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O-glycosylation disorders pave the road for understanding the complex human O-glycosylation machinery

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Over 100 human Congenital Disorders of Glycosylation (CDG) have been described. Of these, about 30% reside in the *O*-glycosylation pathway. *O*-glycosylation disorders are characterized by a high phenotypic variability, reflecting the large diversity of *O*-glycan structures. In contrast to *N*-glycosylation disorders, a generic biochemical screening test is lacking, which limits the identification of novel *O*-glycosylation disorders. The emergence of next generation sequencing (NGS) and *O*-glycoproteomics technologies have changed this situation, resulting in significant progress to link disease phenotypes with underlying biochemical mechanisms. Here, we review the current knowledge on *O*-glycosylation disorders, and discuss the biochemical lessons that we can learn on 1) novel glycosyltransferases and metabolic pathways, 2) tissue-specific *O*-glycosylation mechanisms, 3) *O*-glycosylation targets and 4) structure-function relationships. Additionally, we provide an outlook on how genetic disorders, *O*-glycoproteomics and biochemical methods can be combined to answer fundamental questions regarding *O*-glycan synthesis, structure and function.

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Introduction

Glycosylation, the addition of carbohydrate chains to proteins, is the most common post-translational and co-translational modification. It is initiated by the cytosolic synthesis of activated sugars (with the exception of CMP-*N*-acetylneuraminic acid) that are subsequently transported to the endoplasmic reticulum (ER) and Golgi apparatus, where glycans are assembled and modified on proteins. Glycosylation affects many aspects of protein function, including protein folding, enzyme activity and cell-to-cell and cell-to-extracellular matrix (ECM) interactions. Therefore, it is not surprising that glycosylation disorders present with a broad range of clinical phenotypes.

Currently, over 100 different Congenital Disorders of Glycosylation (CDG) have been described [1,2], the majority affecting the *N*-glycosylation pathway. Broad availability of an adequate screening assay for abnormal *N*-glycosylation, isofocusing of serum transferrin (TIEF), has resulted in the identification of defects in glycosyltransferases, nucleotide sugar transporters and enzymes involved in sugar metabolism, which are all directly linked to glycosylation. In recent years, more complex mechanisms have been identified underlying abnormal *N*-glycosylation related to Golgi trafficking, homeostasis and vesicular transport [3•,4,5].

In contrast to *N*-glycosylation defects, the identification of *O*-glycosylation disorders is much more challenging. In humans, *O*-glycans are initiated by seven different monosaccharides that can be further extended to complex *O*-glycan structures. For mucin *O*-glycosylation (O-linked *N*-acetylgalactosamine, *O*-GalNAc), the most common form of *O*-glycosylation, over 20 polypeptide GalNAc transferases are known with tissue and substrate-specific activities [6,7]. Isofocusing of ApoC-III was developed to detect defects in mucin type *O*-glycosylation [8]. Although many of the Golgi homeostasis disorders showed abnormal mucin type *O*-glycosylation of ApoC-III, only mutations in polypeptide GalNAc transferase 2 (*GALNT2)* could be detected with this test. So far, the complexity of *O*-glycan structures renders it impossible to design a single screening test for diagnostics of *O*-glycosylation disorders.

O-glycans are important for protein structure, folding, stability, recognition, expression, and processing, and they are known to modulate enzyme activity [9,10,11•,12–15]. Furthermore, highly negatively charged *O*-mucin glycans can bind water, forming protective layers and preventing bacterial adhesion [16]. The function of an *O*-glycan can be tissue, protein, and site-specific, alongside mediating different functions throughout development [17]. That, *O*-glycans play not only

important, but also complex roles, is illustrated by the vast amount of *O*-glycan enzymes that upon knockout, caused embryonic lethality or tissue-specific phenotypes in mice [18,19•]. Mice knockout systems have provided invaluable lessons about *O*-glycan function, for example, the role of *O*-fucosylation of thrombospondin type 1 repeats (TSRs) by POFUT2 in epithelial organization and expression of signaling factors during gastrulation [20].

