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Review

N-acetylglucosamine modification in the lumen of the endoplasmic reticulum

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ABSTRACT

Background: O-linked β-*N*-acetylglucosamine (O-GlcNAc) modification of epidermal growth factor (EGF) domains catalyzed by EGF domain O-GlcNAc transferase (EOGT) is the first example of GlcNAc modification in the lumen of the endoplasmic reticulum (ER).
Scope of review: This review summarizes current knowledge on the EOGT-catalyzed O-GlcNAc modification of EGF domains obtained through biochemical characterization, genetic analysis in *Drosophila*, and identification of human *EOGT* mutation. Additionally, this review discusses GTDC2—another ER protein homologous to EOGT that catalyzes the GlcNAc modification of O-mannosylated α-dystroglycan—and other components of the biosynthetic pathway involved in GlcNAc modification in the ER lumen.
Major conclusions: GlcNAc modification in the ER lumen has been identified as a novel type of protein modification that regulates specific protein function. Moreover, abnormal GlcNAc modification in the ER lumen is responsible for Adams–Oliver syndrome and Walker–Warburg syndrome.
General significance: Elucidation of the biological function of GlcNAc modification in the ER lumen will provide new insights into the unique roles of O-glycans, whose importance has been demonstrated in multifunctional glycoproteins such as Notch receptors and α-dystroglyan.

1. Introduction

O-linked β-*N*-acetylglucosamine (O-GlcNAc) modification was first reported as a cell-surface moiety on intact lymphocytes [1]. However, subsequent studies with improved methods failed to detect O-GlcNAc-bearing proteins on the cell surface. Instead, O-GlcNAc was considered restricted to the cytoplasmic, mitochondrial, and nuclear compartments. To date, more than 1000 nuclear, cytosolic, and mitochondrial proteins have been suggested to be O-GlcNAcylated proteins [2]. Intracellular O-GlcNAcylation is dynamically regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) [3–6]. O-GlcNAc modification regulates many cellular processes [7] such as transcriptional control [8], chromatin remodeling [9–11], signal transduction [12], cell differentiation [13–15], and nutrient sensing [16,17]. OGT is the only enzyme that catalyzes the O-GlcNAcylation of intracellular proteins [18]. Recently, extracellular O-GlcNAc was discovered on epidermal growth factor (EGF) repeats of Notch receptor [19] (Fig. 1). The addition of O-GlcNAc to extracellular proteins is catalyzed by EGF domain-specific O-GlcNAc transferase (EOGT) in the endoplasmic reticulum (ER) [20,21]. This is the first example of GlcNAc modification in the lumen of the ER.

2. Extracellular O-GlcNAc on EGF domains

Extracellular O-GlcNAc was first detected on the 20th EGF domain (EGF20) of *Drosophila* Notch receptors. The EGF domain contains six conserved cysteine residues that form three disulfide bond pairs between the first and the third conserved cysteines (C^1–C^3), C^2–C^4 and C^5–C^6. O-GlcNAc modification occurs on the Thr residue located between C^5–C^6 [19]. Moreover, Delta and Serrate, ligands for Notch receptors, are O-GlcNAcylated by EOGT [22,23]. In *Drosophila* cuticles, Dumpy is a major O-GlcNAcylated protein [20]. Dumpy, a giant 2.5-MDa membrane-anchored protein, contains 308 EGF-like repeats. Among those, multiple Thr/Ser residues are O-GlcNAcylated, although the exact modification sites have not been assigned. In mammals, mass spectrometric analysis of mouse O-GlcNAcylated proteins in the brain led to the identification of five EGF domain-containing proteins: Hspg2, Nell1, Lama5, Pamr1, and Notch2 [24]. In platelets, thrombospondin-1 was proposed to be O-GlcNAcylated [25], although it is possible that the CTD110.6 anti-O-GlcNAc antibody used in this study cross-reacted with other terminal GlcNAc residues [23,26,27]. Recent attempts to isolate endogenous EOGT substrates from Chinese hamster ovary (CHO) cells have led to the identification of multiple O-GlcNAcylated cell surface proteins, including Notch3 [23]. Sequence alignment of O-GlcNAcylated proteins has suggested that the predictive consensus sequence for the modification is C^5XXGX(T/S)GXXC^6 [19]. However, whether the C^5XXGX(T/S)

