The dystrophin glycoprotein complex, which connects the cell membrane to the basement membrane, is essential for a variety of biological events, including maintenance of muscle integrity. An O-mannose-type GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man structure of α-dystroglycan (α-DG), a subunit of the complex that is anchored to the cell membrane, interacts directly with laminin in the basement membrane. Reduced glycosylation of α-DG is linked to some types of inherited muscular dystrophy; consistent with this relationship, many disease-related mutations have been detected in genes involved in O-mannosyl glycan synthesis. Defects in protein O-linked mannos-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1), a glycosyltransferase that participates in the formation of GlcNAc-β1,2-Man glycan, are causally related to muscle-eye-brain disease (MEB), a congenital muscular dystrophy, although the role of POMGnT1 in postphosphorylation of GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man glycan remains elusive. Our crystal structures of POMGnT1 agreed with our previous results showing that the catalytic domain recognizes substrate O-mannosylated proteins via hydrophobic interactions with little sequence specificity. Unexpectedly, we found that the stem domain recognizes the β-linked GlcNAc of O-mannosyl glycan, an enzymatic product of POMGnT1. This interaction may recruit POMGnT1 to a specific site of α-DG to promote GlcNAc-β1,2-Man clustering and also may recruit other enzymes that interact with POMGnT1, e.g., fukutin, which is required for further modification of the GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man glycan. On the basis of our findings, we propose a mechanism for the deficiency in postphosphorylation modification of the glycan observed in POMGnT1-KO mice and MEB patients.

α-dystroglycanopathy | muscular dystrophy | glycosyltransferase | lectin | O-mannosyl glycan
Golgi by the addition of unidentified saccharide(s), and finally its terminus is modified by LARGE, which catalyzes the extension of a xylosyl–glucuronol polylaccharide chain (β1,3-Xyl-α,1,3-GlcA, Xyl–GlcA repeat) (6). The synthesized polylaccharide chain of core M3 on α-DG is required for its interaction with laminin and is known as the “IIIH6 epitope” (7). Recently, we identified that fukutin (FKTN) and fukutin-related protein (FKRP) are ribitol 5-phosphate transferases that form the tandem ribitol 5-phosphate structure on the 3-position of the GalNAc residue of the phosphorylated core M3 (8). FKTN, FKRP, TMEM5, and B4GAT1, all of which have been linked to CMD, are thought to be involved in postphosphorylation modification. Although POMGnT1 does not catalyze any reactions involved in the formation or further modification of core M3 to form structure in the Golgi. Together, these findings suggest that the POMGnT1–FKTN complex is important for postphosphorylation modifications of core M3 to form a platform that requires further glycosylation of the Xyl–GlcA repeat by LARGE.

Results

Overall Structure of POMGnT1. A soluble form of POMGnT1 (residues 92–660) was expressed in HEK293T cells, purified, and crystallized in two distinct crystal forms under different conditions (SI Appendix, Table S1). Crystallographic phases were determined by the iodide–single wavelength anomalous dispersion (SAD) method using an NaI-soaked P44222 crystal. The P44222 crystal contains one molecule in each asymmetrical unit, whereas the C2221 crystal contains three molecules in each asymmetrical unit, and the molecular structures obtained from the two crystal forms are nearly identical, with an rmsd value of ~0.5 Å. Because the resolution of the C2221 crystal structure was higher than that of the P44222 crystal structure, here we describe the POMGnT1 structure by referring to chain A of the C2221 crystal as the apo form structure (SI Appendix, Table S1).

Soluble POMGnT1 consists of a stem domain, a catalytic domain, and an extended linker region connecting the two domains (Fig. 1A). The stem domain (residues 92–250) is composed of two stacked β-sheets and two α-helices. The fold resembles that of murine FAM3B (also known as PANDER (pancreatic-derived factor; PDB ID code 2YQQ) (12)), whose amino acid sequence is 20.4% identical to that of the stem domain (SI Appendix, Figs. S2A and S3). Although FAM3B/PANDER has been shown to be involved in the regulation of glucose homeostasis, the molecular function of the protein remains elusive (13). The rmsd value of Ca atoms between the two structures, determined using DaliLite (14), is 1.7 Å. The T176P, S198R, and E223K mutations in POMGnT1 are present in congenital muscular dystrophy–dystroglycanopathy with brain and eye anomalies A3 (MDDGA3) (15–18). The Ser198 and Glu223 residues are located on the surface of the stem domain, and their side chains interact with each other via hydrogen bonds. Mutations at Ser198 or Glu223 are likely to disrupt these hydrogen bonds and destabilize the protein structure, thereby contributing to MDDGA3 pathology.