In humans, a more complete understanding of the human *O*-glycosylation machinery can be accomplished by studying genetic defects in *O*-glycosylation. Identification of an increasing number of genetic *O*-glycosylation disorders has been facilitated by the emergence of next generation sequencing (NGS) [2]. Furthermore, recent developments in glycopeptide analysis revealed previously unidentified *O*-glycosylation enzymes and their targets, which can be linked to disease. 3D structural models of human glycosyltransferases are rare, especially since these types of proteins are embedded in the membrane of the ER and Golgi apparatus making crystallization extremely daunting. However, in recent years, some structures have been resolved and modeled. Taken together, new opportunities arise to link findings from genetic disease with fundamental research to increase our understanding of the mechanisms of *O*-glycosylation. In this review, we illustrate the importance of inherited *O*-glycosylation disorders to elucidate the structural aspects of the *O*-glycosylation machinery (Figure 1). Glycosaminoglycan biosynthesis disorders are not discussed and have been described in great detail by others [21]. For elaborate descriptions of *O*-glycosylation disorder phenotypes, we recommend the reviews of Wopereis *et al.* [22], Hennet [21] and Jaecken and Péanne [3•].

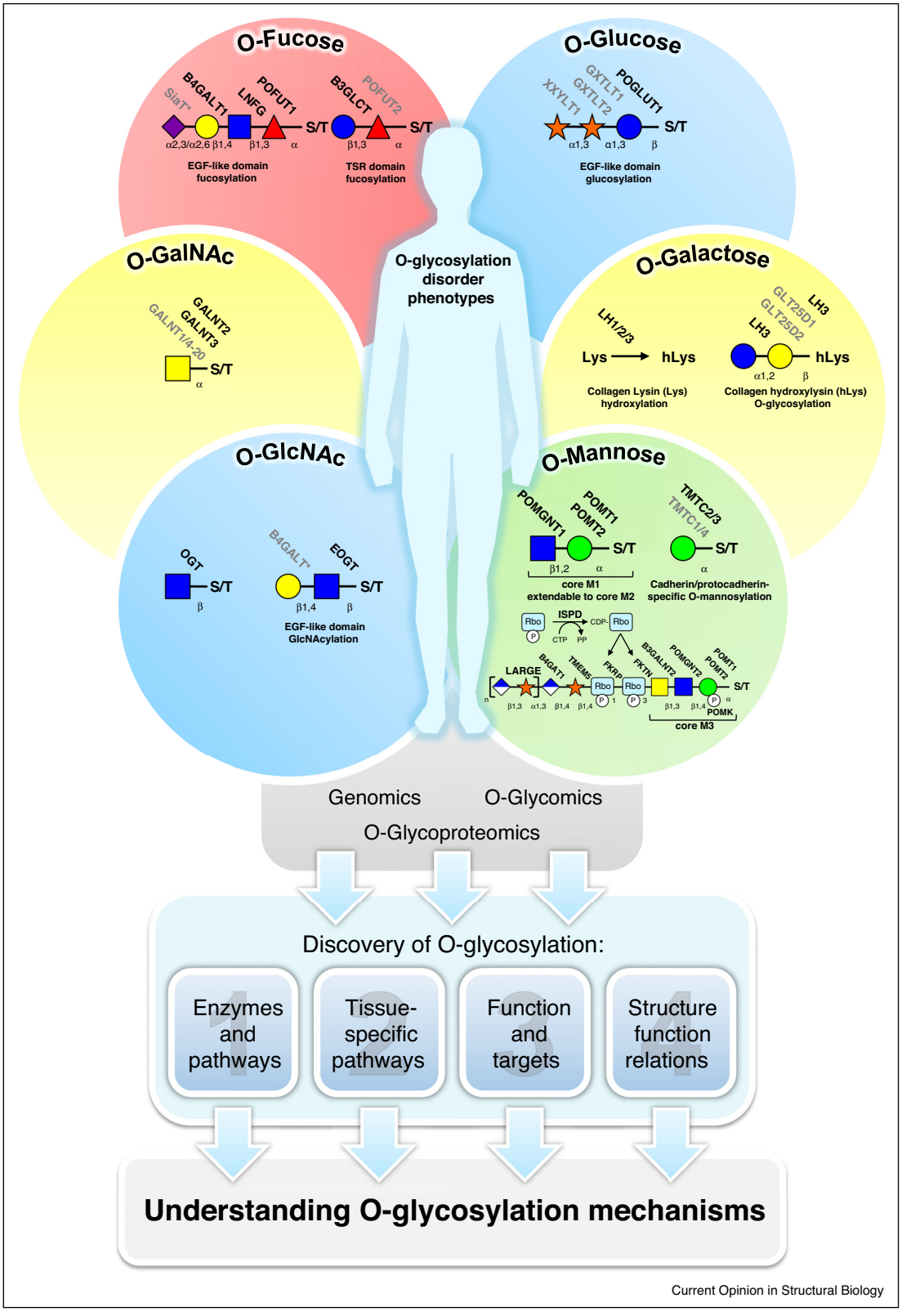
O-glycosylation disorders: current status and screening methods