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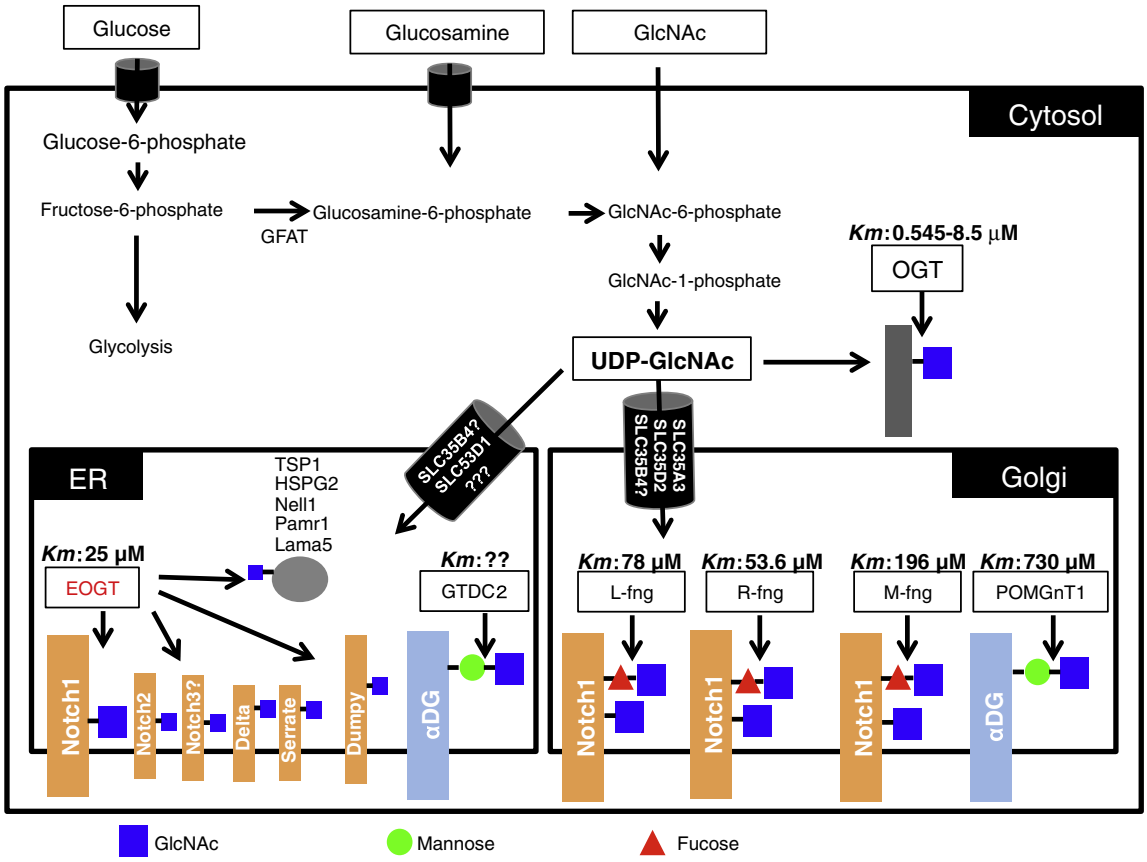


