Notch EGF repeats occurs in the secretory pathway. Consistent with this, RNAi-mediated reduction of OGT did not decrease *O*-GlcNAc levels on *Drosophila* Notch. Furthermore, OGT activity was detected in a membrane fraction prepared from S2 cells (Matsuura *et al.*, 2008). Thus, it appears that the *O*-GlcNAc modification on EGF domains occurs independently of the action of OGT. The *O*-GlcNAc on Notch is found at Ser or Thr located between the fifth and sixth cysteines of a Notch EGF domain, C-terminal to the site of Ofut modification (Matsuura *et al.*, 2008). For the structure of an EGF domain see Chapter 2. Notch EGF domains like those of Factors VII, IX, and XII as well as plasminogen activators and Protein Z are *O*-glycosylated by fucose and/or glucose as discussed above (Rampal *et al.*, 2007). However, with the exception of Factor XII, these plasma glycoproteins do not contain Ser or Thr at the corresponding site that might receive *O*-GlcNAc. By contrast, potential *O*-GlcNAc sites are present in many EGF repeats of Notch receptors (Fig. 4.1) and Notch ligands, Delta, and Serrate. In fact, it was shown

that the *O*-GlcNAc modification occurs at multiple sites in Notch EGF repeats and the ECD of Delta (Matsuura *et al.*, 2008; Table 4.1). This extracellular *O*-GlcNAc might be employed to modulate specific biological processes during animal development.

The *O*-GlcNAc modification is present on mammalian Notch EGF repeats secreted from CHO cells and preliminary data have detected an EGF repeat OGT activity in a membrane fraction from mammalian cells (C. Saito, Y. Tashima, P. Stanley, and T. Okajima; unpublished observations). This is consistent with the presence of Thr/Ser residues at conserved consensus sites (C^5XXXXS/T^6) in mammalian Notch receptors and DSL ligands (Matsuura *et al.*, 2008). In addition, it was previously reported that *O*-GlcNAc is present at the luminal face of the ER (Abeijon and Hirschberg, 1988). It should be noted that in mammals *O*-GlcNAc glycans on secreted or membrane proteins are likely to be elongated since *O*-GlcNAc is readily modified by β1,4galactosyltransferase in the Golgi (Whelan and Hart, 2006).

2.6. General overview

It is now clear that the ECDs of Notch and the DSL Notch ligands are coated with sugars (Fig. 4.1). For the most part, these sugars are transferred to specific motifs recognized by an initiating glycosyltransferase. Subsequently, glycosyltransferases like Fringe may recognize the initial sugar in the context of the EGF motif. Based on knowledge of the specificity of the glycosyltransferases for EGF repeats, biochemical properties of Notch, ligands, and recombinant mutants lacking individual glycosylation sites, and structural analyses of Notch fragments, a general picture of mature, glycosylated, *Drosophila* Notch, and mammalian Notch1, as they would be expected to be expressed *in vivo,* has emerged (Fig. 4.1). In the case of *Drosophila* Notch, concrete structural information has been obtained in several instances by mass spectrometry of tryptic peptides (Table 4.1). The other mammalian Notch receptors and the DSL *Drosophila* and mammalian Notch ligands should be similarly glycosylated on their EGF repeats. For example, mouse Notch1 EGF4 (C^1A**S**NPC^2) has been shown to be O-glucosylated (Bakker *et al.*, 2009; Sethi *et al.*, 2010).

The glycans associated with Notch may confer direct or indirect effects on Notch activity. For example, glycan-binding proteins may bind to Notch glycans and thereby link Notch with other glycoproteins on the same or an adjacent cell surface. Other sources of indirect effects of glycans on Notch signaling are the glycolipids formed by Brainiac and Egghead in *Drosophila* (Muller *et al.*, 2002; Schwientek *et al.*, 2002; Wandall *et al.*, 2003, 2005). Mutants in these genes cannot make the complete glycan part of the glycolipid, and one consequence is that Notch signaling is defective.

However, inactivation of genes encoding GalNAcTs, which transfer GalNAc to the substrates generated by Egghead and Brainiac, does not result in severe Notch signaling defects (Stolz *et al.*, 2008). Glycolipids are presumably important in regulating Notch conformation or stability in the membrane (Pizette *et al.*, 2009; Hamel *et al.*, 2010).

3. Consequences of Glycan Removal for Notch Signaling

3.1. *N*-glycans or *O*-GalNAc glycans

The removal of all *N*-glycans leads to embryonic death in the mouse at the peri-implantation stage (Marek *et al.*, 1999). However, when only two major classes of *N*-glycans are eliminated, the complex (Fig. 4.1E) and hybrid type embryos survive until mid-gestation (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). The phenotype is not identical to a Notch-null phenotype (Bolos *et al.*, 2007) but has some features which suggest that Notch signaling may be partly affected. Thus the heart is underdeveloped and remains as a loop, and some embryos exhibit *situs inversus* which is consistent with inhibition of Notch signaling (Raya *et al.*, 2003). However, this may be an indirect effect. The loss of complex and hybrid *N*-glycans is expected to reduce the time that cell surface glycoproteins interact with the extracellular galectin lattice (Dennis *et al.*, 2009), thereby enhancing the endocytosis of growth factor receptors, and potentially Notch receptors, leading to reduced Notch signaling.

