

[10] Roles of *O*-Fucose Glycans in Notch Signaling Revealed by Mutant Mice

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Abstract

Notch receptor signaling is important for many developmental processes in the metazoa. Insights into how Notch receptor signaling is regulated have been obtained from the characterization of mutants of model organisms in which Notch signaling is perturbed. Here we describe the effects of mutations that alter the glycosylation of Notch receptors and Notch ligands in the mouse. The extracellular domain of Notch receptors and Notch ligands carries *N*-glycans and *O*-glycans, including *O*-fucose and *O*-glucose glycans. Mutations in several genes that inhibit the synthesis of *O*-fucose glycans, and one that also affects the maturation of *N*-glycans, cause Notch signaling defects and disrupt development.

Overview

Notch receptors belong to a family of single transmembrane glycoproteins containing 29–36 EGF repeats in their extracellular domain (ECD). Notch receptor signaling is critical for cell fate determination, cell growth control, and development in metazoans. In mammals, there are four Notch receptors (Notch1–Notch4) and five Notch ligands (Jagged1, Jagged2, Delta1, Delta3, and Delta4). Each Notch receptor is synthesized in the endoplasmic reticulum (ER) as a single polypeptide and later cleaved in the trans-Golgi by a furin-like convertase and expressed on the cell surface as a heterodimer (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). On the binding of Notch ligands expressed in neighboring cells, Notch receptors are successively cleaved—first by a cell surface metalloprotease (ADAM10/ADAM17), leaving approximately 12 amino acids on the extracellular side of the Notch transmembrane portion and subsequently by the presenilin complex that has a γ -secretase activity that cleaves within the membrane. The latter cleavage releases Notch intracellular domain (ICD), which binds to the CSL transcriptional repressor (RBP-J κ in mammals) and recruits other transcriptional co-activators to turn the CSL/NICD complex into a transcriptional activator that induces the expression of target genes, among which are the Hairy-enhancer-of-split or *Hes* transcriptional factors. The overall pathway is presented in a simplified form in Fig. 1, with a focus on the location of the predicted *N*- and *O*-glycans of Notch1. The Notch receptor ECD becomes glycosylated as it transits the