Fig. 1. O-GlcNAcylation occurs in the endoplasmic reticulum (ER). Extracellular O-linked β-*N*-acetylglucosamine (O-GlcNAc) occurs on several epidermal growth factor (EGF) domain-containing proteins, including Notch1, Notch2, Delta, Serrate, Dumpy, Tsp1, Hspg2, Nell1, Lama5, and Pamr1. O-GlcNAcylated proteins related to Notch signaling are shown in *orange*, α-dystroglycan (αDG) in *blue*, and the others in *gray*. Uridine diphosphate (UDP-GlcNAc) is synthesized from fructose 6-phosphate via the hexosamine biosynthesis pathway (HBP). The end product of HBP is UDP-GlcNAc, which is a donor substrate for various GlcNAc transferases, including EGF domain O-GlcNAc transferase (EOGT), OGT, GTDC2, lunatic fringe (L-fng), radical fringe (R-fng), manic fringe (M-fng), and POMGnT1. The K_m values of each GlcNAc transferase are indicated. SLC35A3 and SLC35D2 are localized in the Golgi whereas SLC35D1 is localized in the ER. In contrast, the subcellular localization of SLC35B4 is different depending on the cell type. Note that the requirement of these NSTs for GlcNAcylation of glycoproteins such as Notch receptors and α-dystroglycan has not been experimentally verified. GFAT; glucosamine:fructose-6-phosphate aminotransferase.

GXXC[6] sequence is necessary/sufficient for modification has not been confirmed [21,23]. In human embryonic kidney (HEK) 293T cells, the reactivity of Notch1 EGF repeats to CTD110.6 anti-O-GlcNAc antibody increased upon β1,4 galactosidase digestion, suggesting the presence of a O-GlcNAc-β1,4-Gal structure [21]. However, a similar structure was not detected in Lec1 CHO cells [23].

3. EGF domain-specific O-GlcNAc transferase (EOGT)

EOGT is evolutionarily conserved among various species, from *Caenorhabditis elegans* to humans. EOGT exhibits no similarity to OGT, but it is phylogenetically related to plant xylosyltransferases. *EOGT* encodes a luminal ER protein with a KDEL-like ER-retrieval sequence at its carboxyl terminus (Fig. 2A), and catalyzes O-GlcNAcylation in the ER [28]. Expression of *EOGT* has been detected in all adult mouse tissues, with highest expression in the lungs [21]. During mouse development, high expression levels of EOGT have been detected in the growing edge of the limb buds; the expression is localized to the digits of the four limbs at later stages [29]. *EOGT* is also highly expressed on stem cells derived from human umbilical cord blood [30]. In a previous study, *in vitro* enzyme assay demonstrated that EOGT specifically utilizes uridine diphosphate (UDP)-GlcNAc as the sugar donor, and its activity is enhanced in the presence of Mn^{2+} [21]. Additionally, EOGT has a putative donor binding DXD motif; alanine substitution mutation of this motif markedly decreased the enzyme activity of EOGT [22].

Approximately 2–5% of cellular glucose enters the hexosamine biosynthesis pathway (HBP), a branch of glucose metabolism (Fig. 1). Glucosamine and GlcNAc can activate HBP because they directly enter HBP distal to glucosamine:fructose-6-phosphate aminotransferase, the first and rate-limiting enzyme of HBP. The end product of this pathway is the nucleotide sugar donor UDP-GlcNAc, and UDP-GlcNAc is used as the substrate for O-GlcNAc modification [20,21,28,31]. Because the O-GlcNAcylation level increases with glucose, glucosamine, or GlcNAc treatment [28], the level of extracellular O-GlcNAc may change depending on the availability of UDP-GlcNAc in the ER.

