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Biological Functions of Glycosyltransferase Genes Involved in *O*-fucose Glycan Synthesis

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Rare types of glycosylation often occur in a domain-specific manner and are involved in specific biological processes. Well-known examples of such modification are *O*-linked fucose (*O*-fucose) and *O*-linked glucose (*O*-glucose) glycans on epidermal growth factor (EGF) domains. In particular, *O*-fucose glycans are reported to regulate the functions of EGF domain-containing proteins such as urinary-type plasminogen activator and Notch receptors. Two glycosyltransferases catalyze the initiation and elongation of *O*-fucose glycans. The initiation process is catalyzed by *O*-fucosyltransferase 1, which is essential for Notch signalling in both *Drosophila* and mice. *O*-fucosyltransferase 1 can affect the folding, ligand interaction and endocytosis of Notch receptors, and both the glycosyltransferase and non-catalytic activities of *O*-fucosyltransferase 1 have been reported. The elongation of *O*-fucose monosaccharide is catalyzed by Fringe-related genes, which differentially modulate the interaction between Notch and two classes of ligands, namely, Delta and Serrate/Jagged. In this article, we have reviewed the recent reports addressing the distinctive features of the glycosyltransferases and *O*-glycans present on the EGF domains.

Key words: Fringe, Notch, *O*-fucose, *O*-fucosyltransferase, *O*-glucose.

Abbreviations: EGF, epidermal growth factor; Xyl, xylose; Glu, glucose; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; *O*-linked glucose, *O*-glucose, *O*-linked fucose, *O*-fucose.

Protein glycosylation is a common co- and post-translational modification that has critical biological functions at both the cellular and whole-organism levels. In addition to the generally observed types of glycosylation, such as *N*-glycosylation and mucin-type *O*-glycosylation, several unusual types of glycans have recently been discovered and are considered to play important roles in specific biological processes. Such unusual glycosylations are often observed as specific modifications on certain protein domains (*1*). An example of such domain is the epidermal growth factor (EGF) domain that is characterized as a small domain of 30–40 amino acids stabilized by three disulfide bonds. These disulfide bonds form three intramolecular disulfide bonds in the 1–3, 2–4 and 5–6 patterns and contribute to form the structure with two anti-parallel β-sheets at the N-terminal and C-terminal subdomains (*2*). Three types of unusual post-translational modifications have been found specifically at conserved residues within certain EGF domains. These modifications are β-hydroxylation of Asp or Asn residues, *O*-linked glucose (*O*-glucose) and *O*-linked fucose (*O*-fucose) (*3*).

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O-LINKED FUCOSE

O-fucose glycan has been found in many plasma glycoproteins. It was first described in human urinary plasminogen activator (uPA) (*4*, *5*) and then in tissue plasminogen activator (tPA) (*6*), blood clotting factors VII (*7*), IX (*8*) and XII (*9*) (Fig. 1). In addition, *O*-fucosylation of the salivary plasminogen activator from bats has also been reported (*10*). In contrast to Factor VII that bears only the *O*-fucose monosaccharide (*7*), Factor IX bears *O*-fucose glycans that are elongated into the tetrasaccharide Sia-α-2,6(*3*)-Gal-β-1,4-GlcNAc-β-1,3-Fuc-α-O-Ser/Thr (*8*, *11*).

Recent studies have revealed that EGF-specific *O*-glycans are not only limited to plasma glycoproteins but also exist in less abundantly expressed cell surface glycoproteins that are involved in intercellular signal transduction, such as Notch receptors and Notch ligands—Delta and Serrate/Jagged (*12*, *13*). Notch signalling mediates an evolutionarily conserved signalling pathway that regulates a wide range of developmental processes (*14*). The extracellular domain of Notch receptors is mainly composed of tandemly repeated EGF domains (36 EGF domains in the *Drosophila* Notch and mammalian Notch1), many of which are expected to be *O*-fucosylated (*15*, *16*). In particular, the *O*-fucosylation site at EGF domain 12, which is located at a ligand-binding site, is evolutionarily conserved; this suggests its functional importance. Moreover, Cripto, a membrane-bound co-receptor for Nodal, is also shown

