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Extracellular *O*-linked β-*N*-acetylglucosamine: Its biology and relationship to human disease

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Abstract

The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of cytoplasmic and nuclear proteins regulates basic cellular functions and is involved in the etiology of neurodegeneration and diabetes. Intracellular *O*-GlcNAcylation is catalyzed by a single *O*-GlcNAc transferase, *O*-GlcNAc transferase (OGT). Recently, an atypical *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (EOGT), which is responsible for the modification of extracellular *O*-GlcNAc, was identified. Although both OGT and EOGT are regulated through the common hexosamine biosynthesis pathway, EOGT localizes to the lumen of the endoplasmic reticulum and transfers GlcNAc to epidermal growth factor-like domains in an OGT-independent manner. In *Drosophila*, loss of *Eogt* gives phenotypes similar to those caused by defects in the apical extracellular matrix. Dumpy, a membrane-anchored apical extracellular matrix protein, was identified as a major *O*-GlcNAcylated protein, and EOGT mediates Dumpy-dependent cell adhesion. In mammals, extracellular *O*-GlcNAc was detected on extracellular proteins including heparan sulfate proteoglycan 2, Nell1, laminin subunit alpha-5, Pamr1, and transmembrane proteins, including Notch receptors. Although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated, exome sequencing identified homozygous *EOGT* mutations in patients with Adams-Oliver syndrome, a rare congenital disorder characterized by aplasia cutis congenita and terminal transverse limb defects. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

Key words: Extracellular *O*-linked β-*N*-acetylglucosamine; Notch; Adams-Oliver syndrome

Core tip: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) on extracellular protein domains is the most recently identified *O*-glycosylation of epidermal growth factor repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by an endoplasmic reticulum-resident *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (*EOGT*). In *Drosophila*, Dumpy, a membrane-tethered cuticle protein, was identified as a major *O*-GlcNAcylated protein that mediates the interaction between epithelial cells and the extracellular matrix. In mammals, extracellular *O*-GlcNAc was detected on Hspg2, Nell1, Lama5, Pamr1, and Notch receptors, although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated. However, the recent finding that *EOGT* is a causative gene for Adams-Oliver syndrome provided important insights into the significance of extracellular *O*-GlcNAc in mammals. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

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INTRODUCTION

O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) was first identified in 1984 as a cell-surface saccharide moiety on intact lymphocytes[1]. Later studies, however, revealed that *O*-GlcNAc is present on nuclear, cytosolic, and mitochondrial proteins. This modification is prevalent in multicellular organisms, where more than 1000 *O*-GlcNAcylated proteins have been identified[2]. Intracellular *O*-GlcNAcylation is reversible, and its cycling is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase[3-5]. A large number of studies have indicated that *O*-GlcNAcylation is involved in various cellular functions, including transcription, epigenesis, cellular signaling, cell differentiation, and glucose sensing[6-9]. It had long been believed that *O*-GlcNAc is a unique intracellular modification and that OGT is the sole enzyme catalyzing the *O*-GlcNAc transfer reaction. However, extracellular *O*-GlcNAc was recently discovered on the extracellular domains of Notch receptors (Figure 1A). In this minireview, we will focus on extracellular *O*-GlcNAc and its relevance to human disease.

EXTRACELLULAR *O*-GLCNAC ON EGF DOMAINS

The first example of the *O*-GlcNAc modification of extracellular protein domains was the 20th EGF domain (EGF20) of *Drosophila* Notch expressed in S2 cells. Biochemical analyses revealed that *O*-GlcNAcylation occurs on the threonine located between the fifth and sixth cysteine[10]. Moreover, *in vivo* studies revealed that *O*-GlcNAc is abundantly expressed in the *Drosophila* cuticle[11]. Among cuticle proteins, Dumpy, a giant 2.5-MDa membrane-anchored cuticle protein containing a very large number of EGF-like domains (308 EGF-like repeats), was identified as a major *O*-GlcNAcylated protein[11]. In addition to Notch and Dumpy, Delta and Serrate, ligands for Notch receptors, have been shown to be *O*-GlcNAcylated by extracellular *O*-linked β-*N*-acetylglucosamine (EOGT)[10,12] (Figure 1B) in *Drosophila* S2 cells.