Different GlcNAc transferases exhibit different subcellular localization and affinity for UDP-GlcNAc (Fig. 1). Among the GlcNAc transferases, OGT exhibits highest affinity for UDP-GlcNAc, with the K_m value ranging from 545 nM to 8.5 μM [5,32]. Higher K_m value for UDP-GlcNAc (25 μM) was reported for EOGT [28]. However, this value is still lower than that reported for most Golgi-resident GlcNAc transferases. For example, lunatic fringe, radical fringe, and manic fringe, acting as *O*-fucose β-1,3*N*-acetylglucosaminyltransferases, exhibit K_m values of 78 μM, 53.6 μM, and 196 μM, respectively [33]. POMGnT1, acting as an *O*-mannose β-1,2*N*-acetylglucosaminyltransferase, exhibits K_m value of 0.73 mM [34]. The K_m values of UDP-GlcNAc for Mgat1, Mgat2, Mgat4, and Mgat5 are 40 μM, 0.96 mM, 5.0 mM, and 11.0 mM, respectively [35]. Therefore,

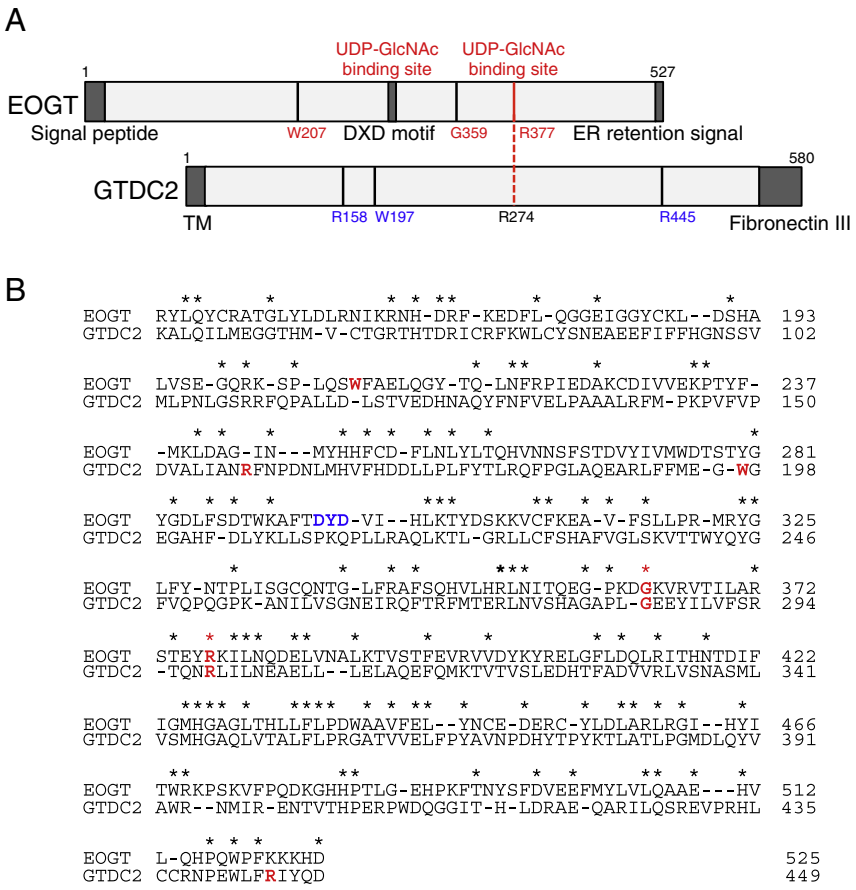


Fig. 2. *EOGT* mutations identified in Adams–Oliver syndrome patients. (A) Schematic representation of the structures of EOGT and GTDC2. EOGT encodes a luminal ER protein containing an amino-terminal signal peptide (signal peptide) and a carboxyl-terminal KDEL-like ER retrieval signal (ER retention signal). EOGT contains two putative UDP-GlcNAc binding sites: R377 (shown in *red line*) and putative DXD (*gray box*). GTDC2 has a transmembrane domain (TM) and a fibronectin domain. The mutations identified in AOS patients are indicated as *red letters* and those identified in WWS patients are indicated as *blue letters*. The putative UDP-GlcNAc binding site for EOGT (R377) is conserved in GTDC2 (R274; *red dotted line*). (B) The amino acid sequence alignment of mouse EOGT (NP_780522) and mouse GTDC2 (Q8BW41) is shown. Amino acid residues conserved between EOGT and GTDC2 are shown by *asterisks*. Amino acid residues associated with AOS or WWS are highlighted by *red letters*. The DXD motif is indicated by *blue letters*.