O-GalNAc glycans are initiated by polypeptide GalNAc transferases (Ten Hagen *et al.*, 2003). There are ~20 ppGalNAcTs in mammals and to date the inactivation of a subset of these enzymes has not led to Notch phenotypes. However, removal of the single core 1 GalT termed T-synthase, which transfers Gal to GalNAc-*O*-Ser/Thr, is embryonic lethal in mouse at ~E14 (Xia *et al.*, 2004). The embryos die of brain hemorrhage and exhibit defective angiogenesis. Conditional deletion of T-synthase in endothelial cells revealed that core 1 (and/or core 2) *O*-GalNAc glycans control the separation between blood and lymphatic vessels, in part by affecting the function of podoplanin (Fu *et al.*, 2008). Interestingly, *Drosophila* has a number of genes potentially encoding a core 1 GalT. Deletion of C1Galt1A that is expressed in the amnioserosa and the central nervous system is lethal (Lin *et al.*, 2008). Larval brain hemisheres are misshapen and the ventral nerve cord is elongated. Thus, the elongation of *O*-GalNAc on glycoproteins in the developing central nervous system is essential for morphogenesis of the larval brain in *Drosophila*. Notch signaling has not been investigated in the mouse or fly *O*-glycan mutants, though it is potentially affected.

3.2. *O*-fucose glycans

3.2.1. Inactivation of Fringe genes

Fringe functions in the Golgi compartment, where it transfers GlcNAc onto Fucose in EGF repeats with the appropriate consensus as discussed above (Fig. 4.1). Catalytic activity is required for Fringe function. In third instar wing discs, Fringe is exclusively expressed in the dorsal compartment and acts in signal-receiving cells. Fringe inhibits Notch activation by Serrate in dorsal cells, which limits Serrate-Notch signaling from dorsal cells to ventral cells. In contrast, Fringe potentiates Notch activation by Delta, which allows Delta-Notch signaling from ventral to dorsal cells. Thus, Fringe is key to the positioning of strong Notch activation at the D-V boundary. Such positioning of Notch activation is also required for boundary formation of the leg and eye imaginal discs. In *Drosophila*, Fringe is required for a subset of Notch-dependent processes including inductive signaling, but it is not required in lateral inhibition or asymmetric cell division processes regulated by Notch signaling (Haines and Irvine, 2003; Irvine, 1999).

In mammals the mouse *Lfng* gene was the first to be inactivated by gene disruption (Evrard *et al.*, 1998; Zhang and Gridley, 1998). Consistent with roles in boundary formation in *Drosophila*, mice lacking Lfng have defective somitogenesis and severe skeletal defects. A missense mutation in human LFNG also gives rise to skeletal defects (Sparrow *et al.*, 2006). The expression of Lfng must be tightly controlled for somitogenesis to proceed correctly. Overexpression or underexpression of Lfng causes similar skeletal defects (Barrantes *et al.*, 1999; Serth *et al.*, 2003). A regulatory element upstream of the Lfng coding sequence termed FCE is required to maintain transcriptional oscillation of Lfng during somitogenesis (Cole *et al.*, 2002; Morales *et al.*, 2002). In mice lacking the FCE in which Lfng is expressed but transcriptional oscillation is lost, it was revealed that Lfng oscillation is critical for the segmentation of the anterior but not the posterior skeleton (Shifley *et al.*, 2008). By rescuing $Lfng^{-/-}$ mice with a chicken Lfng cDNA controlled by up to 5 kb of the mouse Lfng promoter, oscillation of Lfng was found to be necessary for cervical, thoracic and lumbar somite, and vertebrae development, but not for sacral and tail somite or vertebrae development (Stauber *et al.*, 2009). Lfng expression is also regulated at the protein level by processing via a specific proprotein convertase (Shifley and Cole, 2008). Thus, precise timing of Lfng modification of Notch is essential for the proper formation of somites and the skeleton (Cinquin, 2007). Deletion of Mfng (Moran *et al.*, 2009) or Rfng (Zhang *et al.*, 2002) has no discernable effects on somitogenesis or skeletal development. Most importantly, it was found that mice lacking all three *Fringe* genes may be viable, and two females were fertile (Moran *et al.*, 2009). Therefore, unless there is another gene that can substitute for Fringe, it must be concluded that Notch signaling proceeds through embryogenesis, with the exception

of somitogenesis, and postnatal development in the mouse with Notch receptors modified solely by *O*-fucose. Mfng and Rfng do not play obvious roles during these developmental stages.

The consequences of inhibiting Fringe expression have also been investigated during development in chick, fish, and frog. Somitogenesis requires oscillating expression of lunatic fringe in the chick as it does in mammals (Dale *et al.*, 2003). Lunatic fringe is important in zebra fish for induction of mesoderm (Peterson and McClay, 2005), the generation of segmental boundaries (Prince *et al.*, 2001), and development of the notochord (Appel *et al.*, 2003), though its expression does not oscillate. In Xenopus, Notch, Delta, and Lunatic fringes are important in regulating the outgrowth of the tail bud (Beck and Slack, 2002).