The catalytic domain comprises residues 300–646, and the region after Pro646 is disordered in the crystal (Fig. 1A and B). The catalytic domain is composed of two structural motifs, a Rossmann-like fold (the N-lobe, residues 300–299), and the N- (residues 300–327) and C-lobes (residues 499–646) of the catalytic domain are shown in green, yellow, cyan, and blue, respectively. (B) Cartoon representation of POMGnT1 (residues 97–646) with Mn2⁺–UDP and mannosylated peptide. Mn2⁺ and UDP are represented by sphere and stick models, respectively. The two mannosylated peptides are represented by stick models. The first mannosylated peptide is at the catalytic domain, and the second is at the stem domain. The neighboring POMGnT1 molecule is placed on the left side, but its stem domain could not be modeled because of the obscure electron density. (C) Interaction between mannosylated peptide (deep blue) and catalytic domain. Sunburst icons indicate hydrophobic interactions. (D) Interaction between mannosylated peptide (orange) and stem domain (green). Yellow dotted lines indicate ionic interactions or hydrogen bonds.

Fig. 1. Crystal structure of POMGnT1. (A) Cartoon representation of POMGnT1 (residues 97–646) in the apo form. Stem domain (residues 97–250), linker region (residues 251–299), and the N- (residues 300–498) and C-lobes (residues 499–646) of the catalytic domain are shown in green, yellow, cyan, and blue, respectively. (B) Cartoon representation of POMGnT1 (residues 97–646) with Mn2⁺–UDP and mannosylated peptide. Mn2⁺ and UDP are represented by sphere and stick models, respectively. The two mannosylated peptides are represented by stick models. The first mannosylated peptide is at the catalytic domain, and the second is at the stem domain. The neighboring POMGnT1 molecule is placed on the left side, but its stem domain could not be modeled because of the obscure electron density. (C) Interaction between mannosylated peptide (deep blue) and catalytic domain. Sunburst icons indicate hydrophobic interactions. (D) Interaction between mannosylated peptide (orange) and stem domain (green). Yellow dotted lines indicate ionic interactions or hydrogen bonds.
these mutations should affect the protein stabilization or folding and may induce POMGnT1 trapping by ER quality control, resulting in retention of the protein in the ER.

**Mannosylated Peptide Recognition.** Although the flexible loop (residues 505–518) is completely disordered in the apo form structure, it is structured to accommodate a nucleotide sugar in the Mn$^{2+}$, UDP, and mannosylated peptide complex forms (Fig. 1 B and C). The donor substrate-dependent ordering is also observed in the rabbit Gnt I complex structure with UDP-GlcNac or UDP-GlcNAc derivative (19). In the UDP-GlcNAc complex structure of Gnt I (PDB ID code 1FOA), UDP-GlcNac interacts with Arg117, Asp144, His194, Asp212, the main chain of Val321, and Ser322 via ionic interactions or hydrogen bonds. In addition, the 2-aceatoamido methyl group of the GlcNAc moiety interacts with a small hydrophobic pocket formed by Leu269 and Leu311. The interactions with UDP are also observed in the Mn$^{2+}$, UDP, and mannosylated peptide complex of POMGnT1. Thus, these residues are conserved structurally in POMGnT1, except for Glu394 and Asn507 (SI Appendix, Table S2). Glu394, corresponding to the x residue in the DxD motif of the glycosyltransferase, interacts with the O2′ atom of UDP, whereas Asp212 of Gnt I, which corresponds to Glu394 of POMGnT1, interacts with the O2′ and O3′ atoms of UDP-GlcNac (23). Furthermore, it has been proposed that Asp291 is the catalytic base of Gnt I (19). Asp476 of POMGnT1 corresponding to Asp291 of Gnt I is also structurally conserved. Thus, Gnt I and POMGnT1 share a common sugar-transfer mechanism of UDP-GlcNAc, as suggested from the study of Gnt I (19).