the affinity of EOGT toward UDP-GlcNAc is higher than that exhibited by most other GlcNAc transferases, except OGT. Given that EOGT shows high affinity to UDP-GlcNAc but still depends on HBP, the concentration of UDP-GlcNAc in the ER is probably maintained at a low level by specific ER-resident UDP-GlcNAc transporters.

As described below, mutations in *EOGT* have been reported in patients with Adams–Oliver syndrome, a congenital disorder. The reported mutations include missense mutations (W207S and R377Q) and a frame shift mutation that creates a premature stop codon (G359Dfs*28). All the mutations diminish the ability to O-GlcNAcylate EGF domains in the ER. The W207S and R377Q mutations involve changes in amino acids conserved across all species, from *C. elegans* to *Homo sapiens*. Importantly, the R377 residue is conserved in GTDC2, an EOGT-related glycosyltransferase in the ER, as described in the next section (Fig. 2B) [36]. The EOGT ^{R377Q} mutant protein exhibits decreased binding to UDP-GlcNAc; therefore, this Arg residue may be important for nucleotide-sugar recognition by EOGT [36].

4. *O*-mannose β-1,4-*N*-acetylglucosaminyltransferase (GTDC2)

GTDC2 (also called POMGnT2) is highly homologous to EOGT (Fig. 2), and it is similarly localized in the ER (Fig. 1) [27,37]. *GTDC2* is conserved in vertebrates, including chicken, *Xenopus*, zebrafish, mouse, and human. As shown in Fig. 2A, GTDC2 is type II single-pass transmembrane protein containing a carboxy-terminal fibronectin III domain. *GTDC2* was originally reported as one of the glycosyltransferase genes responsible for Walker–Warburg syndrome (WWS). WWS is a severe form of α-dystroglycanopathy characterized by brain and eye anomalies and congenital muscular dystrophy caused by abnormal glycosylation of α-dystroglycan (αDG) [38]. *GTDC2* encodes *O*-mannose β-1,4-*N*-acetylglucosaminyltrasnferase responsible for GlcNAcylation of O-mannose on αDG in the ER [37]. Interestingly, GTDC2-catalyzed GlcNAc modification on O-mannosylated αDG is detectable by anti-O-GlcNAc antibody CTD110.6 [27]. Following GlcNAc modification in the ER, B3GALNT2 adds β1,3-linked GalNAc to synthesize GalNAcβ1,3GlcNAcβ1,4Mannose trisaccharide. POMK (SGK196) recognizes this trisaccharide to phosphorylate the 6-position of O-mannose [37], which is required for LARGE-dependent biosynthesis of laminin-binding glycan [39,40]. *GTDC2*-knockout mice are slightly smaller than wild type mice at birth and die within postnatal day 1. Moreover, the brains of *GTDC2*-defectve mice exhibit abnormal basal lamina and neuronal migration defect due to lack of laminin-binding glycans [41].

GTDC2 mutations that have been reported in WWS patients include R158H, W197Δ, and R445Δ (Fig. 2A). The R158 residue of GTDC2 is conserved among vertebrates and R158H mutation is predicted to be very deleterious. W197Δ mutation causes truncation at the beginning of the putative catalytic domain, whereas the R445Δ mutation deletes the fibronectin III domain [38]. As expected, the R158H and W197Δ mutants failed to produce enzymatic products detectable with the CTD110.6 antibody. In contrast, the R445Δ mutant retains the ability to produce enzymatic products, suggesting that the fibronectin III

domain might have a molecular function distinct from its glycosyltransferase activity [27].