Similar to intracellular *O*-GlcNAc, extracellular *O*-GlcNAc is conserved in mammals but can be subjected to subsequent modification. The co-expression of Notch1 with EOGT in HEK293T cells suggests that the *O*-GlcNAc moiety is further modified with galactose to form *O*-linked *N*-acetyl-lactosamine (*O*-LacNAc)[13]. Recently, five extracellular *O*-GlcNAcylated proteins [Hspg2(Perlecan), Nell1, Lama5, Pamr1, and Notch2] were identified by a modified chemical/enzymatic photochemical cleavage approach for enriching *O*-GlcNAcylated peptides from mouse cerebrocortical brain tissue[14]. Another carbohydrate analysis revealed that *O*-GlcNAcylation occurs in the native thrombospondin-1 (TSP1) purified from platelets as well as in the recombinant TSP1 fragments expressed in insect High Five cells[15] (Figure 1B). The sequence alignment of *O*-GlcNAcylated proteins suggests that the predictive consensus sequence for the modification is C^5XXGX(T/S)GXXC^6, where C^5 and C^6 are the fifth and sixth conserved cysteines of the EGF domain, respectively. It should be noted, however, that no experimental data are available to indicate whether the C^5XXGX(T/S)GXXC^6 sequence is necessary or sufficient for the modification[10].

EOGT IS RESPONSIBLE FOR EXTRACELLULAR *O*-GLCNAC

In contrast to the OGT-catalyzed intracellular modification, the addition of *O*-GlcNAc onto extracellular proteins is mediated by a distinct *O*-GlcNAc transferase, the EGF-domain specific *O*-GlcNAc transferase (EOGT)[11,13]. *Eogt* is evolutionarily conserved from *Caenorhabditis elegans* to humans. EOGT contains a hydrophobic region corresponding to a signal peptide and a KDEL-like ER-retrieval sequence at the carboxyl terminus (Figure 2A)[11]. EOGT exhibits no similarity to OGT, but it is phylogenetically related to plant xylosyltransferases. EOGT possesses a putative UDP-GlcNAc-binding DXD motif[12]. EOGT specifically utilizes uridine diphosphate (UDP)-GlcNAc as a sugar donor, and its *in vitro* enzyme activity is enhanced in the presence of divalent cations, especially Mn^{2+}[11,13].

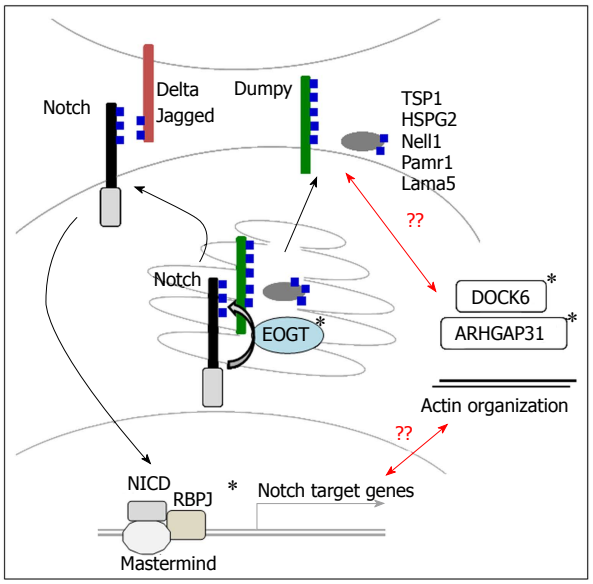
Because the levels of *O*-GlcNAcylation on Notch are increased by treatment with glucosamine or GlcNAc[8], it is suggested that the hexosamine biosynthesis pathway (HBP) is upstream of extracellular *O*-GlcNAc modification. The end product of the HBP is UDP-GlcNAc, which is utilized by EOGT as a donor substrate to modify proteins with *O*-GlcNAc in the ER. The transport of UDP-GlcNAc across the ER or Golgi membrane is mediated by nucleotide-sugar transporters[16-19]. However, it remains unclear which UDP-GlcNAc transporters are required for *O*-GlcNAcylation by EOGT.

Although *EOGT* expression has been detected in all adult mouse tissues, its expression is highest in the lung and lowest in the skeletal muscles[13]. During mouse development, high expression was detected in the growing edge of the limb buds; the expression was localized to the digits of the four limbs at later stages[20].