Most of the currently known *O*-glycosylation disorders have been identified through genetic techniques. The clinical phenotypes are highly variable, which is linked to the large number of different *O*-glycan types. *O*-glycosylation defects have now been identified for each type of *O*-glycan, and an overview of the known *O*-glycosylation disorders is provided in Figure 1 and Table 1. Thus far, assays for functional validation of mutations are largely lacking, except for the dystroglycanopathies. This is a group of disorders affecting the *O*-mannosyl glycan on the α-dystroglycan (αDG) protein that is essential for binding to extracellular matrix components (Table 1; *O*-mannose). Functional confirmation of *O*-mannosylation defects is possible by histochemical detection of the *O*-mannosyl glycan of αDG in muscle biopsies [23]. Together with NGS of patients, this has resulted in the identification of novel Golgi glycosyltransferases, while mass spectrometry of recombinant αDG has recently resolved the complete *O*-mannose glycan structure [24,25•,26•].

Thus, together with NGS, functional tests are highly warranted for a more rapid identification of inherited *O*-glycosylation disorders, and to increase our understanding of *O*-glycosylation mechanisms. *O*-glycomics, the profiling of the complete set of glycans produced by specific cell types, offers potential as a generic functional test. Methods have been developed for the comparative analysis of *O*-glycans from complex samples [27–30]. Unfortunately, *O*-glycomics has thus far not contributed to the functional confirmation of *O*-glycosylation disorders. This can be explained by the fact that *O*-glycosylation is highly dependent on the specific attachment site, and *O*-glycans do not have a general consensus sequence with the exception of *O*-fucose glycans ($C^2X_{3\text{-}5}S/TC^3$ and $WX_5CX_{2/3}S/TCX_2G$; C = conserved cysteines of epidermal growth factor (EGF)-like or TSRs, S/T = serine or threonine, X = any residue) and *O*-glucose glycans (C^1XSXPC^2). Therefore, it is essential to study *O*-glycan structures in their protein context. Identification of aberrant *O*-glycopeptides by direct LC–MS/MS analysis of intact *O*-glycopeptides in patient samples or model systems would be preferred, thus providing a complete overview of the affected *O*-glycans and *O*-glycosylation sites. Despite the challenges in the field of *O*-glycoproteomics (reviewed in Ref. [31]), first studies have demonstrated the potential of LC–MS/MS for holistic *O*-glycopeptide profiling. In 2016, Hoffmann *et al.* [32••] analyzed intact *O*-glycopeptides in human blood plasma in an untargeted fashion by analyzing HILIC enriched and fractionated glycopeptides by reversed phase LC–MS/MS using multistage collision induced dissociation (CID) and electron transfer dissociation (ETD) fragmentation experiments. In total, 31 *O*-GalNAc sites and regions from 22 proteins were identified, which included 11 novel *O*-glycosylation sites and regions. More recently, King *et al.* [33••] performed high collision energy dissociation (HCD) and ETD LC–MS/MS analysis of de-sialylated glycopeptides purified by lectin chromatography from AB RhD-positive platelets and blood plasma. Their analysis detected 1123 *O*-GalNAc sites from 649 glycoproteins, which not only provided novel biological insights but above all demonstrated the feasibility of holistic *O*-glycoproteomics.

Although functional tests still need to be developed, the *O*-glycosylation disorders that have been identified have aided structural biology in a number of ways. Despite the fact that *O*-glycan disorders are very heterogeneous, patients generally show tissue-specific phenotypes, hinting toward tissue-specific *O*-glycan targets and function. Studying *O*-glycosylation disorders has 1) led to the discovery of new glycosyltransferases and metabolic pathways, 2) provided insight in tissue-specific glycosylation pathways, 3) aided in the discovery of *O*-glycosylation targets and 4) elucidated structure-function relations of *O*-glycosyltransferases and nucleotidyltransferases (Figure 1). Below, we will provide recent examples of

Figure 1



Characterization of *O*-glycosylation disorders is indispensable to accomplish a better understanding of the human *O*-glycosylation mechanisms. Phenotypic heterogeneity of the *O*-glycosylation disorders reflects the high diversity of *O*-glycan structures with a high tissue-specificity. Phenotypic characterization and modern omics techniques such as genomics, glycomics, and glycoproteomics complement each other in the

each type of discovery in the *O*-glycosylation field, covering the majority of the *O*-glycosylation disorder core types.