In the mannosylated peptide complex, the mannose moiety and five residues of a mannosylated nonapeptide [Ac–AAPT(–GlcNAc, as suggested from the study of GnT I (19)] could be modeled near the UDP-binding site (Fig. 1 B and C). The peptide-binding site consists of both the N- and C-lobes, including the flexible loop. Residues from Trp473 to Glu506 (in the N-lobe), from Asn507 to Phe512 (in the C-lobe), and from Trp600 to Arg605 (also in the C-lobe) contribute to the formation of the peptide-binding site. Asp474, Met477, Met481, Asn507, Phe512, and Trp600 engage in hydrophobic interactions with the peptide moiety. In addition, although the asymmetrical unit contains two molecules, the electron density of the stem domain in chain B was obscure, because this region does not interact with other molecules in the crystal structure. This lack of interaction also suggests the linker region is flexible.

Because Asn507 and Phe512 are disordered in the apo state, molecular modeling might facilitate substrate or binding, as suggested by Ushikoshi and Rini (23). On the other hand, the mannose moiety interacts with Arg605 in the C-lobe. The catalytic domain of POMGnT1 recognizes substrate peptide residues via hydrophobic interactions, and there are neither hydrogen bonds nor charged interactions between the peptide and the catalytic domain. The observation that mutant proteins of POMGnT1 (D474A, M481A, Trp473A, Trp473M, and W473/M477A, transfected with the indicated mutant POMGnT1. POMGnT1-deficient cells; WT, transfected with WT POMGnT1; R129A, Glu394, corresponding to the x residue in the DxD motif of the glycosyltransferase, interacts with the O2′ atom of UDP, whereas Asp212 of Gnt I, which corresponds to Glu394 of POMGnT1, interacts with the O2′ and O3′ atoms of UDP-GlcNac (23). Furthermore, it has been proposed that Asp291 is the catalytic base of Gnt I (19). Asp476 of POMGnT1 corresponding to Asp291 of Gnt I is also structurally conserved. Thus, Gnt I and POMGnT1 share a common sugar-transfer mechanism of UDP-GlcNAc, as suggested from the study of Gnt I (19).

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Another mannosylated peptide is bound at the stem domain of POMGnT1 (Fig. 1 B and D). Arg29, Asp179, and Arg207 interact with this mannose moiety through hydrogen bonds. The carbonyl atoms of Pro3 and Ala7 also form hydrogen bonds with Arg207 and Tyr152, respectively. The other parts of the peptide moiety interact with Ser153, Phe183, His184, Trp206, Leu231, and Ser232. Because the interaction between the second mannosylated peptide and the neighboring protein molecule is likely to contribute to crystal packing, this binding may be a crystallographic artifact. However, because there are some ionic or hydrogen bonds between the mannose moiety and the stem domain via Arg129, Asp179, and Arg207, whose residues are conserved among mammalian POMGnT1 and also among FAM superfamily (SI Appendix, Fig. S3), the structure also suggests another condition in which the stem domain can recognize the mannose moiety. To test these two possibilities (crystal artifact vs. biological relevance), we analyzed the saccharide-binding capacity of the stem domain; the results are described in the next section.

**Analysis of Saccharide Binding at the Stem Domain.** We analyzed the affinity between the stem domain (residues 92–288) of POMGnT1 and a panel of saccharides by frontal affinity chromatography (FAC) (SI Appendix, Fig. S4 and Table S3) (24). The stem domain specifically bound Glc-β-pNP, Man-β-pNP, and GlcNAc-β-pNP, with KD values of 0.19, 0.36, and 0.23 mM, respectively. However, no binding was detected for high-mannose, hybrid, agalactosylated (including GlcNac-β1,2-Man–O-glycans), sialylated (Siaα2,3,6,7,8-Gal–N-glycans), or polyacylated sugars. Moreover, the stem domain could not bind GlcNAc-β1,3-Gal containing glycolipid-type saccharide (720, 721, and 724–734 in SI Appendix, Fig. S4), O-GalNAc–type sugar structures (Gal–β1,3-GalNAc; 911 in SI Appendix, Fig. S4), or polyacylated glycans (902–906 in SI Appendix, Fig. S4).

Furthermore, surface plasmon resonance (SPR) analysis revealed that the KD values for Man-α-pNP and GlcNAc-α-pNP were more than 50 times larger than those for Man-β-pNP and GlcNAc-β-pNP, respectively (Table 1). Therefore, the endogenous ligand of the stem domain must be a β-linked GlcNAc moiety, which is an enzymatic product of POMGnT1. These KD values suggested that the binding of α-linked mannose in the crystal is a crystallographic artifact.