BIOLOGICAL FUNCTION OF EXTRACELLULAR *O*-GLCNAC IN *DROSOPHILA*

The biological function of extracellular *O*-GlcNAc was first suggested by the phenotype of the *Eogt* mutant in

A



B

***O*-GlcNAcylated proteins**	***O*-GlcNAc site**	**Protein function**	**Species**	**Ref.**
Notch1	LNAFSCQCMPGYTGQKC	Receptor for Notch signaling	*Drosophila*	[10]
Dumpy	Unspecified	Apical extracellular matrix protein	*Drosophila*	[11,12]
Hspg2 (Perlecan)	ACAPGYTGRRCES	heparan sulfate proteoglycan/extracellular matrix component	Mouse	[14]
Nell1	VCPSGFTGSHCEK	Glycoprotein involved in bone physiology	Mouse	[14]
Lama5	TCPPGLSGERCDT	One of the vertebrate laminin alpha chains	Mouse	[14]
Pamr1	ACLAGYTGQRCEN	Regeneration-associated Muscle protease homolog	Mouse	[14]
Notch2	VCSPGFTGQRCNI (?)	Receptor for Notch signaling	Mouse	[14]
thrombospondin-1	CGACPPGYSGNGIQCTLELVPR	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions	Human	[15]
Delta	Unspecified	Ligand for Notch signaling	Drosophila	[11,12]
Serrate	Unspecified	Ligand for Notch signaling	Drosophila	[12]

Figure 1 Extracellular *O*-linked β-*N*-acetylglucosamine. A: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of extracellular protein domains is a newly identified translational modification of epidermal growth factor (EGF) domains, including Notch, HSPG2, Pamr1, and Lama5. Extracellular *O*-GlcNAc is mediated by EOGT in the endoplasmic reticulum (ER). Mutations in EOGT were recently identified in patients with Adams-Oliver syndrome (AOS). The role of EOGT in the pathogenesis of AOS is currently unknown. Given that RBPJ, a transcriptional factor for Notch signaling, is a causative gene for AOS, *O*-GlcNAcylation of Notch receptors by EOGT might regulate Notch receptor trafficking or Notch-ligand interactions. ARHGAP31 or DOCK6, another causative gene for AOS, affects the actin cytoskeleton by regulating Cdc42 and Rac1 activity. Thus, another possibility is that the *O*-GlcNAcylation of unidentified cell adhesion molecules by EOGT affects actin dynamics. It should be noted, however, that Dumpy homologues are not present in mammals. The *O*-GlcNAcylation of Notch ligands was reported in Drosophila. The causative genes for AOS are shown by asterisks; B: Summary of proteins with extracellular *O*-GlcNAc identified to date.

Drosophila[11]. Although the *Eogt* mutant does not exhibit the classical Notch phenotype, it shows defects in the wings, notum, and cuticle (*i.e.*, wing blistering, vortex, and cuticle detachment), similar to the *dumpy* mutant[11,12]. As mentioned above, Dumpy is a membrane-tethered protein that represents a major *O*-GlcNAcylated protein in the cuticle[11]. Moreover, the genetic interaction and phenotypic similarity between *Eogt* and *dumpy* suggests that EOGT is required for Dumpy-dependent epithelial cell-matrix interactions.

Previous studies using *Eogt* mutant embryos suggested that *O*-GlcNAc is required for the correct targeting of Dumpy into the chitinous matrix, possibly by mediating interactions with other components in the extracellular matrix (ECM)[11]. Currently, the molecular mechanisms by which Dumpy mediates cell adhesion are unknown, and thus the precise mechanism by which *O*-GlcNAc mediates cell adhesion must await the functional characterization of Dumpy. However, it is intriguing to speculate that multiple *O*-GlcNAc moieties arranged regularly along the EGF repeats of Dumpy have the ability to associate with unidentified chitin (a polymer of GlcNAc)-binding lectins in the ECM, thereby enabling the cuticle assembly/maintenance required for epidermis adhesion.