Discovery of new glycosyltransferases and metabolic pathways

Firstly, genetic defects in *O*-glycosylation with a characteristic phenotype have aided the discovery of new *O*-glycosylation gene candidates. For example, NGS has resulted in the identification of new genes causing dystroglycanopathy that is characterized by muscular dystrophy and, in severely affected individuals, eye and brain abnormalities. Dystroglycanopathies are caused by defective *O*-mannosylation of αDG, leading to aberrant cell-to-ECM connections. Genetic analysis of patients with dystroglycanopathy features has revealed mutations in *ISPD (CRPPA)*, *FKTN* and *TMEM5(RXYLT1)* [34–37] (Table 1; *O*-mannose). The function of these proteins has been elucidated the last three years [24,26•,38,39•,40]. Identification of ISPD as a cytosolic cytidyltransferase even led to the discovery of a new mammalian nucleotide sugar: CDP-ribitol [38]. Soon after, FKTN and FKRP were identified as ribitol 5-phosphate (Rbo5P) glycosyltransferases, and Rbo5P moieties were detected on the functional *O*-mannosyl glycan of αDG [24,26•,39•]. TMEM5 was identified as a β1,4-xylosyltransferase, adding xylose onto the second Rbo5P of unique *O*-mannosyl glycans on αDG [24,40]. Subsequently, mass spectrometry of genetically engineered αDG led to the discovery of glycerol 3-phosphate (Gro3P) on the glycan, indicating the existence of a CDP-glycerol biosynthesis pathway [25•]. This was further supported by the finding that FKRP and FKTN can use CDP-glycerol as substrates for glycosylation [41•]. If CDP-glycerol and Gro3P have a regulatory role in *O*-mannosyl glycan extension remains to be investigated [25•]. An interesting observation is that these findings on αDG show high overlap with the wall teichoic acids in gram-positive bacteria, that contain repeating units of Rbo5P and Gro3P [42]. The *O*-mannosylation disorders are a classical example of how we learn about novel mechanisms and even completely new human metabolic pathways, initiated by genetic screening of patients with distinct *O*-glycosylation disorder phenotypes.

O-glycosylation disorders can provide insight in tissue-specific pathways

Secondly, the phenotypes associated with *O*-glycosylation disorders can provide important insights about tissue-specific glycosylation mechanisms. This is nicely illustrated by *POFUT1* (Table 1; *O*-fucose) and *POGLUT1* (Table 1: *O*-glucose) deficiency, both resulting in Dowling-Degos disease. The shared phenotype, characterized by reticular pigmentation of the skin [43,44], indicates a similar underlying pathomechanism. Indeed, both enzymes are involved in the regulation of Notch signaling, however, by the addition of different glycan types. POFUT1 stabilizes EGF-like repeats by the addition of *O*-fucose glycans, and POGLUT1 through the addition of *O*-glucose glycans [11•]. Interestingly, POGLUT1 also shows *O*-xylosyltransferase activity, but the function of *O*-xylose on EGF-like domains remains to be investigated [45,46•]. The *O*-fucose glycan initiated by POFUT1 is extended with *N*-acetylglucosamine (GlcNAc) by LNFG [reviewed in Ref. 47]. Interestingly, *LNFG* patients present with a completely different phenotype of vertebral malsegmentation, spondylocostal dysostosis (SDO) [48,49]. This phenotype is also associated with defects in Notch signaling, and other types of SDO are all caused by defects in proteins involved in Notch1 signaling [50]. Another recent article reports that the *POGLUT1* D233E mutation causes muscular dystrophy [51]. Investigating other POGLUT1 targets could shed light on the mechanisms underlying the different phenotypes. The different phenotypes for *POFUT1* and *POGLUT1* deficiency provide opportunities to investigate tissue-specific targets and *O*-glycosylation mechanisms.