Because *Lfng* null mice survive poorly, conditional mutants and bone marrow or fetal liver transfer experiments were used to identify requirements for Lfng in T-cell and marginal zone B (MZB) cell development (Stanley and Guidos, 2009; Visan *et al.*, 2006b). Lfng is expressed in double-negative (DN) T cells but not in double-positive (DP) T cells (Visan *et al.*, 2006a). Misexpression of Lfng in DP T cells blocks T-cell development by preventing DN T cells from interacting with thymic stroma and allows B cells to develop in the thymus (Koch *et al.*, 2001). In the spleen, not only Lfng but also Mfng is required for the maximal generation of MZB cells (Tan *et al.*, 2009). This interesting result shows that Lfng and Mfng are not redundant but play complementary roles in generating MZB cells. In a disease-related model of Alagille syndrome, removal of one copy of Mfng (or Rfng or Lfng) along with one copy of Jagged1 causes proliferation of bile ducts in mouse liver (Ryan *et al.*, 2008). This was the first indication of a role for Rfng *in vivo*. However, based on expression levels, Rfng may also play a role in angiogenic sprouting of tip cells during vascularization of the retina (Benedito *et al.*, 2009). The three Fringe genes are expressed in tip cells and loss of Lfng leads to an increase in sprouting.

3.2.2. Overexpression or misexpression of fringe

Both temporal and spatial regulation of Fringe expression is necessary for appropriate control of Notch signaling and cell fate determination. Misexpression of Fringe in the *Drosophila* ventral wing disc inhibits Notch signaling and results in wing loss (Irvine and Wieschaus, 1994). Ectopic expression of Fringe in the fly rescues neurogenic defects induced by overexpression of Serrate but gives reduced viability when ubiquitously overexpressed under a heat-shock promoter (Fleming *et al.*, 1997). Expression of Fringe throughout the wing from early development results in wing loss (Klein and Arias, 1998), and an overexpression screen for modulators of Notch signaling identified Fringe (Hall *et al.*, 2004). In the mouse, misexpression of Lfng in the thymus causes T cell precursors to become B cells (Koch *et al.*, 2001). The mechanism

is non cell-autonomous. Competition experiments showed that DP T cells expressing Lfng take up the stromal niche of DN cells and prevent their interaction with the stroma, thereby preventing their development into DP cells (Visan *et al.*, 2006a). During somitogenesis, Fringe expression must oscillate in a tight cycle or skeletal formation is disrupted (Serth *et al.*, 2003). As noted above, Fringe proteins are secreted (Irvine and Wieschaus, 1994). Although it was shown that tethering Fringe in the Golgi so that it cannot be secreted preserves its functions in wing development (Bruckner *et al.*, 2000) and that most probably Fringe is secreted in order to reduce its intracellular concentration (Shifley and Cole, 2008), it is also possible that Fringe has extracellular function(s).

3.2.3. Inactivation of protein *O*-fucosyltransferase

The functional significance of *O*-fucosylation was investigated by mutation or RNAi-mediated suppression of Ofut1 in *Drosophila* (Okajima and Irvine, 2002; Sasamura *et al.*, 2003) and by targeted mutation in the mouse (Shi and Stanley, 2003). In both mouse and fly the loss of Ofut1/Pofut1 leads to phenotypes characterized by the absence of all Notch signaling. Not only fringe-dependent inductive signaling but also fringe-independent lateral inhibition and lineage decision processes were impaired in *Drosophila*, suggesting that Ofut1 is universally required for Notch signaling. Similarly in mouse, the phenotype of *Pofut1*$^{-/-}$ embryos is like that of embryos defective in global Notch signaling (Lu and Stanley, 2006). A spontaneous mutation in the mouse *Pofut1* gene that gives a milder phenotype has also been described (Schuster-Gossler *et al.*, 2009). This mouse revealed that Pofut1 expression is most important in the paraxial mesoderm during skeletal development.

3.2.4. Inhibition of GDP-fucose synthesis

The donor substrate for Ofut1 and Pofut1 is GDP-fucose which is synthesized by two enzymes termed GMD (GDP-mannose-4-6-dehydratase) and FX (3-5-epimerase/4-reductase). A GMD mutant cell line Lec13 with markedly reduced Notch signaling first indicated that the addition of fucose to Notch is necessary for optimal Notch signaling (Moloney *et al.*, 2000a). Like Fringe mutants, *Gmd* mutants in *Drosophila* show impairment of Notch activation at the D-V boundary of wing discs (Okajima *et al.*, 2005; Sasamura *et al.*, 2007). FX mouse embryos are partially rescued by GDP-fucose from maternal sources (Becker *et al.*, 2003; Smith *et al.*, 2002). However, when FX$^{-/-}$ bone marrow cells were used to form chimeras, myelopoiesis (Zhou *et al.*, 2008), and intestinal development (Waterhouse *et al.*, 2010) were impaired due to defective Notch signaling. Therefore, the transfer of fucose to Notch, not just the presence of Pofut1, is required for optimal Notch signaling (Stahl *et al.*, 2008).