Interestingly, comprehensive genetic interaction studies revealed an interaction between *Eogt* and pyrimidine metabolism in the wing blister phenotype[12]. Thus, an alternative possibility is that loss of *Eogt* directs the increased UDP-GlcNAc pool in the cytoplasm. This will lead to elevated pyrimidine synthesis, such as uracil, that is likely to promote wing blistering[12]. If this is the case, EOGT might regulate pyrimidine metabolism by *O*-GlcNAcylating Dumpy. The contribution of pyrimidine metabolism to the *Eogt* phenotype was also suggested by the genetic interaction between *Eogt* and the Notch signaling genes,

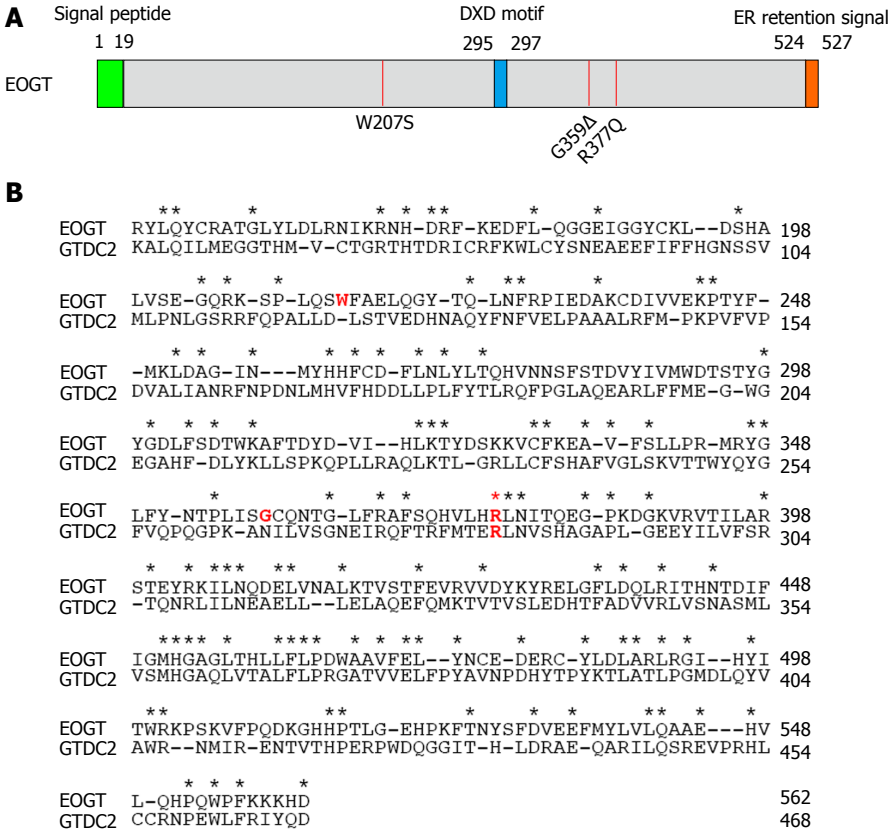


Figure 2 Extracellular *O*-linked β-*N*-acetylglucosamine mutations found in Adams-Oliver syndrome. A: A schematic representation of the primary structure of EOGT. The amino-terminal signal peptide is shown in yellow and the carboxyl-terminal Lys-Asp-Glu-Leu-like endoplasmic reticulum (ER) retrieval signal is in orange. The putative DXD motif involved in binding the nucleotide sugar is shown in blue. The position of each mutation is indicated by a red line; B: The amino acid sequence alignment of mouse EOGT (NP_780522, 149-562 aa) and mouse GTDC2/EOGT-L (Q8BW41, 55-468 aa). Identical amino acid residues are indicated by asterisks. Amino acid residues corresponding to the mutations in patients with Adams-Oliver syndrome are highlighted by red letters. EOGT: Extracellular *O*-linked β-*N*-acetylglucosamine.

which are involved in pyrimidine synthesis regulation[12].

EXTRACELLULAR *O*-GLCNAC AND ITS RELATIONSHIP TO ADAMS-OLIVER SYNDROME

The significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function, and the physiological roles of *O*-GlcNAc in mammals have not been investigated. However, exome sequencing in Adams-Oliver syndrome (AOS) patients provided important insights into the significance of extracellular *O*-GlcNAc in mammals. AOS is a rare congenital disorder characterized by vertex scalp defects [aplasia cutis congenital (ACC)] and terminal transverse limb defects (TTLDs)[21]. Recently, homozygous mutations in *EOGT* were identified in some patients with AOS[20,22]. These mutations include missense mutations (W207S and R377Q) and a frame shift mutation that creates a premature stop codon (G359Dfs*28) (Figure 2A). Currently, the blood levels of extracellular *O*-GlcNAc, the sugar moiety and its metabolites in the patients have not yet been investigated. However, the frame shift mutation in *EOGT* likely abolishes the enzyme activity because the truncated form of EOGT lacks the putative catalytic region containing the sequences conserved between EOGT and GTDC2, another ER-resident GlcNAc transferase modifying α-dystroglycan[23-25] (Figure 2B). The biochemical properties of the W207S and R377Q mutations have not yet been addressed. However, the R377 residue of EOGT is conserved in GTDC2. Thus, it is likely that the R377 residue may be important for GlcNAc transferase activity in EOGT and GTDC2 and that the R377Q mutation impairs the *O*-GlcNAc transferase activity of EOGT.