Patient phenotypes aid in the identification of *O*-glycosylation targets

The phenotype of some *O*-glycosylation deficiencies resembles the phenotype of genetic defects in potential target proteins. Hence, phenotypic characterizations can point to potential glycosylation targets. For instance, mutations in *FGF23* cause familial tumoral calcinosis paired with increased re-adsorption of phosphate by the renal proximal tubule. Interestingly, patients carrying mutations in the polypeptide GalNAc transferase *GALNT3* present with exactly the same phenotype [52–57] (Table 1: *O*-GalNAc), suggesting a shared mechanism of disease. Indeed, Kato *et al.* [13] demonstrated that the phosphatonin FGF23 is *O*-glycosylated at Thr^{178} by GALNT3, preventing the furin protease cleavage of FGF23 and regulating phosphate re-absorption [13]. There are over 20 polypeptide GalNAc transferases. Some share substrate specificities and have overlapping expression in different tissues [6,7]. Nevertheless, *O*-GalNAc glycosylation of FGF23 seems a non-redundant function of GALNT3 [13]. Despite the large number of GALNTs, only *GALNT3* and *GALNT2* deficiency have been reported. Khetarpal *et al.* showed that loss of function of *GALNT2* lowered high-density lipoprotein

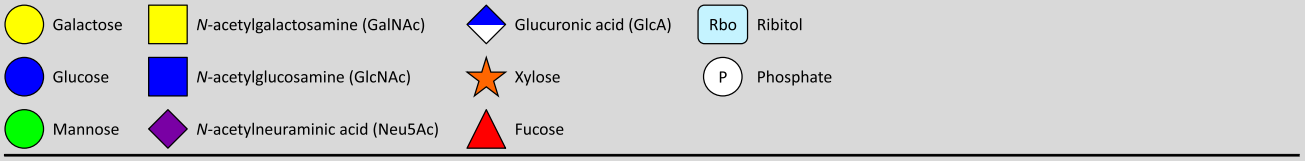
(Figure 1 Legend Continued) discovery of (tissue-specific) *O*-glycosylation transferases, pathways, targets and *O*-glycan function. Enzymes causing known *O*-glycan disorders are in black. Enzymes in grey have not been associated with an *O*-glycosylation disorder. *Hypothesized enzyme or multiple possible glycosyltransferases. GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; Rbo = ribitol; P = phosphate. A legend for the glycan symbols is presented in Table 1.

cholesterol (HDL-C) levels in human, mice, rats and cynomolgus monkeys. GALNT2 exhibited species-specific glycosylation targets, including PLTP, a regulator of HDL metabolism in plasma [58•]. PLTP activity was altered by absence of GALNT2 *O*-GalNAc modifications, explaining the findings in *GALNT2* patients. The involvement of additional GALNT2 targets in the disease phenotype remains to be investigated.

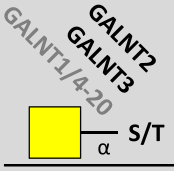
Likewise, mutations in OGT (*O*-GlcNAc-transferase) and HCF1 (host cell factor 1, a transcriptional regulator of the cell cycle) (cause similar intellectual disability (ID) phenotypes [59•,60•,61,62•] (Table 1: *O*-GlcNAc). OGT is a unique *O*-GlcNAc transferase that modifies nucleocytoplasmic proteins, a process that can be reversed by OGA (*O*-GlcNAcase) [63–65]. All five patient mutations that have been described so far reside in the N-terminal tetratricopeptide (TPR) repeats of OGT, which are involved in the substrate recognition and specificity of OGT [66]. *OGT* patient-derived cells and model cell lines with patient mutations showed normal *O*-GlcNAcylation [59•,60•,62•]. This homeostasis was suggested to be maintained by a reduced expression of *OGA* [59•,60•] or by temporal dynamics in *O*-GlcNAcylation kinetics [62•]. In addition, OGT is involved in proteolytic maturation of HCF1 [14,67], and it has been suggested that the