We also examined the ability of the stem domain to bind mannosylated peptide (Man-O-peptide) and GlcNAc-β1,2-Man peptide (Table 1 and SI Appendix, Table S3), which are a substrate and product of POMGnT1, respectively. The results were similar to those obtained with pNP sugar derivatives. Mannosylated peptide did not bind to the stem domain, but GlcNAc-β1,2-Man peptide bound with a KD value of 0.5 mM. The KD values for GlcNAc-β1,2-Man peptide or β-linked pNP derivatives were
three to six times lower than the $K_m$ for mannosylated peptide [Ac-AAPT(-α-Man)PVAAAP-NH$_2$] of POMGnT1 (1.84 mM) (4). It is difficult to compare these $K_d$ and $K_m$ values directly. However, these observations suggest that recognition of such carbohydrates by the stem domain may affect POMGnT1 activity.

The results of our comprehensive binding analyses by FAC and SPR demonstrate that the stem domain can recognize β-linked monosaccharide structures. In the Golgi, both core M1 (GlcNAc-β1,2-Man-O-Ser/Thr) and core M3 [GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man-O-Ser/Thr] structures are present. Therefore, we analyzed the affinity of the stem domain for GalNAc-β1,3-GlcNAc-β- and β-pNP, which mimics the core M3 structure. The stem domain could bind to this saccharide derivative with a $K_d$ value of 0.8 mM but not to an isomer with an L-acidNac structure, GalNAc-β1,4-GlcNAc-β-pNP (Table 1). This result suggests that the stem domain of POMGnT1 recognizes core M3 as well as its enzymatic product.

**Crystal Structures of Stem Domain with Saccharides.** To analyze differences between α- and β-linked monosaccharides, we determined crystal structures of the stem domain (residues 92–250) in complex with its ligands. In these experiments, we used pNP-sugars (Man-α-pNP, Man-β-pNP, GlcNAc-α-pNP, GlcNAc-β-pNP, and GalNAc-β1,3-GlcNAc-β- and β-pNP) and GlcNAc-β1,2-Man- and GalNAc-pNPs. The results revealed that all pNP sugars bound at nearly identical sites on the stem domain, as observed in soluble POMGnT1, although the positions of the pNP groups differed slightly between the α- and β-linked pNP structures (Fig. 3A–D). In both α- and β-linked pNPs, the pNP groups interact with a hydrophobic hollow of the stem domain composed of Tyr152, Phe183, and Trp206; the interactions between the sugar moieties and the stem domain are almost identical. Arg129 interacts with O4 atoms, Asp179 with O4 and O6, and Arg207 with O5, O6, and O1 by ionic or hydrogen-bond interactions. The interaction between O1 and Arg207 is present only in β-linked pNP derivatives. This interaction effectively explains the observation that the stem domain binds β-linked sugar moieties more strongly than α-linked ones. Arg129, Asp179, and Arg207 are conserved within the FAM3 superfamily (SI Appendix, Fig. S3). FAM3/PANDER is capable of disrupting insulin signaling and promoting increased hepatic glucose production (13), although the underlying molecular mechanism has not been elucidated. Our findings suggest that members of the FAM3 superfamily, including PANDER, also have saccharide-binding ability.

The GlcNAc moiety of GlcNAc-β1,2-Man peptide, an enzymatic product of POMGnT1, also binds to the same site of the stem domain as pNP sugars (Fig. 3C). The details of the interaction are similar to those of pNP sugar binding: Arg129 interacts with O4, Asp179 with O4 and O6, and Arg207 with O5, O6, and O1 by ionic or hydrophobic interactions. The interaction between the carbonyl atom of Pro3 and Trp152 is a hydrophobic interaction between Pro5 and Phe183. In the GalNAc-β1,3-GlcNAc-β-pNP complex, the GlcNAc moiety interacts with the stem domain, as observed in the GlcNAc-β-pNP complex, but the GalNAc moiety engaged in no interactions with the stem domain (Fig. 3D). This observation agrees closely with the results of the SPR analysis; i.e., there is little difference between GlcNAc-β1,2-Man-α-peptide and GlcNAc-β1,3-GlcNAc-β-pNP (Table 1).