AOS is genetically heterogeneous, and its molecular pathology appears complex. In addition to *EOGT*, homozygous mutations of *DOCK6,* gain-of-function mutations of *ARHGAP31*, and heterozygous mutations for *RBPJ* were reported in AOS[26-28] (Figure 1A). *ARHGAP31* and *DOCK6* encode proteins that regulate the activity of key regulators of the actin cytoskeleton, RAC1 and CDC42. Accordingly, patient fibroblasts harboring disease-causing *ARHGAP31* or *DOCK6* mutations exhibited disorganized cytoskeletons and morphologies[27,28]. By contrast, *EOGT* mutant fibroblasts showed a typical spindle appearance comparable to that of control fibroblasts[22]. Therefore, it appears that EOGT does not directly affect the actin cytoskeleton, although the pos-

sibility remains that EOGT affects actin dynamism in restricted cell-types other than fibroblasts.

EXTRACELLULAR *O*-GLCNAC AND NOTCH SIGNALING

Another intriguing possibility for the role of EOGT in the pathogenesis of AOS involves Notch regulation because *RBPJ* encodes the transcriptional factor for Notch signaling. It has been reported that disease-causing *RBPJ* mutations decrease binding to the Notch target promoter, *HES1*[26]. Therefore, if EOGT and RBPJ act through a common signaling pathway in AOS, EOGT might positively regulate Notch signaling by the *O*-GlcNAcylation of Notch receptors. It should be noted, however, that no experimental data are available to support this hypothesis.

In *Drosophila*, *O*-GlcNAcylated EGF domains could be simultaneously modified with other *O*-glycosylations, namely *O*-fucose and *O*-glucose. *O*-fucosylation and *O*-glucosylation are catalyzed by ER-resident glycosyltransferases, POFUT1/Ofut1[29] and POGLUT1/Rumi[30]. These enzymes play indispensable roles for Notch signaling by affecting the trafficking, processing, and ligand-binding ability of Notch receptors[30-37]. In contrast, *O*-GlcNAc is dispensable for the majority of Notch receptor functions because *Eogt* mutants failed to exhibit apparent defects in most Notch-dependent biological processes, including embryonic neurogenesis, wing margin formation, and wing vein specification[11]. Given that the mutation of *Ofut1* or *rumi* does not produce Dumpy-like phenotypes, *O*-GlcNAcylation and *O*-fucosylation/*O*-glucosylation appears to be significant for the separate protein functions and distinct developmental processes in *Drosophila*. Nonetheless, there remains the possibility that these *O*-glycosylations may have partially redundant roles for Notch function, which would be revealed by genetic interaction studies between *Eogt* and *rumi*/*Poglut1* or *Eogt* and *Ofut1*/*Pofut1*.

Currently, no animal models for AOS have been established, and no AOS-related phenotypes were reported in *RBPJ* heterozygous mice[38]. In this regard, it would be interesting to investigate whether *EOGT* mutant mice would serve as a disease model for AOS.

CONCLUSION

The *O*-GlcNAc on extracellular protein domains is the most recently identified *O*-glycosylation of EGF repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by EOGT in the ER. In *Drosophila*, Dumpy was identified as a major *O*-GlcNAcylated protein that contributes to the interaction between epithelial cells and cuticles. Recent reports revealed that the mutations in *EOGT* cause AOS. However, the significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function in *Drosophila*, and the roles of *O*-GlcNAc in mammals have not been elucidated. In mammals, extracellular *O*-GlcNAc was detected on the TSP1, Hspg2, Nell1, Lama5, Pamr1, and Notch receptors[14,15]. Considering that a number of extracellular and transmembrane proteins are potentially *O*-GlcNAcylated by EOGT, additional studies will be required to address the roles of extracellular *O*-GlcNAc in Notch-dependent and independent biological processes in mammals as well as the molecular pathogenesis of human disease.

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