Table 1

List of reported congenital *O*-glycosylation disorders with their associated OMIM numbers, phenotypes and method of diagnostics. Affected glycan structures on serine or threonine (S/T) residues are given for each enzyme deficiency. Enzymes in black: reported *O*-glycan defects. Enzymes in grey: no reported patients. *Hypothesized enzyme or multiple possible glycosyltransferases. **Putative *O*-glycosylation disorders. LGMD = Limb-Girdle Muscular Dystrophy; MEB = Muscle Eye Brain disease; WWS = Walker-Warburg Syndrome; ID = Intellectual Disability. This table does not include the glycosaminoglycan biosynthesis disorders, or disorders known to affect multiple glycosylation pathways, including *O*-glycosylation

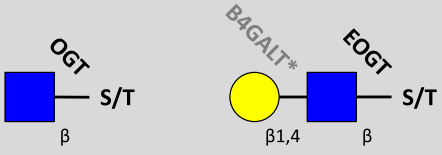


O-GalNAc



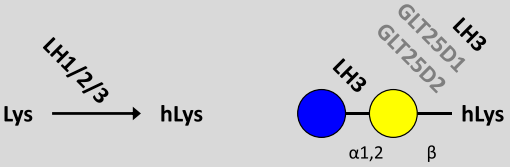
Gene	Function	OMIM	Disease	Tissue	Current diagnostics
GALNT2	Polypeptide GalNAc transferase	602274	Reduced high-density lipoprotein cholesterol	Brain	Genetics, ApoC-III IEF
GALNT3	Polypeptide GalNAc transferase	601756	Familial tumoral calcinosis	Subcutaneous tissues, skin, bone	Genetics

O-GlcNAc



Gene	Function	OMIM	Disease	Tissue	Current diagnostics
OGT	*O*-GlcNAc transferase proteolytic processing	300255	X-linked ID	Brain, skeleton, heart, face, genitalia, eye	Genetics
EOGT	EGF-domain-specific *O*-GlcNAc transferase	614789	Adams-Oliver syndrome	Skin, skeleton	Genetics

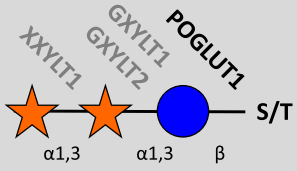
O-Galactose



Collagen Lysin (Lys) hydroxylation | **Collagen hydroxylysin (hLys) O-glycosylation**

Gene	Function	OMIM	Disease	Tissue	Current diagnostics
PLOD1	Helical lysyl hydroxylase (LH1)	153454	Ehlers-Danlos syndrome, kyphoscoliotic type, 1	Skeleton, skin, muscle	Lysyl-protocollagen hydroxylase activity in skin fibroblasts, genetics
PLOD2	Telopeptide lysyl hydroxylase (LH2)	601865	Bruck-syndrome 2	Skeleton, skin	Genetics
PLOD3	Lysyl hydroxylase/ hydroxylysyl		galactosyltransferase/ galactosylhydroxylysyl glucosyltransferase (LH3)	603066	Lysyl hydroxylase 3 deficiency
Skeleton, skin, eye, cochlea or auditory nerve	Glucosyltransferase activity in serum or lymphoblastoid cells, genetics				

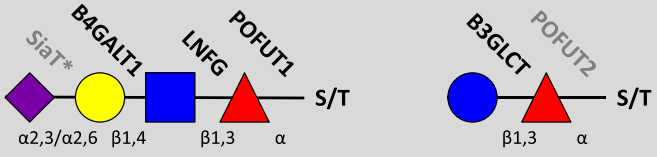
O-Glucose



EGF-like domain glucosylation

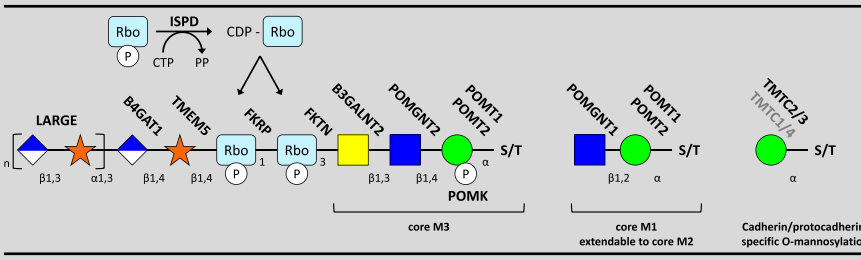
Gene	Function	OMIM	Disease	Tissue	Current diagnostics
POGLUT1	*O*-glucosyltransferase	615618	1) Dowling-Degos disease 4 2) Muscular dystrophy	1) Skin 2) Muscle	Genetics