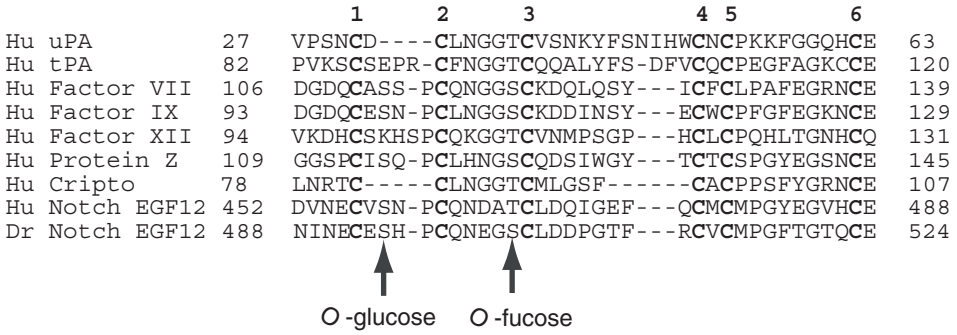

Fig. 1. **Amino acid sequence alignment of EGF domains modified with *O*-fucose and *O*-glucose glycans.** Amino acid sequence of EGF domains of human uPA, tPA, blood clotting factors (VII, IX and XII), Protein Z, Cripto and EGF domain 12 from *Drosophila* and human Notch1 are aligned. The sites for *O*-fucosylation or *O*-glucosylation are indicated. The conserved cysteine residues are numbered.

to be *O*-fucosylated (*17*, *18*). By comparing the amino acid sequences surrounding the modification site of the *O*-fucosylated proteins that have been discovered thus far, the consensus sequence for *O*-fucosylation is proposed to be $C^2XXX(A/G/S)S/TC^3$ (C^2 and C^3 are the second and third conserved cysteines and X is any amino acid) (*15*).

O-LINKED GLUCOSE

O-glucose was initially identified as an unknown serine derivative located between the first and second cysteine residues within the first EGF domain of bovine factor VII (*19*, *20*). A similar modification was subsequently reported in the bovine and human Factor IX (*20*, *21*) and Protein Z (*21*) (Fig. 1). The carbohydrate structure of the completely elongated form is reported as the trisaccharide Xyl-α-1,3-Xyl-α-1,3-Glu-β-O-Ser (*22*). A similar structure was later found in the bovine thrombospondin (*23*) and Pref-1 (*24*). The consensus sequence for *O*-glucosylation is predicted to be C^1XSXPC^2 (C^1 and C^2 are the first and second conserved cysteines and X is any amino acid) (*23*). A considerable number of EGF repeats on Notch receptors conform to *O*-glucosylation sites, and they were experimentally confirmed to be *O*-glucosylated (*12*).

FUNCTIONAL SIGNIFICANCE OF *O*-FUCOSE GLYCANS

The first implication of the biological relevance of *O*-fucose glycans was obtained from the study of uPA. The defucosylated and bacterially expressed uPA could bind to the uPA receptors but failed to activate them, suggesting that the monosaccharide *O*-fucose is required for uPA-induced signalling (*25*). In the case of Cripto, *O*-fucosylation was initially proposed to be essential for Nodal signalling. This is based on the observation that substitution of Ala for Thr to which *O*-fucose is attached led to the functional inactivation of both human and mouse Cripto (*17*, *18*). However, it was subsequently discovered that the Thr residue and not *O*-fucose alone is required for facilitating Nodal signalling (*26*).

Functional significance of multiple *O*-fucose modifications on Notch receptors has extensively been studied. The Thr to Ala mutation in EGF domain 12 decreases the responsiveness of Notch1 to Jagged1 and Delta1 (*27*), suggesting that this site is positively required for Notch signalling. In contrast, a similar mutation at *Drosophila* Notch EGF domain 12 resulted in Notch activation by Serrate even in the presence of Fringe, suggesting that this site is essential for the inhibition of Fringe-induced Serrate-to-Notch signalling (*28*). These conflicting observations might be explained by the presence of an adjacent *O*-fucosylation site in *Drosophila* Notch EGF domain 13, which is not conserved in Notch1. Nonetheless, these studies clearly demonstrate that EGF domain 12 is the physiologically relevant site for *O*-fucose glycan modification. The roles of other highly conserved *O*-fucose sites were reported: mutation of the *O*-fucose site in EGF domains 26 and 27 resulted in hyperactivation and impaired cell surface trafficking of Notch receptors, respectively (*27*).