3.2.5. Inactivation of nucleotide sugar transporters

GDP-fucose and UDP-GlcNAc must be imported from the cytosol into the secretory pathway to be utilized by Ofut1/Pofut1 or Fringe, respectively. In *Drosophila*, two GDP-fucose transporters, GFR (Golgi GDP-fucose transporter) and EFR (ER GDP-fucose transporter), are required (Ishikawa *et al.*, 2005, 2010). *EfrGfr* double mutants exhibit loss of Notch activation at the D-V boundary, whereas single mutants have only temperature-sensitive Notch signaling defects. EFR is a multifunctional nucleotide sugar transporter that also contributes to heparan sulfate biosynthesis. In the mouse, mutants that lack the Golgi GDP-Fuc transporter GFR homologue Slc35c1 do not have markedly defective Notch signaling, but mimic the symptoms of a human leukocyte adhesion deficiency termed LADII (Hellbusch *et al.*, 2007; Yakubenia *et al.*, 2008). Consistent with this, the synthesis of *O*-fucose glycans on Notch1 EGF fragments was shown not to be impaired in fibroblasts from LADII patients (Sturla *et al.*, 2003). However, knockdown of the mouse Golgi GDP-fucose transporter in C2C12 muscle cells caused a slight decrease in Notch signaling in a co-culture assay (Ishikawa *et al.*, 2005). Nevertheless, it is clear that one or more additional transporters are required to *O*-fucosylate mammalian Notch receptors. The human homologue of *Drosophila* EFR, SLC35B4, would seem not to be a candidate for the ER GDP-fucose transporter in mammals, since it transports only UDP-sugars and specifically did not transport GDP-fucose in a cell-free assay (Ashikov *et al.*, 2005).

Another transporter which is directly involved in the synthesis of *O*-fucose glycans on Notch is the UDP-sugar transporter fringe-connection (FRC). This transporter, discovered in *Drosophila*, is multifunctional and transports UDP-glucuronic acid, UDP-GlcNAc and possibly UDP-xylose and UDP-glucose (Selva *et al.*, 2001). The homologue is SLC35D2 in humans and it transports UDP-GlcNAc (Ishida *et al.*, 2005; Suda *et al.*, 2004). *Drosophila frc* mutants exhibit defective Notch signaling as well as heparan sulfate-defective phenotypes (Selva *et al.*, 2001). They display a neurogenic phenotype as well as Notch processing defects (Goto *et al.*, 2001). Thus, a subset of Notch phenotypes observed in the *frc* mutant may in part be attributable to other glycosylation defects, including O-glucose glycosylation.

A transporter termed Slc35a3 that may be more specific for UDP-GlcNAc (Ishida *et al.*, 1999) is mutated in cattle with congenital skeletal malformations (Thomsen *et al.*, 2006; Fig. 4.3), a phenotype typical of mice lacking Lfng (Evrard *et al.*, 1998; Zhang and Gridley, 1998) and the human spondylocostal disease due to mutated *LFNG* (Sparrow *et al.*, 2006). Slc35a3 must have a predominant role in delivering UDP-GlcNAc to Fringe.

3.2.6. Inactivation *of* β1,4galactosyltransferase 1

Investigations of Notch signaling in a co-culture assay using CHO glycosylation mutants identified a requirement for Gal on *O*-fucose glycans for

the inhibition of Jagged1-induced Notch signaling by Lfng or Mfng (Chen *et al.*, 2001). Consistent with a biological function for Gal *in vivo*, reduced expression of a subset of Notch target genes involved in somitogenesis in the mouse was observed in mice lacking β4galt1 (Chen *et al.*, 2006). The effects were quite subtle, perhaps because there are several other β4galts that may modify *O*-fucose glycans in mammals (Lo *et al.*, 1998).

Based on the fact that *O*-fucose glycans from mammalian cells may carry a tetrasaccharide (Fig. 4.1), it was expected that *Drosophila* β1,4galactosyltransferase and sialyltransferase genes might affect Notch signaling. Although there is no evidence that the GlcNAcβ1,3Fuc disaccharide is elongated by galactose or SA in *Drosophila*, genes that could encode the relevant glycosyltransferases are present in the genome. Two *Drosophila* homologoues of mammalian β1,4galactosyltransferases (β4GalNAcTA and β4GalNAcTB) turn out to be β1,4*N*-acetylgalactosaminyltransferases and do not transfer Gal *in vitro* (Chen *et al.*, 2007; Haines and Irvine, 2005; Stolz *et al.* 2008). They are involved in the biosynthesis of insect-specific glycosphingolipids but not glycoproteins and transfer GalNAc to GlcNAcβ1-3Manβ1-4Glcβ1-Ceramide (Chen *et al.*, 2007; Stolz *et al.*, 2008). Mutations in these genes affect ventralization of ovarian follicle cells due to defective EGFR signaling between the oocyte and dorsal follicle cells. Mutations affecting other steps in this biothynthetic pathway block the generation of Manβ1-4Glcβ1-Cer from Glcβ1-Cer (*egghead*), or the subsequent generation of GlcNAcβ1-3Manβ1-4Glcβ1-Cer from Manβ1-4Glcβ1-Cer (*brainiac*) (Wandall *et al.*, 2003, 2005). Interestingly, *egghead* and *brainiac* mutations cause abnormal neurogenesis during embryogenesis and compound egg chambers and dorsal appendage fusion during oogenesis. These phenotypes have been explained by defects in Notch and EGFR signaling and suggest that the extended form of glycosphingolipids may play a role in the modulation of receptor activities or the distribution of signaling molecules (Pizette *et al.*, 2009). Consistent with this proposal, recent evidence shows that DSL Notch ligand signaling is modulated by the composition of glycosphingolipids in a membrane (Hamel *et al.*, 2010). Like *egghead* and *brainiac*, β4GalNAcTB mutant animals display ventralization of ovarian follicle cells due to defective EGFR signaling (Chen *et al.*, 2007), whereas the β4GalNAcTA mutant exhibits abnormal neuromuscular system and behavioral defects (Chen *et al.*, 2007; Haines and Irvine, 2005). Both mutations do not give a neurogenic phenotype, although the possibility remains that these two enzymes are functionally redundant during embryogenesis (Chen *et al.*, 2007).