O-Fucose



EGF-like domain fucosylation | **TSR domain fucosylation**

Gene	Function	OMIM	Disease	Tissue	Current diagnostics
POFUT1	*O*-fucosyltransferase	607491	Dowling-Degos disease 2	Skin	Genetics
LFNG	*O*-fucose β-1,3-GlcNAc transferase	602576	Spondylocostal Dysostosis 3	Axial skeleton	Genetics, enzyme activity assay
B3GLCT	*(B3GALTL)*	*O*-fucose β-1,3-glucose transferase	610308	Peter's Plus Syndrome	Eye, face, skeleton, lips
Genetics, ELISA of	properdin serum levels				



Gene	Function	OMIM	Disease	Tissue	Current diagnostics
ISPD (CRPPA)	CDP-ribitol synthase	614631	WWS, MEB, LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
POMT1	Protein *O*-mannosyltransferase	607423	WWS, MEB,LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy, *O*-mannosyltransferase activity assay
POMT2	Protein *O*-mannosyltransferase	607439	WWS, MEB, LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
POMK (SGK196)	Protein *O*-mannosyl kinase	615247	WWS, MEB, LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
POMGNT2 (GTDC2)	*O*-mannose β-1,4-GlcNAc transferase	614828	WWS	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
B3GALNT2	β-GlcNAc β-1,3-GalNAc transferase	610194	WWS, MEB, ID	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
FKTN	β-GalNAc-3 Rbo5P transferase	607440	WWS, MEB, LGMD, FCMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
FKRP	Rbo5P-1 Rbo5P transferase	606596	WWS, MEB, LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
TMEM5(RXYLT1)	Rbo5P β-1,4-xylose transferase	605862	WWS, MEB	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
B4GAT1	xylose β-1,4-GlcA transferase	605517	WWS	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
LARGE	α-xylose β-1,3-GlcA transferase β-GlcA α1,3-Xyl transferase	603590	WWS, MEB, LGMD	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy
POMGNT1	*O*-mannose β-1,2-GlcNAc transferase	606822	WWS, MEB, LGMD, nonsyndromic retinitis pigmentosa	Muscle, brain, eye, heart	Genetics, IIH6/VIA4-I on muscle biopsy, *O*-Man β-1,2 GlcNAc-transferase activity assay
*TMTC3***	Putative *O*-mannosyltransferase	617218	Cobblestone lissencephaly Periventricular nodular heterotopia	Brain, minimal muscle and eye involvement	Genetics
*TMTC2***	Putative *O*-mannosyltransferase	615856	Sensorineural hearing loss	Cochlea or auditory nerve	Genetics

X-linked ID in some *OGT* patients is linked to insufficient activated HCF1 [60•]. Taken together, it is plausible that ID genes are regulated or glycosylated by OGT, and this should be addressed in further studies for a better understanding of the disease mechanisms.

For a long time, POMT1 and POMT2 were believed to be the only human *O*-mannosyltransferases. In 2017, glycoproteomics in HEK293 knock-out cells revealed that the *O*-mannosylation of cadherins and protocadherins is independent of these two enzymes [68]. Using a similar approach, four new *O*-mannosyltransferases were identified. TMTC1-4, which specifically glycosylate cadherins and protocadherins, and thus have different targets than the POMT1/POMT2 glycosyltransferases (Table 1: *O*-mannose) [68,69••]. Interestingly, patients with *TMTC2* and *TMTC3* mutations have very different phenotypes. *TMTC3* mutations are associated with lissencephaly (6 families, 9 patients) and periventricular nodular heterotopia with ID and epilepsy (three siblings) [70,71]. Both phenotypes are associated with deficient neuronal migration. *TMTC2* deficiency is associated with sensorineural hearing loss [72,73], suggesting that the TMTCs have different, tissue-specific targets. Mutations in *Cadherin-23* and *Protocadherin-15* cause Usher syndrome, which is characterized by deafness and blindness, and can cause non-syndromic recessive hearing loss [74–76]. Hence, it is tempting to speculate that TMTC2 is involved in the *O*-mannosylation of these proteins. However, direct demonstration of enzyme activity of the TMTCs is still lacking and whether the *TMTC3* and *TMTC2* disease phenotypes are directly related to

hypomannosylation of cadherins, protocadherins or other proteins remains to be investigated.