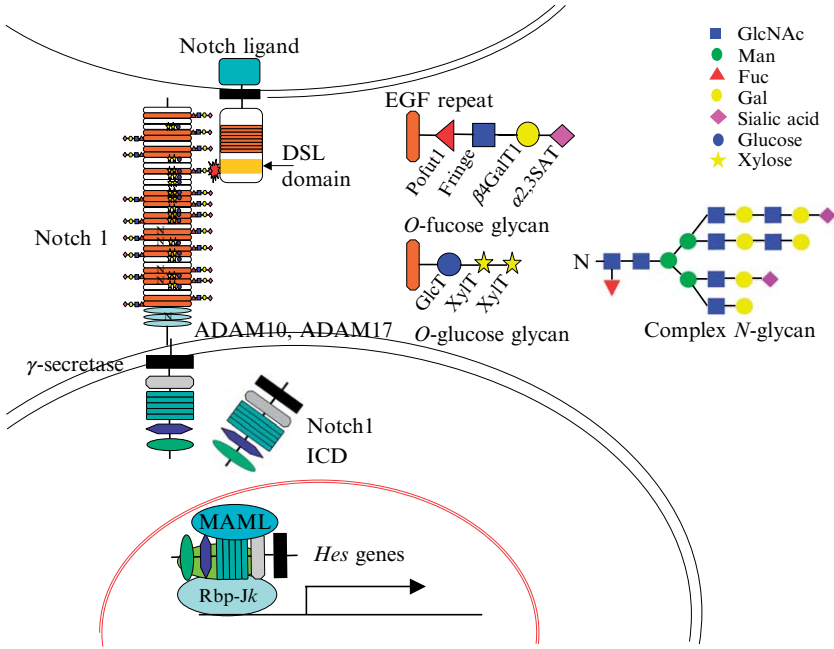


FIG. 1. Notch signaling occurs when a Notch ligand binds to a Notch receptor. Both ligands and receptors have multiple EGF repeats in their extracellular domain. The EGF repeats of both Notch receptors and ligands contain consensus sites for the addition of *O*-fucose and *O*-glucose glycans (see text) and AsnXSer/Thr consensus sequons for the addition of *N*-glycans. The EGF repeats that carry these glycans are identified for the Notch1 receptor because the glycosylation of this receptor is best characterized. EGF repeats with an *N*-glycan consensus are marked N; *O*-fucose and *O*-glucose glycans are shown with symbols. Notch/ligand binding is indicated by a red symbol at the ligand-binding domain of Notch1 (EGF repeats 11 and 12). When ligand binds, the dimeric Notch1 receptor is cleaved at a position 12 aa residues outside the membrane by an ADAM protease. The released ECD is endocytosed by the ligand-expressing cell, and there is an intramembrane cleavage of Notch by a complex with γ -secretase activity. The released NICD forms a complex with the transcriptional repressor CSL/Rbp-J κ and activators (master-mind like; MAML) and induces the expression of target genes such as *Hes* genes.

ER and Golgi compartments. Experiments in *Drosophila*, mice, and cultured cells have revealed functional roles for individual sugars on the ECD of Notch receptors (Haines and Irvine, 2003; Haltiwanger and Lowe, 2004).

Glycosylation of Notch Receptors and Notch Ligands

The extracellular domain of Notch receptors is glycosylated with *N*-glycans (Johansen *et al.*, 1989) and *O*-glycans, including *O*-fucose and *O*-glucose glycans (Moloney *et al.*, 2000b). *O*-glycosylation occurs at Ser or

TABLE I
MOUSE MUTANTS IN THE *O*-FUCOSE GLYCAN PATHWAY

Gene	Chromosome position	Mutation	References
<i>Pofut1</i>	Chr 2	Floxed exon 2 is removed. No <i>Pofut1</i> transcripts.	Shi and Stanley, 2003
<i>Lfng</i>	Chr 5	Replacement of exon1 with neo selection cassette.	Evrard <i>et al.</i> , 1998
		Replacement of partial exon1 with LacZ fused with neo selection cassette.	Zhang and Gridley, 1998
<i>Rfng</i>	Chr 11	Replacement of entire protein exon 1–8 with PGKneo selection cassette, then removed PGKneo cassette.	Moran <i>et al.</i> , 1999
		Deletion of exon 2–7 and part of exon1 replaced by PGKneo cassette.	Zhang <i>et al.</i> , 2002
<i>B4galt1</i>	Chr 4	Deletion of exon1, replaced with PGKneo selection cassette	Lu <i>et al.</i> , 1997
		Replacement of exon1 with PGKneo selection cassette	Asano <i>et al.</i> , 1997

Thr in the consensus sequence C_1XXPS/TC_2 between the first and second Cys residue in Notch EGF repeats. A trisaccharide may be present at *O*-glucose sites. Although the structure of the trisaccharide is unknown, it is predicted to contain xylose in the structure $Xyl\beta 1,3Xyl\beta 1,3Glc$ (Fig. 1). *O*-fucosylation occurs at Ser or Thr in the consensus sequence $C_2X_{4-5}S/TC_3$ between the second and third Cys of Notch EGF repeats. Fucose is transferred by the enzyme protein *O*-fucosyltransferase-1 (*Pofut1*), and the *O*-fucose may be extended with *N*-acetylglucosamine (GlcNAc) transferred by a Fringe $\beta 1,3GlcNAcT$, and subsequently by galactose and sialic acid in mammals (Moloney *et al.*, 2000b). Notch ligands also contain EGF repeats with the consensus sequence for *O*-fucose and *O*-glucose and the *O*-fucose residues are modified by Fringe (Panin *et al.*, 2002; Shao *et al.*, 2002). Targeted mutations of glycosyltransferases responsible for the synthesis of *O*-fucose glycans have been made in the mouse (Table I).