The *Drosophila* genome encodes a sole α2,6-sialyltransferase (SiaT) (Koles *et al.*, 2004). This enzyme acts on oligosaccharides and glycoproteins *in vitro* and *in vivo* (Koles *et al.*, 2007). *Drosophila SiaT* is expressed in a limited number of cells in the late stages of the developing embryonic central nervous system. Thus, it appears that modification with SA in *Drosophila* does not

affect general functions of glycoproteins, but rather it affects specific glycoprotein functions in the nervous system (Repnikova *et al.*, 2010).

3.2.7. Elimination or addition of an *O*-fucose site

An *O*-fucose site highly conserved across the metazoa resides in EGF12 of all Notch receptors (Haines and Irvine, 2003). Deletion experiments in *Drosophila* Notch identified EGF11 and EGF12 as the DSL Notch ligand-binding site (Rebay *et al.*, 1993; Xu *et al.*, 2005), suggesting that the *O*-fucose in EGF12 may be important in Notch ligand binding. Deletion of this ligand-binding region in mouse Notch1 reduced binding of Delta1 and Jagged1 and the ability of both ligands to induce Notch1 signaling (Ge *et al.*, 2008). Elimination of solely the *O*-fucose site in EGF12 of *Drosophila* Notch prevented inhibition by Fringe of Serrate-induced Notch signaling (Lei *et al.*, 2003). This was reflected in the inability of Fringe to inhibit Notch binding to S2 cells expressing Serrate, leading to the conclusion that Fringe action at Notch EGF12 is important for downregulation of Notch signaling by Serrate at the dorsal/ventral wing boundary. Mutation of three other *O*-fucose sites in the *Abruptex* region of Notch (EGF24, EGF26, EGF24, and EGF26, or EGF31) did not affect Notch activation (Lei *et al.*, 2003).

Interestingly, similar experiments in mouse Notch1 gave rise to rather different results. First, the EGF12 mutation to remove *O*-fucose gave Notch1 that was inactive in co-culture signaling assays (Rampal *et al.*, 2005a; Shi *et al.*, 2007). Removal of *O*-fucose in EGF26 gave a hyperactive Notch1 for both Delta1 and Jagged1 ligands and removal of *O*-fucose from EGF27 reduced cell surface expression of Notch1 (Rampal *et al.*, 2005a). *In vivo*, the consequences of mutating EGF12 was also different. Mice homozygous for Notch1 lacking *O*-fucose in EGF12 are viable and fertile (Ge and Stanley, 2008). However, the $Notch1^{12f}$ allele is hypomorphic, as shown by its inability to rescue a Notch1 ligand-binding domain mutant allele. T-cell development is markedly compromised in $Notch1^{12f}$ homozygotes due to reduced Notch1 signaling and ligand binding to T cells. This hypomorphic allele is of interest because it affects only Notch1 signaling in the context of a viable mouse.

There is also the *split* mutation in *Drosophila* Notch which results in the introduction of an *O*-fucose site in EGF14 (Li *et al.*, 2003). This mutation causes activation of Notch in proneural cells of the ommatidium, thereby preventing their differentiation which normally follows after Delta inhibits Notch signaling by lateral inhibition. This phenotype is not dependent on Fringe, indicating that the addition of *O*-fucose to EGF14 is enough to activate Notch inappropriately during eye development.

3.3. *O*-glucose glycans

The first insight into biological functions of *O*-glucose glycans (Fig. 4.1B) in Notch signaling was obtained by the identification of the *Drosophila* mutant

rumi (Acar *et al.*, 2008) which encodes a protein *O*-glucosyltransferase. Rumi mutations cause a global Notch pathway defective phenotype. However, the requirement for Rumi is temperature dependent; severe Notch signaling defects are observed when flies are raised at elevated temperatures (28°C), but not the lower temperature of 18°C. Like Ofut1, Rumi is a soluble protein of the ER. Although both Notch and Notch ligands can be modified with *O*-glucose (Moloney *et al.*, 2000b), Rumi acts cell-autonomously in Notch signal-receiving cells and not in signal-sending cells that present Notch ligands, suggesting that O-glucosylation is required for Notch functions. To date there are no mutants in Rumi in mammals, nor in the xylosyltransferases that subsequently add xylose to Glc-*O*-EGF (Sethi *et al.*, 2010).

3.4. Glycosaminoglycans

There are a variety of mutants in GAG synthesis in *Drosophila*, mice, and *C. elegans* (Bulow and Hobert, 2006). While many of these mutations affect developmental processes, none give rise to strong Notch signaling mutant phenotypes. However, targeted knockdown of a specific heparan sulfate sulfotransferase in *Drosophila* (Hst3b) causes neurogenic phenotypes indicative of a role for a specific form of heparan sulfate in Notch signaling (Kamimura *et al.*, 2004).