Finally, *B3GLCT* deficiency leads to Peter's Plus syndrome, a severe disorder characterized by anterior eye chamber defects (Table 1: *O*-fucose). B3GLCT attaches glucose via a β-1,3 linkage to *O*-fucose (synthesized by POFUT2) on TSRs of proteins. In search for B3GLCT targets linked to the eye defects, Dubail *et al.* [77•] found that *ADAMTS9* haploinsufficient mice showed a similar eye phenotype [77•]. Glycosylation with glucose-β-1,3-fucose by POFUT2 and B3GLCT ensures proper secretion of ADAMTS9 during development. Taken together, the identification of new genetic *O*-glycosylation disorders can provide important insights about the targets and functions of specific *O*-glycans.

Modeling mutations to study structure-function relations of *O*-glycosylation proteins

In the last few years, crystal structures have been solved of enzymes related to *O*-glycosylation disorders, for example of OGT [78], POMK [79], POMGNT1 [80] and ISPD [38]. Known disease-causing mutations can be modeled in 3D structures, helping to understand the function of specific enzymatic domains and with it, underlying disease mechanisms. For example, the crystal structure of ISPD revealed a N-terminal cytidyltransferase domain and a C-terminal domain connected via a linker helix [38]. Surprisingly, the C-terminal domain did not share homology with any known enzyme domains. No missense mutations have been reported in the C-terminal domain, but the c.1114_1116del (p.Val372del) mutation is reported for five patients. The absence of the Val residue leads to relatively mild phenotype (LGMD) compared to larger deletions like a deletion of exon 6–8 or 9–10 (WWS). Taken together, this demonstrates that the C-terminal domain is important for ISPD function, either contributing to the stability of the enzyme, or having a enzymatic function on its own [38], a question that so far remains unanswered. For *POMGNT1*, one study has reported a correlation between mutations closer to the 5′ end of the gene with more severe hydrocephalus than mutations near the 3′ end. However, correlations with enzymatic activity or structure have not been established yet [81]. Taken together, much work remains to elucidate the 3D structure of many *O*-glycosylation enzymes. However, if such models are accomplished, structure-function relationships can be studied utilizing described *O*-glycosylation patient mutations. Additionally, this will lead to a better understanding of disease mechanisms, and will hopefully be accompanied by the emergence of new treatment opportunities.

Conclusions

We illustrated that studying the complex phenotypes of *O*-glycosylation disorders has enabled the elucidation of *O*-glycosylation proteins, targets, and *O*-glycan structure and function. Nevertheless, many questions remain to be answered about the *O*-glycosylation machinery. Although we know in many diseases which *O*-glycan core structure is affected, for most, their exact attachment site and tissue-specific protein targets remain to be elucidated. In the future, the development of more advanced *O*-glycopeptide profiling methods is essential to facilitate these discoveries. Ideally, untargeted *O*-glycoproteomics LC–MS/MS technology will evolve to enable robust high-throughput analysis for the in-depth characterization of intact *O*-glycopeptides in biological samples. The screening of patient groups with similar clinical presentations or with different genetic *O*-glycosylation defects (e.g. in different *GALNTs*) with genomics and *O*-glycoproteomics will lead to the discovery of glycosylation genes and tissue-specific targets, respectively. As illustrated in this review, comparing the phenotypes of other known disorders to the phenotype of *O*-glycosylation disorders can hint to the respective targets.

So far, most *O*-glycosylation defects that have been identified affect the core sugar of *O*-glycans. In the last few years, NGS has been applied more frequently, and probably will lead to the identification of additional disorders that affect more distal monosaccharides on *O*-glycan structures. Functional validation of these disorders will require developments in the glycoproteomics field, since large scale in-depth characterization of the exact glycan structure of intact glycopeptides is still challenging. Furthermore, it is important to develop *in-silico* approaches to identify differential *O*-glycopeptides and interpret complex glycobiology by novel bioinformatic approaches. Combined analysis of *O*-glycopeptide data and patient meta data by machine learning is of particular interest to associate protein specific *O*-glycosylation changes to the physiopathology of *O*-glycosylation disorders. Taken together, understanding the disease mechanisms of the *O*-glycosylation disorders will contribute to our understanding of *O*-glycosylation mechanisms, while vice versa, new mechanistic insights are highly warranted to develop new therapeutic strategies.

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Conflict of interest statement

Nothing declared.

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