To investigate further the functional importance of the observed interactions, we analyzed the glycosyltransferase activity of soluble POMGnT1 (residues 62–660, long construct (L)) in the presence of GlcNAc pNPs. The results revealed that GlcNAc-pNP binding did not affect the activity even in the presence of 10 mM GlcNAc-β-pNP. Furthermore, we performed SPR analyses of the R129A and D179A mutants of the stem domain (residues 92–288); the R207A mutant of the stem domain (92–288) could not be prepared because of its instability in vitro. The R129A (92–288) and D179A (92–288) mutants had drastically reduced binding affinities for GlcNAc-β-pNP (Table 1). Next, we examined the glycosyltransferase activities of these mutants in soluble form. The R129A (L) and R207A (L) mutants decreased the catalytic activity, whereas the D179A (L) mutant had no effect on catalysis (Fig. 2A). It is not clear why the glycosyltransferase activities of R129A (L) and D179A (L) mutants are so different from each other, although both the mutations diminish the saccharide-binding activity. We hypothesized that the stability of the stem domain affects the folding of the catalytic domain. Accordingly, we analyzed the thermal stability of the mutants (SI Appendix, Fig. S8). We found the R129A mutant stem domain (92–250) decreased the melting temperature (Tm) by 10.8 °C relative to WT (92–250), whereas the melting temperature (Tm) of the D179A (residues 92–250) was comparable to that of the WT (92–250). These observations suggest that, consistent with our hypothesis, the stability rather than the saccharide-binding activity of the stem domain affects the glycosyltransferase activity of the catalytic domain.

**Role of Carbohydrate-Binding Ability.** To investigate the requirement for the stem and catalytic domains in postphosphoryl modification of core M3, we established POMGnT1-deficient cells by CRISPR/Cas9 gene editing. Both the α-DG core protein and the IIH6 epitope (Xyl-Glca repeat) were detected at about 120 kDa in the parental cells (HEK293; Fig. 2B (2, 25)). We confirmed the defect in the IIH6 epitope in POMGnT1-deficient cells (mock; Fig. 2B). The expression of WT POMGnT1 and the W473A/M477A mutant rescued the IIH6 epitope and the molecular weight of α-DG (WT and W473A/M477A; Fig. 2B). However, expression of the R129A

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**Table 1. Binding affinity between the stem domain and sugar derivatives by SPR**

<table>
<thead>
<tr>
<th>Stem domain</th>
<th>Sugar derivative</th>
<th>$K_d$, mM</th>
<th>SE, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>GlcNAc-β-pNP</td>
<td>0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>WT</td>
<td>GlcNAc-α-pNP</td>
<td>16.52</td>
<td>2.20</td>
</tr>
<tr>
<td>WT</td>
<td>Man-β-pNP</td>
<td>0.29</td>
<td>0.03</td>
</tr>
<tr>
<td>WT</td>
<td>Man-α-pNP</td>
<td>17.78</td>
<td>2.80</td>
</tr>
<tr>
<td>WT</td>
<td>GlcNAc-β1,2-Man-α-peptide</td>
<td>0.50</td>
<td>0.02</td>
</tr>
<tr>
<td>WT</td>
<td>GalNAc-β1,3-GlcNAc-β-pNP</td>
<td>0.80</td>
<td>0.04</td>
</tr>
<tr>
<td>WT</td>
<td>GalNAc-β1,4-GlcNAc-β-pNP</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>R129A</td>
<td>GlcNAc-β-pNP</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>D179A</td>
<td>GlcNAc-β-pNP</td>
<td>N.D.</td>
<td>—</td>
</tr>
</tbody>
</table>

N.D., not detected; pNP., p-nitrophenyl.
mutant did not rescue the IIH6 epitope or the molecular weight of α-DG (R129A; Fig. 2F). Expression of the D179A mutant significantly increased the molecular weight of α-DG but did not fully rescue the reactivity of anti-IIH6 antibody (D179A; Fig. 2F). This result indicates that the carbohydrate-binding activity of the stem domain, rather than the glycosyltransferase activity of the catalytic domain, is required for IIH6 epitope formation in vivo.