FRINGE-RELATED GENES

The first evidence for the functional significance of *O*-fucose glycans for Notch signalling is the discovery of the *fringe* mutant in *Drosophila*, which is required for positioning of the dorsoventral boundary in developing wing discs (*29*). Later, Fringe was demonstrated to encode an *O*-fucose-specific *N*-acetylglucosamine (GlcNAc) transferase (*30*, *31*). Expression of *fringe* in *Drosophila* is highly regulated during development. Moreover, Fringe positions Notch activation at its expression border, and such positioning is required for boundary formation of the wing, leg and eye imaginal discs (*15*). In mammals, inactivation of *Lfng* led to defects in somitogenesis (*32*, *33*). In *Drosophila* wing discs, Fringe is shown to modulate the Notch–ligand interaction: Fringe potentiates Delta–Notch signalling but inhibits Serrate–Notch signalling. This effect has been explained by the alternation of the Notch–ligand physical interaction: Fringe promotes Notch–Delta binding but inhibits Notch–Serrate binding (*30*, *34*). In mammals, the observed effect appears more complicated since the effect could vary under different combinations among the four Notch receptors (Notch1–4), five ligands (Delta1, 3 and 4, and Jagged1 and 2) and three types of Fringe proteins (Lfng, Rfng and Mfng). However, in