5. GlcNAc modification in the luminal ER

UDP-GlcNAc, synthesized through HBP in the cytoplasm, is transported by nucleotide sugar transporters (NSTs) into the ER or Golgi apparatus. Although UDP-GlcNAc transporter activity has been detected in the ER [42], the specific UDP-GlcNAc transporters responsible for GlcNAcylation in the luminal ER have not been identified. A potential candidate for ER UDP-GlcNAc transport is SLC35D1. This protein transports UDP-GlcUA, UDP-GalNAc, and UDP-GlcNAc [43,44]. *SLC35D1* knockout resulted in diminished chondroitin sulfate synthesis and defective cartilage and skeletal development in mice [44]. However, this phenotype does not overlap with that exhibited by *GTDC2*-deficient mice, suggesting the presence of other UDP-GlcNAc transporters responsible for GlcNAcylation in the luminal ER.

Another candidate for UDP-GlcNAc transport in the ER is SLC35B4, a multi-substrate specific nucleotide-sugar transporter. In CHO cells, human SLC35B4 transports both UDP-GlcNAc and UDP-Xyl, but it is strictly localized in the Golgi [45,46]. In contrast, in Madin–Darby canine kidney cells, SLC35B4 was shown to be located in the ER [47], suggesting the possibility of cell-type specific contribution of SLC35B4 for UDP-GlcNAc transport in the ER. Unfortunately, no biological function or association with EOGT and GTDC2 activity has been reported in SLC35B4.

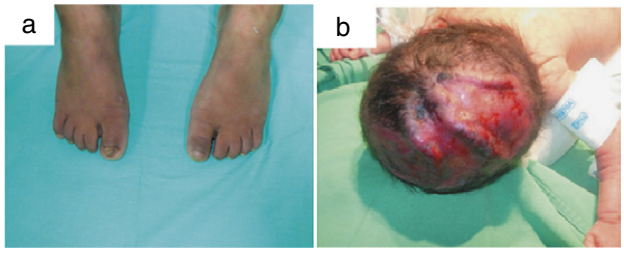
Another potential mechanism for the delivery of UDP-GlcNAc to the ER is that a fraction of the UDP-GlcNAc imported into the Golgi by Golgi UDP-GlcNAc transporters (i.e. SLC35A3 and SLC35D2; Fig. 1) is transported to the ER by retrograde vesicle transport. The importance of retrograde transport of nucleotide sugars has been demonstrated in the case of GDP-fucose, which is required for Notch O-fucosylation [35]. Thus, multiple UDP-GlcNAc transporters localized at the ER and Golgi might be involved in GlcNAcylation in the luminal ER. It is also likely that different species have evolved different mechanisms for UDP-GlcNAc transportation in the ER, as suggested by the different transporter activities reported for SLC35B4 orthologs [45–48].

6. Role of extracellular O-GlcNAc

6.1. Drosophila Eogt mutant

The biological role of extracellular O-GlcNAc was first identified by analyzing the phenotype of *Drosophila Eogt* mutants [20]. Although *Eogt* mutants do not display the classical Notch phenotype, they exhibit defects in the wings, notum, and cuticle, similar to *dumpy* mutants. Dumpy, a membrane-tethered protein, is a major O-GlcNAcylated protein in *Drosophila* cuticles. Moreover, the genetic interaction and phenotypic similarity between *Eogt* and *dumpy* suggests that extracellular O-GlcNAc is required for Dumpy-dependent epithelial cell–matrix interactions. However, comprehensive genetic interaction studies have revealed association between *Eogt* and pyrimidine metabolism in the wing blister phenotype [22]. Moreover, pyrimidine biosynthetic activities are increased in *dp* mutant larvae [49,50]. Thus, an alternative mechanism underlying Eogt-mediated cell adhesion is that, the lack of O-GlcNAcylation of Dumpy in *Eogt* mutants leads to an increased UDP-GlcNAc pool in the ER and cytoplasm. The increased cytoplasmic UDP-GlcNAc concentration activates *de novo* pyrimidine synthesis, leading to overproduction of a toxic UMP metabolite such as uracil, which likely promotes wing blistering [22]. If this is the case, Eogt might regulate pyrimidine metabolism by O-GlcNAcylating Dumpy. The contribution of pyrimidine metabolism to the *Eogt* phenotype has also been suggested by the genetic interaction between *Eogt* and Notch signaling genes, which are involved in pyrimidine synthesis regulation [22].