3.5. General overview

Mutant organisms lacking the ability to initiate or elongate *O*-fucose glycans display Notch signaling defects that reflect cell-autonomous effects of Notch receptor functions (Figs. 4.2 and 4.3). Notch receptors lacking *O*-fucose glycans (Fig. 4.1A) altogether are functionally inactive, but do not behave in a dominant-negative manner in heterozygotes. Overexpression of Ofut1 gives a similar phenotype to loss of Ofut1 in *Drosophila*. The loss of *Drosophila* Fringe, or Lfng in other organisms, gives rise to defects in the formation of segmental boundaries, a subset of the developmental fate decisions under the control of Notch signaling. Again, overexpression or misexpression of Fringe may also give Notch mutant phenotypes. By contrast, the loss of *O*-glucose glycans (Fig. 4.1B) generates milder, temperature-sensitive Notch signaling phenotypes in *Drosophila*. Nevertheless, all these phenotypes appear to arise from the altered glycosylation of Notch because they mimic Notch signaling phenotypes generated by mutations in Notch receptors themselves, or in downstream members of Notch signaling pathways. The same cannot be said for Notch phenotypes related to removal of other glycans such as *N*-glycans (Fig. 4.1E), *O*-GalNAc (mucin) glycans (Xia *et al.*, 2004) or GAGs (Fig. 4.1D), which are known to play key roles in embryogenesis, but may not directly affect Notch signaling. One way to address roles for known glycans is to generate Notch mutants that lack a particular site of

glycosylation. To date this has been done with *O*-fucose sites only and care must be taken in interpreting results. For example, mutation of the *O*-fucose site in Cripto inactivates its ability to stimulate Nodal signaling (Schiffer *et al.*, 2001). However, this was found to be due to the amino acid change rather than to the loss of the *O*-fucose glycan (Shi *et al.*, 2007). Also, roles for an *O*-fucose glycan may be inhibitory, as in *Drosophila* Notch EGF12 (Lei *et al.*, 2003) or muscle agrin (Kim *et al.*, 2008), or stimulatory, as in mammalian Notch1 EGF12 (Ge and Stanley, 2008).

4. Mechanisms of Glycan Regulation of Notch Signaling

Identifying biological roles for glycans by targeted knockdown of glycosyltransferase genes is effective and a necessary first step, but rarely identifies the key substrate of the missing glycosyltransferase responsible for a given phenotype. All activities involved in glycosylation act on multiple substrates. Thus, Notch phenotypes arising from defective *N*-glycan, *O*-GalNAc or glycosaminoglycan synthesis are difficult to investigate at a mechanistic level because these glycans are ubiquitously expressed on many cell surface receptors. Determining whether their removal causes an effect on Notch signaling is a challenge. Nevertheless, it is possible to identify specific substrates that give rise to a particular phenotype. For example, loss of the *N*-glycan branching transferase GlcNAcT-V causes reduced signaling from certain growth factor receptors (Partridge *et al.*, 2004); loss of a different branching GlcNAcT causes reduced glucose transport by Glut2 (Ohtsubo *et al.*, 2005); and removal of SA by Klotho from the ion channel TRPV5 increases its activity (Cha *et al.*, 2008). All of these effects reflect changes in cell surface retention time due to interactions with cell surface galectins. Alterations in Gal or SA residues of the *N*-glycans of Notch might likewise alter cell surface residence time.

The number of glycoproteins modified by *O*-fucose, *O*-glucose or *O*-GlcNAc glycans are far fewer because these *O*-glycans are found only at specific sites in certain EGF repeats (Fig. 4.1). Nevertheless, there are numerous glycoproteins that possess such EGF repeats (Matsuura *et al.*, 2008; Rampal *et al.*, 2007). Identification of the glycoprotein whose altered activity leads to a mutant phenotype involves determining whether an effect is cell-autonomous or non-cell-autonomous and carefully characterizing the phenotype of various mutant alleles. It is by these approaches that *O*-fucose and *O*-glucose glycans have been associated directly with Notch receptor signaling activity. The important question is—how exactly do the individual sugars of *O*-fucose and *O*-glucose glycans regulate signaling by Notch receptors?

4.1. *O*-fucose glycans

Further analysis of *Drosophila Gmd* mutants revealed that, unlike *Ofut1* mutants, Notch-dependent lateral inhibition and cell lineage decision processes are not affected during embryogenesis, as evidenced by the lack of a neurogenic phenotype in maternal and zygotic *Gmd* mutants (Okajima *et al.*, 2008). The different phenotypes of *Gmd* and *Ofut1* mutant embryos suggested that Ofut1 might possess additional functions besides acting as a fucosyltransferase. To test this possibility, rescue experiments were performed using the $Ofut1^{R245A}$ allele that lacks fucosyltransferase activity but is expressed at normal levels. Expression of $Ofut1^{R245A}$ in $Ofut1^{-/-}$ embryos results in robust neurogenesis, suggesting that O-fucosylation may be dispensable for Notch receptor function. Moreover, as in the case of *fringe* mutant clones, clones of cells expressing only $Ofut1^{R245A}$ show ectopic *wg* expression in the dorsal wing disc. Thus, O-fucosylation of Notch is not absolutely required for Notch to signal in *Drosophila*.

In mammalian ES cells lacking Pofut1, partial rescue of Notch signaling and Notch ligand binding was observed with a cDNA encoding $Pofut1^{R245A}$ (Stahl *et al.*, 2008). However, similar levels of rescue were obtained following transfection with an unrelated ER glucosidase, suggesting a non-specific effect of upregulating the unfolded protein response. Nevertheless, these results show that Notch1 lacking *O*-fucose can signal. However, cells having a normal amount of Pofut1 but reduced GDP-fucose levels, and therefore reduced O-fucosylation of Notch receptors, exhibit markedly reduced Notch signaling (Moloney *et al.*, 2000a; Chen *et al.*, 2001). Thus mammalian Notch receptors may signal poorly when they do not carry *O*-fucose.