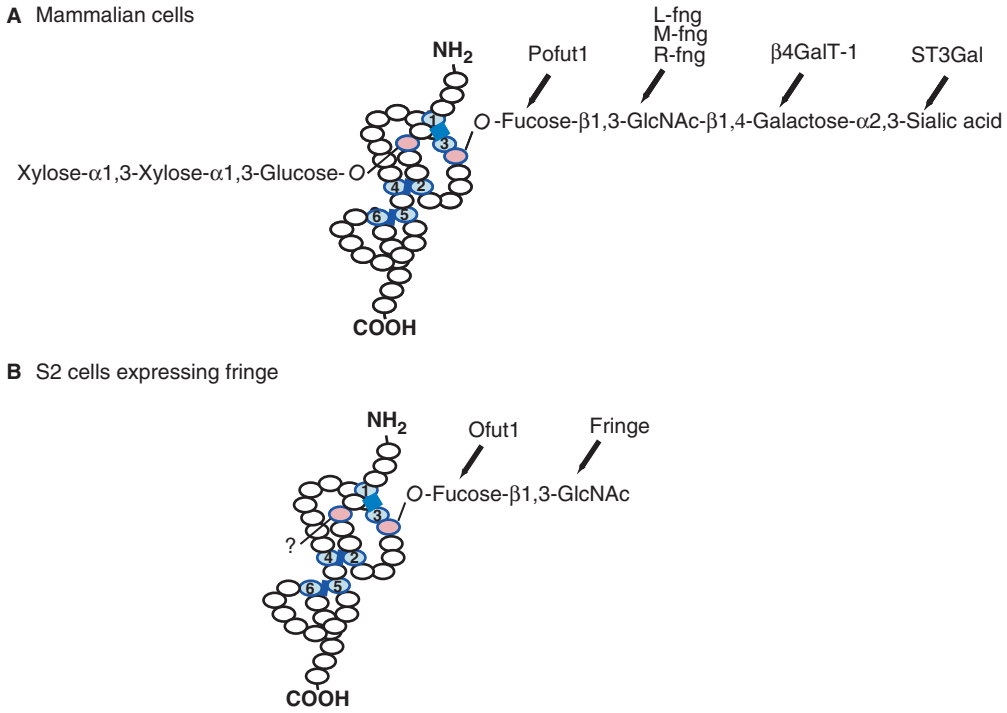

Fig. 2. **Glycosyltransferases involved in EGF-domain specific *O*-glycosylations.** (A) The structure of *O*-glycans on the EGF domains in mammals and the glycosyltransferase genes corresponding to each glycosylation step are illustrated. *O*-fucosyltransferase 1 (*Pofut1*) (*40*, *47*) and Fringe-related genes (*Lunatic Fringe*, *Radical Fringe* and *Manic Fringe* in mammals and *fringe* in *Drosophila*) (*29*, *48*, *49*) are specifically employed to synthesize *O*-fucose glycans on Notch receptors. In contrast, genes for β1, 4-galactosyltransferase and sialyltransferase are identical to those involved in *N*- and *O*-glycan biosyntheses. For the biosynthesis of *O*-glucose trisaccharide, three different glycosyltransferases (Protein *O*-glucosyltransferase, *O*-glucose α-1, 3-xylosyltransferase and xylose α-1,3-xylosyltransferase) are predicted to be involved (*50–53*). (B) The structure of *O*-glycans on the EGF domains in *Drosophila* S2 cells. Fringe expression was not detected in the wild-type Drosophila S2 and *O*-fucose glycans were terminated as monosaccharides. On Fringe expression, their structure was extended to disaccharides. *O*-glucosyltransferase activity was demonstrated in the S2 cells; however, the detailed structure remains elusive (*53*).

general, the effect of mammalian Fringe is similar with *Drosophila* Fringe; it inhibits Jagged1-induced Notch signalling, and promotes Delta1-induced Notch signalling as well as the binding of Delta1 with Notch1 and 2 [refer to the recent review by Pamela Stanley (*35*)].

β-1,4-GALACTOSYLTRANSFERASE

In mammals, GlcNAc-β-1,3-Fuc-α-*O*-Ser/Thr is further modified with galactose (Gal) and sialic acid by the action of β-1,4-galactosyltransferase and sialyltransferases (Fig. 2). To address whether the addition of Gal and sialic acid is required to produce the effect of Fringe, Notch signalling activity was measured in a variety of glycosylation mutants of Chinese hamster ovary (CHO) cells. A comparison of Lec2, Lec8, Lec13 and Lec20 CHO cells showed that the trisaccharide Gal-β1,4-GlcNAc-β1,3-Fuc is the minimal *O*-fucose glycan required to support *fringe*-dependent modulation of Notch signalling (*36*). In contrast, in *Drosophila* S2 cells expressing *fringe*, the structure of *O*-fucose glycans was terminated as disaccharide GlcNAc-β1,3-Fuc (*37*) (Fig. 2), and further elongation was not required to produce the modulatory effect of Fringe on Notch–ligand binding. Thus, at least in *Drosophila*, the simple addition of GlcNAc is sufficient to produce the effects of Fringe on Notch–ligand binding. Further studies are required to determine whether the addition of Gal in CHO cells influences Notch–ligand binding or other processes required for Notch signalling, and whether the effect of β-1,4-galactosyltransferase is mediated by either Notch or other glycans involved in the Notch signalling pathway (*37*).

O-FUCOSYLTRANSFERASE 1

O-fucosyltransferase 1 is a soluble glycosyltransferase that is localized in the endoplasmic reticulum (ER), where it transfers *O*-fucose to the EGF domains (*38*, *39*). The functional significance of *O*-fucosylation has been studied in *Pofut1*/*Ofut1* mutants. In both *Drosophila* and mammals, the loss of *Pofut1*/*Ofut1* resulted in the phenotype that is characteristic of Notch loss of function (*40–42*). These results consistently suggest that *Pofut1*/*Ofut1* is essential for Notch signalling. However, the molecular mechanisms underlying the absolute requirement of *Pofut1*/*Ofut1* are not fully understood. In *Drosophila*, the loss of OFUT1 led to the loss of cell surface expression and the intracellular accumulation of Notch receptors (*39*, *43*). With regard to the molecular mechanism, it was proposed that OFUT1 is required for the correct folding of Notch receptors in the ER and their subsequent trafficking to the cell surface (Fig. 3). This is