A



B

	Functions
ARHGAP31	Cdc42/Rac1 regulatory protein
DOCK6	Cdc42/Rac1 regulatory protein
RBPJ	Transcriptional repressor of Notch signaling
NOTCH1	Notch receptor
EOGT	O-GlcNAc transferase modifying EGF domains

Fig. 3. Candidate genes for Adams–Oliver syndrome (AOS). (A) Representative clinical images of individuals from families of AOS caused by EOGT mutation (Reprinted by permission from Macmillan Publishers Ltd: European Journal of Human Genetics, Ref. [49], copyright 2014). AOS is a rare congenital disorder characterized by terminal transverse limb defects (Shown in Fig. 3A[a]) and vertex scalp defects (Shown in Fig. 3A[b]). (B) Summary of the genetic mutations identified in AOS patients to date. ARHGAP31 and DOCK6 regulate the activity of a key molecule in the actin cytoskeleton (shown in *blue*). *RBPJ* and *NOTCH1* are associated with Notch signaling (shown in *pink*). The biological function of *EOGT* has not been clarified (shown in *yellow*).

6.2. EOGT mutation in Adams–Oliver syndrome

Although the biological roles of extracellular O-GlcNAc in mammals have not been clarified, *EOGT* mutations have been reported in patients with Adams–Oliver syndrome (AOS) [29,51]. AOS is a rare congenital disorder characterized by aplasia cutis congenita (ACC) of the scalp vertex and terminal transverse limb defects (TTLDs) (Fig. 3A). The basic pathophysiologic mechanism in AOS remains unknown, but these defects are assumed to be due to a vasculopathy, because cardiovascular defects are occasionally observed in AOS patients. Indeed, cutis marmorata telangiectatica congenita (CMTC), atrial septal defect (ASD), and ventricular septal defect (VSD), caused by *EOGT* mutations, have been reported in some AOS patients [29,51].

Three homozygous mutations for *EOGT* have been reported to date. All these mutations diminish the ability to O-GlcNAcylate EGF domains. Moreover, an $EOGT^{R377Q}$ mutant lost enzyme activity without affecting the ability to bind to EGF domains. These results suggested that impaired glycosyltransferase activity in mutant EOGT proteins and consequent defective O-GlcNAcylation in the ER constitutes the molecular basis for AOS [28].

In addition to *EOGT*, homozygous mutations of *DOCK6* and *ARHGAP31*, and heterozygous mutations of *RBPJ* and *NOTCH1* have been reported in AOS patients (Fig. 3B) [52–56]. Cardiovascular defects have also been noted in *Notch1*- and *DOCK6*-related AOS. Both ARHGAP31 and DOCK6 regulate the activity of key regulatory proteins of the actin cytoskeleton, such as RAC1 and CDC42. Accordingly, fibroblasts isolated from patients with *ARHGAP31*-related or *DOCK6*-related AOS exhibited disorganized cytoskeleton and morphology [53,54]. In contrast, *EOGT* mutant fibroblasts exhibited typical spindle appearance, comparable to control fibroblasts [51]. Therefore, EOGT does not appear to affect actin cytoskeleton, although EOGT might affect actin dynamism in restricted cell-types other than fibroblasts.