Therefore the mechanisms by which Ofut1/Pofut1 affects Notch signaling are multifaceted. In both flies (Ahimou *et al.*, 2004; Okajima *et al.*, 2005; Sasaki *et al.*, 2007; Sasamura *et al.*, 2007) and mouse somites (Okamura and Saga, 2008), loss of Ofut1/Pofut1 causes Notch to be expressed at reduced levels at the cell surface. $Ofut1^{R245A}$ partially restores the localization of Notch to the apical cell surface (Okajima *et al.*, 2005), whereas extracellular Ofut1 is proposed to stabilize Notch at the cell surface (Sasamura *et al.*, 2007). Moreover, Ofut1 expression rescues defective secretion and ligand binding of *Drosophila* Notch EGF point mutations (Okajima *et al.*, 2005). Accumulation of Notch in the ER of *Drosophila Ofut1* mutant cells has been identified as one mechanism preventing cell surface localization (Okajima *et al.*, 2005), whereas accumulation in novel endocytic vesicles following normal trafficking to the cell surface has been identified as another (Sasamura *et al.*, 2007). While these observations are hard to reconcile, it is possible that the endocytic compartment is closely apposed to the ER. It is difficult to understand why Ofut1 is not also a required chaperone of other glycoproteins such as Crumbs which, like Notch, has many EGF repeats (Okajima and Irvine, 2002). In contrast to *Drosophila* wing disc and mouse

somites, surface expression levels of Notch are unaffected by the removal of Pofut1 in ES or CHO cells (Stahl *et al.*, 2008).

Ofut1/Pofut1 appears to be required for Notch to acquire the correct conformation for recognition by ligands (Okajima *et al.*, 2005; Stahl *et al.*, 2008). Interestingly, when Ofut1 is overexpressed, Notch signaling is inhibited both inside and outside of the regions where it is expressed. This non-autonomous effect of Ofut1 does not depend on its enzyme activity (Sasamura *et al.*, 2007). It is not known whether, under physiological conditions, Ofut1 is secreted and acts outside the cell. Nonetheless, this possibility is of potential interest from a pharmacological point of view, since secreted Ofut1 might serve as a soluble inhibitor of Notch signaling. Thus, it may be that Ofut1 possesses a third activity, which depends on neither its enzyme nor its chaperone activities. As an example, Ofut1 promotes transcytosis of Notch from the apical plasma membrane to the adherens junctions (Sasaki *et al.*, 2007).

In summary, both enzymatic and non-enzymatic activities of Ofut1 contribute to the absolute requirement of Ofut1 for Notch signaling in *Drosophila*. Non-enzymatic activities of Ofut1 are involved in folding and endocytosis of Notch receptors, and these activities are sufficient for a subset of Notch receptor functions. It is conceivable that *O*-glycans such as *O*-glucose and *O*-GlcNAc rescue requirements for *O*-fucose monosaccharide in Notch signaling. Removal of these *O*-glycans might reveal roles of O-fucosylation of Notch receptors. By contrast, it is clear that mammalian Notch receptors in cells unable to transfer fucose but containing normal levels of Pofut1 function poorly (Stahl *et al.*, 2008). The glycosyltransferase activity of Ofut1/Pofut1 is essential to provide the substrate for Fringe and it has been suggested that this is the major function of *O*-fucose on Notch in *Drosophila* (Okajima *et al.*, 2008). On the other hand, *O*-fucose may be required for Fringe-independent Notch signaling. *In vitro* binding assays show that Drosophila Notch fragments lacking fucose bind to Delta and Serrate expressed by S2 cells, albeit at low levels (Okajima *et al.*, 2003, 2005). In addition, a human Notch1 EGF fragment EGF11-13 lacking post-translational modifications can bind to Notch ligand-expressing cells (Hambleton *et al.*, 2004). However, tetramerization of the Notch fragment was necessary to observe binding. Subsequent studies identified calcium as a key requisite and EGF12 to be the major Delta1-binding site (Cordle *et al.*, 2008b). The X-ray structure of a Jagged1 N-terminal fragment DSL-EGF3 that binds to Notch1 revealed a conserved face, and mutations designed to alter this face caused *cis*-inhibition and *trans*-regulation Notch phenotypes in *Drosophila* (Cordle *et al.*, 2008a). Based on an NMR structure of the Notch1 ligand-binding domain fragment that also revealed a conserved face, the nature of the complex was proposed. Interestingly, the model places the *O*-fucose glycan in EGF12 on the opposite side to the Jagged1-binding face. This makes it difficult to understand how Fringe could alter Jagged1-induced

Notch signaling even indirectly, because previous NMR studies indicate that the presence of *O*-fucose would not be expected to change the conformation of an EGF repeat (Kao *et al.*, 1999).

The addition of GlcNAc to *O*-fucose on Notch EGF repeats by Fringe markedly enhances Delta binding to *Drosophila* Notch and inhibits Serrate binding (Lei *et al.*, 2003; Okajima *et al.*, 2003; Xu *et al.*, 2005, 2007). At least in *Drosophila*, the simple addition of GlcNAc is sufficient to produce the effects of Fringe on Notch-ligand binding (Xu *et al.*, 2007). However, the trisaccharide GlcNAc[GlcA]Fuc is found in flies and is reduced in *Drosophila* Fringe mutants (Aoki *et al.*, 2008). If the trisaccharide occurs on Notch, the function of GlcA in Notch ligand binding is of interest to determine.