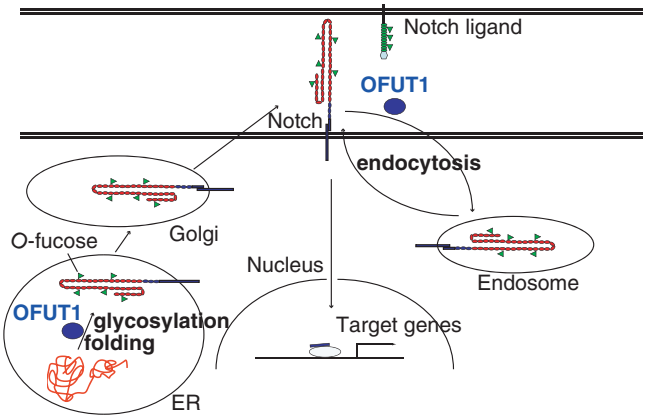

Fig. 3. **Notch signalling pathway and the components involved in the synthesis of *O*-fucose glycans in *Drosophila*.** The canonical Notch signalling pathway and the proposed models for the action of *Drosophila* OFUT1 on Notch receptors are illustrated. It was initially proposed that OFUT1 is required in the ER for the correct folding and subsequent trafficking of Notch receptors (*39*). However, another study showed that OFUT1 associates with Notch at the cell surface and promotes the constitutive endocytosis of Notch receptors and transcytosis of Notch from the apical plasma membrane to the adherens junctions (*44*).

based on the observation that the secretion of Notch was impaired in OFUT1-depleted S2 cells and the abnormal ER accumulation of Notch receptors was observed in *Ofut1* mutant clones in *Drosophila* wing imaginal discs. Moreover, the ability of OFUT1 to promote Notch secretion does not depend on its enzyme activity, suggesting the chaperon-like role of OFUT1 (*39*). More recently, it was demonstrated that the chaperone activity of OFUT1 is sufficient to generate functional Notch receptors (*43*).

In contrast, another model was recently proposed in which OFUT1 associates with Notch at the cell surface and promotes the constitutive endocytosis of Notch receptors (Fig. 3). This is based on the observation that the secreted OFUT1 promotes endocytosis of Notch receptors and that the administration of Notch antibodies to *Ofut1* mutant cells labels an unknown intracellular compartment in which Notch is accumulated. More recently, Sasaki *et al*. (*44*) further suggested that OFUT1 promotes transcytosis of Notch from the apical plasma membrane to the adherens junctions. Although these two models are not mutually exclusive, there is apparent discrepancy in the intracellular location where Notch receptors localize in the *Ofut1* mutant cell. Further studies addressing the conformational change in the Notch receptor in *Ofut1* mutant cells, the presence of OFUT1 at the cell surface and in the endocytic compartment, and the exact location of the accumulated Notch receptors in *Ofut1* mutant cells are required to understand the proposed roles of OFUT1 in the ER and endocytic pathway.

FUTURE PERSPECTIVE

Although extensive studies addressing the functions of *O*-fucose glycans and glycosyltransferases genes involved in the glycosylation pathway have been conducted, little is known regarding the biological function of *O*-glucose glycans. It has been reported that the substitution of Ser by Ala at the *O*-glucosylation site of Factor VII results in decreased coagulant activity (*7*), implicating the functional importance of *O*-glucose modification. The glycosyltransferases involved in the biosynthesis of *O*-glucose glycans have been purified (Fig. 2). The identification of glycosyltransferase genes and analyses of these genes in *Drosophila* and mice would provide greater insights into the biological contributions of the modification of *O*-glucose glycans. It has been reported that the mutation in *fringe-connection* (*frc*), wherein global GlcNAc modifications on Notch receptors are impaired due to the defect in the specific UDP-sugar transporter activity, displayed more severe phenotypes than the *fringe* mutants (*45*, *46*). Thus, a subset of Notch phenotypes observed in the *frc* mutant may in part be attributable to the uncharacterized sugar modification on Notch receptors that contains the GlcNAc moiety. Identification of other EGF domain-specific glycans that are relevant to Notch receptor functions will be essential for a comprehensive understanding of glycan-dependent regulation of Notch signalling activity.

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