Pofut1 Is an Essential Component of the Canonical Notch Signaling Pathway

O-Fucose on Notch receptors was first shown to play a role in Notch signaling in Lec13 Chinese hamster ovary (CHO) cells that make very low

amounts of GDP-fucose (Moloney *et al.*, 2000a; Chen *et al.*, 2001). Jagged1-induced Notch signaling in a co-culture reporter assay is markedly reduced in Lec13 cells. These cells transfer little fucose to glycoproteins but can be corrected by exogenous fucose and thus have normal function of fucosyl-transferases including Pofut1. The fact that Lec13 cells exhibit little Notch signaling suggests that fucose is necessary for optimal Notch signaling in mammalian cells. In the fly, knockdown of OFUT1 causes Notch to accumulate intracellularly in the endoplasmic reticulum, and an inactive form of OFUT1 facilitates the secretion of Notch ECD fragments leading to the conclusion that *Drosophila* OFUT1 functions as a Notch chaperone (Okajima *et al.*, 2005). By contrast, Notch receptors are equivalently expressed on the surface of mammalian cells lacking Pofut1 (Stahl, M. Uemura, K. Shi, S. Ge, C., and Stanley, P., manuscript in preparation).

Targeted mutation of the mouse *Pofut1* gene leads to embryonic lethality at \sim E9.5 with severe defects in somitogenesis, vasculogenesis, cardiogenesis, and neurogenesis, a phenotype typical of mutants lacking downstream effectors that are required for signaling through all four Notch receptors (Shi and Stanley, 2003). The *Pofut1* gene has also been specifically ablated in mouse oocytes (Shi *et al.*, 2005). However, pre-implantation embryos lacking maternal and zygotic *Pofut1* gene transcripts develop through blastogenesis, implant, and progress through the formation of the three germ layers (Shi *et al.*, 2005). This is surprising, because lower organisms use Notch signaling in the determination of cell fates during pregastrulation development, suggesting co-option of Notch signaling during evolution for use in early cell fate decisions and embryogenesis (Shi and Stanley, 2006).

Mice unable to synthesize GDP-fucose would also be expected to have a phenotype that reflects inhibition of Notch signaling. However, embryonic lethality in homozygous null mutants is observed at variable stages *in utero* with the earliest being E12.5 (Smith *et al.*, 2002). Homozygous mutant pups are also born, but survival depends on the provision of fucose in the diet. The variability in the phenotype of mice with a null mutation in the FX gene is strain dependent (Becker *et al.*, 2003). Thus, there is one or more modifier locus that corrects the GDP-fucose deficiency. In strains with highly penetrant embryonic lethality, it seems that Notch signaling is at least partially rescued by fucose obtained from maternal or fraternal sources (Becker *et al.*, 2003; Smith *et al.*, 2002).

Fringe Is a Modulator of Notch Signaling

Fringe is a β 1,3N-acetylglucosaminyltransferase (β 1,3GlcNAcT) that transfers GlcNAc to *O*-fucose on EGF repeats of Notch receptors (Haines and Irvine, 2003). In mammals, there are three Fringe homologs,

Lunatic, Manic, and Radical Fringe (Lfng, Mfng, and Rfng). They function in the Golgi, but all of them are also secreted.

Lfng is expressed in many cell types during embryonic stages, but its dynamic expression in mouse somites indicated a functional role during segmentation (Johnston *et al.*, 1997). Lunatic fringe is the only fringe gene expressed during somitogenesis (Johnston *et al.*, 1997). Targeted mutations that inactivate the mouse *Lfng* gene cause somites to have irregular sizes and shapes and their anterior-posterior patterning to be disrupted. A severely disorganized axial skeleton is the result (Evrard *et al.*, 1998; Zhang and Gridley, 1998). Mutants die perinatally or in early adulthood. The expression patterns of somitogenic genes are altered, such as Myogenin, *Mox1*, *Pax1*, *Pax3*, *Pax9*, and *Uncx4.1*. Furthermore, the expression of Notch signaling pathway genes, such as *Dll1*, *Dll3*, *Notch1*, and *Notch4*, is reduced. Some *Lfng* null mutants may survive to adulthood, and in at least one mutant strain *Lfng* null females are infertile (Hahn *et al.*, 2005). These females exhibit aberrant folliculogenesis with altered expression of Notch pathway genes, and their eggs are arrested in meiosis II and thus are not developmentally competent (Hahn *et al.*, 2005). The two strains of *Lfng*^{-/-} mutant mice differ in the degree of their somitic defects and in the penetrance of their defect in female infertility (Hahn *et al.*, 2006; Xu *et al.*, 2006).