In mammals, there are also effects on the binding of Jagged1 and Delta1 by Fringe modification of Notch. However, the effects vary for different Notch receptors and ligands such that the binding of Delta ligands is not always increased by the action of Fringe, nor is the binding of Jagged ligands always reduced (Hicks *et al.*, 2000; Ladi *et al.*, 2005; Yang *et al.*, 2005). In addition, co-culture signaling assays in which cells expressing a Notch reporter activated by the released intracellular domain of Notch are stimulated by cells expressing DSL Notch ligands are not always affected by Fringe in a manner directly reflected by changes in soluble ligand binding (Yang *et al.*, 2005). This may be because soluble Notch ligands do not bind with the same properties as membrane-bound ligands. For example, initial assays could only detect soluble Jagged1 binding after clustering (Hicks *et al.*, 2000). A decrease in Jagged1 binding could not be observed under conditions in which Fringe inhibited Jagged1-induced Notch1 cleavage (Yang *et al.*, 2005). In addition, Lfng, Mfng, and Rfng have been reported to have different effects on signaling through the same exogenous Notch receptor (Shimizu *et al.*, 2001). There is also evidence of a requirement for the Gal residue on *O*-fucose glycans to observe the effects of Lfng or Mfng on Jagged1-induced Notch signaling (Chen *et al.*, 2001). In this case, Fringe action was necessary but not sufficient to modulate ligand-induced Notch signaling. Ligand-binding assays support a role for Gal in Jagged1 binding to endogenous Notch receptors acted on by Lfng or Mfng (Y. Tashima and P. Stanley; unpublished observations).

In summary therefore, it is clear that Notch and Delta/Jagged ECDs physically interact (Shimizu *et al.*, 1999, 2000; Xu *et al.*, 2007) and that in *Drosophila* Fringe increases binding of Notch to Delta and reduces binding to Serrate (Okajima *et al.*, 2003; Xu *et al.*, 2005, 2007). This suggests that the mechanism by which *O*-fucose glycans regulate Notch signaling is by directly altering the ability of DSL Notch ligands to bind to Notch. However, the situation may be more complicated. For example, it is proposed that in order to bind to the ligand-binding domain of *Drosophila* Notch, Delta must displace the Abruptex region of Notch EGF repeats, and it is not known if Fringe affects this intramolecular interaction (Pei and Baker,

2008). Structural studies of bacterially produced Notch1 and Jagged1 ECD fragments suggest that sugars are not essential to their interaction, but this is hard to reconcile with *in vitro* effects of Fringe and the effects of *O*-fucose glycans on Notch signaling in co-culture assays. Only when structures of complexes between Notch and ligand ECD fragments have been compared before and after Fringe modification will it be possible to begin to understand how *O*-fucose glycans regulate Notch/ligand interactions. In this regard it is encouraging that mutations that eliminate or add a single site of O-fucosylation affect Notch signaling and, in the case of EGF12, cause altered Notch ligand binding (Ge and Stanley, 2008; Lei *et al.*, 2003; Xu *et al.*, 2005). Understanding how the loss of one *O*-fucose glycan affects Notch signaling when 22 other *O*-fucose glycans are presumably present, is a challenge for the future.

4.2. *O*-glucose glycans

Although the loss of protein *O*-glucosyltransferase in *Drosophila rumi* mutants results in a slight accumulation of Notch intracellularly, cell surface expression of Notch is maintained and rather elevated compared to wild-type cells (Acar *et al.*, 2008). Thus, unlike *Ofut1, rumi* is not required for the folding of Notch receptors. Furthermore, RNAi-mediated reduction of *rumi* in a cell-based assay suggests that *O*-glucose is not required for Notch binding to the Delta ligand. Based on comparisons of cleaved Notch forms in *rumi* mutants, it appears that *O*-glucosylation may be required for conformational changes in Notch that occur subsequent to ligand binding, which make Notch a substrate for S2 cleavage by an ADAM protease (Acar *et al.*, 2008). This is a cell-autonomous effect of the signal-receiving cell. Notch ligands lacking *O*-glucose appear to function normally. There is currently no mouse *rumi* mutant, nor are there *Drosophila* or mouse mutants lacking the xylose residues added to *O*-glucose on Notch (Sethi *et al.*, 2010). Finally, no *in vitro* assays of ligand binding to Notch ECD lacking *O*-glucose have been performed.

4.3. General overview

In terms of mechanistic studies, roles for the *O*-fucose glycans on Notch have been those most investigated to date. Removal of single sites of O-fucosylation alters those Notch signaling and the action of Fringe alters DSL Notch ligand binding in *in vitro* assays. However, it is not clear if Notch ligands bind directly to the *O*-fucose glycans of Notch and thereby regulate Notch activation. The structures of complexes between modified and unmodified Notch and its ligands will be necessary to know if *O*-fucose glycans modulate Notch signaling directly or indirectly.

4.4. Conclusions

It is now clear that *O*-fucose and *O*-glucose glycans modulate Notch signaling events critical to cell fate determination and tissue development. However, much work remains to understand exactly how this occurs and also to identify roles for xylose and *O*-GlcNAc on Notch. Meanwhile, it is clear that the glycans of Notch are not just the icing on the cake!

ACKNOWLEDGMENTS

The authors wish to thank their collaborators and lab members for their many contributions over the years. This work was supported by National Caner Institute grant RO1 95022 to P.S. and grants from the Japanese Ministry of Education, Science, Sports and Culture and Human Frontier Science Program to T.O.

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