Among the AOS-causative proteins, Notch1, one of four members of the Notch family of receptors, is the only protein that is modified by EOGT. Upon binding to Notch ligands, Notch receptors undergo proteolytic cleavage, releasing Notch intracellular domain (NICD). In the absence of NICD, the DNA binding protein, RBPJ, acts as a transcriptional repressor. In the nucleus, NICD binds RBPJ and Mastermind-like (MAML), forming the RBPJ/NICD/MAML transcriptional activation complex to induce the transcription of Notch target genes [57,58]. Two disease-causing *RBPJ* mutations have been identified, which lead to decreased binding to the Notch target promoter, *HES1* [52]. Moreover, five different heterozygous Notch1 mutations have been reported: an 85-kb deletion, including a part of the promoter and the non-coding first exon; a splice site mutation; a cysteine substitution in the EGF-like 11 domain; a cysteine substitution in the LNR domain; and an Asp1989Asn mutation in ankyrin repeats. However, the effects of these mutations on Notch signaling have not been investigated. It has been reported that both reduced and elevated Notch signaling can lead to vascular abnormalities [59]. Thus, elucidating the mechanism by which *NOTCH1* mutations and EOGT-catalyzed O-GlcNAcylation affect the structure and function of Notch receptors might provide insights into the pathophysiology of AOS.

Notch1 EGF domains are simultaneously modified with other EGF-domain specific glycosylations (with O-fucose and O-glucose) [60]. O-fucosylation and O-glucosylation are catalyzed by POFUT1/Ofut1 and POGLUT1/Rumi, respectively [61,62]. Both types of O-glycosylation play key roles in Notch signaling, such as trafficking, processing, and ligand binding [63–67]. Interestingly, heterozygous mutations (presumably haploinsufficiency) for POFUT1 or POGLUT1 cause Dowling-Degos disease, an autosomal-dominant genodermatosis characterized by reticular-pigmented anomaly [68–70]. Therefore, O-fucose and O-glucose play important roles in Notch-dependent melanocyte lineage development. In contrast, similar anomalies have not been reported in AOS patients. The different contributions of O-fucose/O-glucose and O-GlcNAc in human pathology imply that different O-glycans on Notch receptors have distinct functions in the regulation of Notch receptors.

7. Conclusions and perspectives

In this review, we summarized current knowledge of GlcNAc modification in the luminal ER. It has been established that GTCD2-catalyzed GlcNAc modification is required for subsequent synthesis of laminin-binding glycans. Although the biochemical properties of GTDC2 have not been fully investigated, O-mannose residues on αDG are partially modified with GTDC2 [27,37,71], and overexpression of GTDC2 could increase the GlcNAc modification required for laminin-binding glycan biosynthesis [41]. Thus, if the enzymatic activity of GTDC2 depends on the HBP pathway, activation of HBP might be a potential therapeutic approach for α-dystroglycanopathy.

In contrast to GTCD2, much less is known regarding EOGT-catalyzed O-GlcNAcylation and the components of the biosynthetic pathway involved in GlcNAc modification in the ER lumen. Particularly, the roles of O-GlcNAc in Notch signaling might be of potential interest in terms of the involvement of Notch signaling in the characteristic anomalies observed in AOS and for the development of rational therapies to interrupt progressive vascular dysfunction. Although the roles of Notch signaling in vascular development and maintenance have been established [72–74] and the vascular anomalies observed in AOS could be attributable to Notch signaling defects, wide varieties of developmental processes are associated with Notch signaling, and only a subset of the Notch-related defects were reported in AOS. Since Notch signal strength required for distinct developmental processes varies in a context-dependent manner [75], it is interesting to speculate that the Notch-dependent processes sensitive to reduced signal strength were selectively compromised in Notch1-related AOS and that EOGT is required to achieve optimal Notch signaling strength in such sensitive biological processes. Such fine-tuning by EOGT might partially underlie the molecular mechanism of genetic interaction between *Eogt* and Notch in wing blistering in *Drosophila* [22]. Careful examination of *EOGT* mutant mice and biochemical characterization *in vitro* might help to elucidate the roles of extracellular O-GlcNAc in this important signaling pathway.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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