In mice lacking both the Notch ligand Jagged2 and Lfng, the generation of supernumerary hair cells in the inner hair cell row that occurs when Jagged2 is not present is suppressed by the concomitant loss of Lfng, whereas supernumerary hair cells in the outer hair cell rows are unaffected (Zhang *et al.*, 2000). *Lfng* modulation of Notch signaling is also thought to be important in tooth development. Although *Lfng*^{-/-} mice do not show abnormal tooth development, Lfng is expressed in the epithelium surrounding the enamel knot signaling center that controls tooth size and shape (Mustonen *et al.*, 2002). A human patient with spondylocostal dysostoses (SCDs) with vertebral malsegmentation has been found to carry an autosomal recessive mutation (F188L) in the LFNG gene (Sparrow *et al.*, 2006). This mutation of a conserved amino acid in LFNG causes mouse Lfng F187L to be mislocalized in transfected cells, to have no enzyme activity, and to be unable to modulate Notch signaling in a co-culture assay (Sparrow *et al.*, 2006).

Interestingly, the targeted mutation of the mouse *Rfng* gene did not result in an obvious abnormal phenotype (Moran *et al.*, 1999; Zhang *et al.*, 2002). Double null mice null for both *Lfng* and *Rfng* resemble *Lfng* mutant mice. The double mutants exhibited no obvious synergistic or additive effects to the *Lfng*^{-/-} somitogenic phenotype, showing there is no functional redundancy between mouse Lfng and Rfng during somitogenesis (Zhang *et al.*, 2002).

Lfng is expressed in a dynamic, repetitive, and complex wave pattern within the mouse presomitic mesoderm (PSM; [Forsberg *et al.*, 1998]). A wave takes 2 h. The mechanism of Lfng cyclic gene expression in the PSM has been investigated using LacZ reporter transgenes fused with *Lfng* promoter regions (Cole *et al.*, 2002; Morales *et al.*, 2002). A conserved *Lfng* promoter fragment is required for *Lfng* cyclic gene expression in the PSM. The oscillatory expression of the *Lfng* gene is controlled by a negative feedback that seems to be regulated by the *Hes7* gene (Bessho *et al.*, 2003; Chen *et al.*, 2005). Cyclic Lfng expression is critical for somitogenesis to proceed normally. Thus, an *Lfng* transgene under the control of a portion of the *Dll1* promoter termed *msd* exhibits constitutive expression in the PSM (Cordes *et al.*, 2004; Serth *et al.*, 2003). Mice carrying this transgene in a background with or without endogenous Lfng have a similar phenotype to *Lfng* null mice exhibiting severe defects in skeleton formation. Although the noncyclic exogenous expression of Lfng did not abolish cyclic expression of endogenous Lfng in the PSM, the fact that constitutive expression in the anterior PSM causes defective somitogenesis shows that transcriptional oscillation of Lfng is essential for somitogenesis (Cordes *et al.*, 2004; Serth *et al.*, 2003). Overexpression of Lfng by the SPC-Lfng transgene in distal epithelial cells of the developing mouse lung did not affect spatial or temporal expression of the *Hes1* Notch target gene or other differentiation markers (van Tuyl *et al.*, 2005). These mice have no detectable lung defects, suggesting that Lfng does not play a significant role in determining cell fate in fetal airway epithelium (van Tuyl *et al.*, 2005). Misexpression of Lfng in the thymus under the *lck* promoter inhibits T-cell development that is dependent on Notch signaling and results in the generation of B cells in the thymus (Koch *et al.*, 2001). The increased Lfng expression in T cells enhances their binding to stromal epithelial cells expressing Delta ligands and thus inhibits their ability to mature as T cells (Tan *et al.*, 2005; Visan *et al.*, 2006).