Discussion

We solved structures for two states of the catalytic domain of POMGnT1, the apo state and the Mn²⁺, UDP, and mannosylated peptide complex state. In both structures, the N-lobe of the catalytic domain has a typical glycosyltransferase fold that resembles those of other glycosyltransferases, including GnT I (SI Appendix, Fig. S2B). However, the C-lobe of the catalytic domain is different from that of GnT I, potentially explaining the difference in substrate specificity between these two proteins. Among 15 disease-related mutations reported previously, six sites are located at the interface between the N- and C-lobes of the catalytic domain (SI Appendix, Fig. S5). Because the interface is somewhat distant from the active site, the mutant residues probably are not directly involved in the enzymatic machinery and instead may contribute to protein folding by connecting the N- and C-lobes. The interaction between substrate peptide and catalytic site is mediated mainly by hydrophobic interactions. These findings agree with the results of our previous studies demonstrating that the hydrophobic tendency is important for glycosyltransferase activity (26). A recent study found that the O-mannosylation on protocadherins was not elongated with GlcNAc (27). The mucin-like domain of α-DG has little secondary structure, whereas the O-mannosylation sites of protocadherins are located on the β-strand (28). Based on such comparisons, we believe that POMGnT1 barely recognizes the β-stranded structure. In fact, the mannosylated peptide in our crystal structure had no secondary structure. However, further analysis is needed to determine the secondary structure definitively.

Several previous studies showed that O-mannosylation sites are clustered at the N-terminal region of the mucin-like domain (residues 313-485) in α-DG, even though this region contains weak glycosylation motifs (29-31). Another study showed that Thr317, Thr319, and Thr379 of α-DG are glycosylated with the core M3-type structure (5). The rest of the O-mannosylation sites (15 sites identified to date, including two that are modified with either O-Man or O-GalNAc) are thought to have core M1-type structures. Although the precise sugar structure of each O-mannosylated site remains unknown, it is assumed that almost all sites are extended with β1,2-GlcNAc by POMGnT1 (32). We found that the stem domain of POMGnT1 is a lectin with specificity for the β-linked GlcNAc moiety in O-mannosyl glycan, which is itself a product of POMGnT1. This binding of the stem domain would facilitate GlcNAcylation of neighboring (or nearby) O-mannose moieties (Fig. 4A).

Our structural and biochemical analyses revealed that both the catalytic and stem domains interact with sugar moieties of the glycosylated peptide but do not recognize specific peptide sequences (except for the hydrophobic tendency noted above). The sugar-recognition mechanism of POMGnT1 is likely to facilitate the addition of GlcNAc to clustered O-mannose sites in α-DG. Many glycosyltransferases do not have such an additional lectin domain; one notable exception is the family of UDP-GalNAc:polypeptide α-N-acetylgalactosaminyltransferases (ppGalNACTs). The domain organization of ppGalNACTs is opposite to that of POMGnT1; in these proteins, the C-terminal lectin domain is a β-trefoil fold also found in R-type lectins. The lectin domain binds GalNAc, a product of the catalytic domain of ppGalNACTs, and modulates glycosylation of glycopeptide substrates (33, 34). This mechanism resembles that of POMGnT1.

The proposed facilitation mechanism raises another question about how POMGnT1 determines the first site of β1,2-GlcNAcylation. We observed that the stem domain recognizes GalNAc-β1,3-GlcNAc-β, which is part of the core M3 structure. The

![Fig. 4. Model of POMGnT1 function in α-DG glycosylation.](image-url)
postphosphoryl modification (8). It is noteworthy that LARGE overexpression can compensate for defects in the postphosphoryl modification pathway. LARGE overexpression produces hyperglycosylated α-DG, and the Xyl-GlcA repeat also forms on non-α-DG glycoprotein or N-glycans in POMT2- and POMGnT1-KO cells (35, 36). Thus, the mechanism underlying compensation by LARGE overexpression may be mediated by a pathway other than that described in our model.

Our finding that the stem domain recognizes core M3 helps explain why POMGnT1 mutations affect postphosphoryl modification of the core M3 structure. Moreover, this hypothesis is more consistent with the model than that described in our model.

**Methods**

The secreted form of human POMGnT1 (residues 92–660) was cloned into pBebTag2Hygro (Thermo Fisher Scientific) with a C-terminal HRV3C protease recognition sequence and a His-tag and was purified from stable HEK293T cell lines for crystallization. Stem domains of human POMGnT1 (residues 92–250 or 92–288) were cloned into pGEX-6P1 (GE Healthcare) and expressed in Escherichia coli BL21(DE3). Site-directed mutants were made by PCR-based site-directed mutagenesis. Additional descriptions of materials and methods used in the study are provided in SI Appendix, Materials and Methods.