The mechanisms by which Fng genes modulate canonical Notch signaling have been investigated in model organisms including *Drosophila*, *Xenopus*, and Zebrafish (Haines and Irvine, 2003). In mammals, mechanisms of Fng actions have been investigated in co-culture Notch signaling assays using reporter constructs that respond to NICD. Overexpression of mammalian Fng proteins has been shown to differentially modulate ligand-induced Notch signaling. Lfng and Mfng inhibit Jagged1-induced Notch1 signaling and potentiate Delta1-induced Notch1 signaling in co-culture systems (Hicks *et al.*, 2000). However, Rfng was found to enhance Notch1 signaling induced by either Delta1 or Jagged1 (Yang *et al.*, 2005). Interestingly, it seems Fng proteins may have different effects on different Notch receptors. For example, Lfng potentiates both

Jagged1- and Delta1-induced signaling by Notch2 (Hicks *et al.*, 2000). Binding assays with soluble ligands and Notch fragments or to cells expressing transfected Notch receptors generally correlate with predictions from signaling assays; that is, Fng effects that inhibit Notch signaling correlate with decreased ligand binding, whereas Fng effects that potentiate Notch signaling correlate with increased ligand binding (Hicks *et al.*, 2000; Shimizu *et al.*, 2001; Yang *et al.*, 2005). The three Fng β 1,3GlcNAcTs have different *in vitro* catalytic efficiencies and may also have different specificities for particular EGF repeats of Notch1 (Rampal *et al.*, 2005a,b; Shao *et al.*, 2003). Thus *in vivo* effects of Fng actions in the modulation of Notch signaling by all four Notch receptors induced by the five Notch ligands may be extremely complex.

β 4GalT-1 Is a Novel Regulator of Notch Signaling

The elongation of the GlcNAc β 3Fuc disaccharide on Notch EGF repeats by β 4GalT-1 was found to be required for Lfng and Mfng inhibition of Jagged1-induced Notch signaling in a co-culture assay (Chen *et al.*, 2001). Neither the Lec20 CHO mutant that lacks β 4GalT-1 and β 4GalT-6 nor the Lec8 CHO mutant that cannot transport UDP-Gal into the Golgi exhibits Lfng or Mfng modulation of Notch signaling. When corrected with a cDNA encoding β 4GalT-1, Lec20 cells are rescued for Fng effects. To investigate *in vivo* mouse embryos lacking β 4GalT-1 were examined (Chen *et al.*, 2006). Although mutant embryos do not have obvious skeletal defects as would be predicted if Fng modulation of Notch signaling were inhibited, many have severely reduced expression of certain Notch target genes such as *Hes5* and *Mesp2* and altered expression of the Notch ligand genes *Dll1* and *Dll3*. Furthermore, the number of lumbar vertebrae in 12 of 13 perinatal mutant embryos differed significantly from control litter mates (Chen *et al.*, 2006). This finding is consistent with known effects of Notch signaling on *Hox* gene functions during mouse skeletal development (Cordes *et al.*, 2004). The subtlety of the defect in mice lacking β 4GalT-1 may reflect the fact that there are six β 4GalT genes, several of which are expressed during embryogenesis.

Mutations in Other Glycosyltransferase Genes

There are now many strains of mice that have a null mutation in a glycosyltransferase gene that affects the synthesis of *N*-glycans or mucin *O*-glycans or *O*-Mannose glycans (Lowe and Marth, 2003). Although Notch receptors and their ligands carry *N*-glycans, none of the mutant mice defective in *N*-glycan synthesis with the exception of the mice lacking

β 4GalT-1 described previously have been found to exhibit an overt phenotype consistent with defective Notch signaling. However, they may well have a subtle defect in Notch signaling that affects functions that would require specific analyses or biological challenges to uncover. Mucin *O*-glycans or *O*-mannose glycans may also be present on Notch receptors and their ligands, but no mutants in the glycosyltransferases that synthesize these *O*-glycans have given a Notch signaling phenotype. Similarly, mice with defective proteoglycan synthesis have not been described to have Notch signaling defects.

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