**ACKNOWLEDGMENTS.** We thank the beamline staffs of the Photon Factory, High Energy Accelerator Research Organization, the SPring-8 synchrotron radiation facility, and the National Synchrotron Radiation Research Center for X-ray diffraction and data collection and Dr. Y. Yamaguchi and Dr. M. Nagae of the RIKEN Structural Glycobiology Team for teaching us the method for making stable cell lines. This work was supported in part by the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from the Ministry of Education, Sports, Science and Technology of Japan and the Japan Agency for Medical Research and Development. This work was also supported by Japan Society for the Promotion of Science Grants-in-Aid for Young Scientists (B) JP26860029 (to N.K.), JP26860682 (to T.Y.), JP15K07284 (to T.E.), and JP26570053 (to H.M.); Scientific Research on Innovative Areas Grant 26110727 (to H.M.); Mizutani Foundation for Glycoscience Grant 150171 (to H.M.); and Grant-in-Aid for Intramural Research 26–8 from the Japan National Center Hospital for Neurological and Psychiatric Disorders (to T.E.).

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N. Kuwabara et al.

SI APPENDIX MATERIALS AND METHODS

1. Protein expression and purification

The soluble secreted form of human POMGnT1 (residues 92–660) was cloned into pSecTag2/Hygro (Thermo Fisher Scientific) with a C-terminal HRV3C protease recognition sequence and His-tag. HEK293T cells were transfected with the POMGnT1 plasmid to establish stably expressing cell lines. The soluble secreted form of POMGnT1 (residues 92–660) was purified as described by Xin et al. (1). In order to purify the soluble secreted form of POMGnT1 for crystallization, we prepared a series of N-terminally truncated mutants of POMGnT1 according to secondary structure prediction based on the primary protein sequence. These mutants contained residues 66–660, 92–660, 204–660, 247–660, or 282–660. We tried to purify all mutants, but only succeeded in purifying and crystallizing POMGnT1 (92–660) from a stable HEK293T cell line. Stem domains of human POMGnT1 (residues 92–250 or 92–288) were cloned into pGEX-6P1 (GE Healthcare) and expressed in Escherichia coli BL21(DE3). Recombinant stem domain proteins were purified with glutathione–Sepharose 4B (GE Healthcare) affinity chromatography, and the GST-tag was removed on the column with PreScission protease (GE Healthcare). The sample was passed through a mono Q column (GE Healthcare), and then loaded onto a Superdex75 column (GE Healthcare). The purified protein was concentrated to 15–30 mg/ml, frozen in liquid nitrogen, and stored at −80°C. Site-directed mutants were made by PCR-based site-directed mutagenesis.

2. Preparation of mannosylated or GlcNAc-β1,2-mannosylated peptide

$^1$H NMR spectra were recorded on a JEOL JMN-600 (600 MHz) spectrometer. Mass numbers were determined by MALDI-TOF-MS using a Voyager® RP (PerSeptive Biosystems). Mass numbers were calculated as averages. MALDI-TOF-MS was performed in positive-ion mode using α-cyano-4-hydroxycinnamic acid (α-CHCA)
as a matrix. Column chromatography was performed on silica gel (Silica Gel 60; Kanto Chemical). RP-HPLC was performed on Inertsil ODS-3 (20 × 250 mm) (GL Sciences). Solvent system: A, 0.1 % TFA in water; B, 0.1 % TFA in acetonitrile. Detection was at 214 nm. Flow rate was 10.0 mL/min. Mannosylated peptide was synthesized as described previously (2), and GlcNAc-β1,2-mannosylated peptide was prepared as follows.

\[ \text{N-(9-Fluorenlymethoxycarbonyl)-O-[2-acetamino-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl]-}(1→2)-(3,4,6-tri-O-acetyl-α-D-mannopyranosyl)-L-threonine benzyl ester (2) \]

A mixture of glycosyl imidate 1 (3) (209 mg, 0.27 mmol) and N-9-fluorenlymethoxycarbonyl-L-threonine benzyl ester (173 mg, 0.40 mmol) in CH\(_2\)Cl\(_2\) (4 mL) containing molecular sieves (4 Å; 0.3 g) was stirred at 0°C. After 30 min, TMSOTf (14 µL, 80 µmol) was added at 0°C, the mixture was stirred for 1 h at 0°C, and then the molecular sieves were removed by filtration through Celite, washing with EtOAc. The filtrate was washed with 1 M aq HCl, sat. NaHCO\(_3\), brine, dried over anhydrous Na\(_2\)SO\(_4\), and concentrated. The residue was purified by column chromatography (CH\(_3\)Cl/MeOH 100:1) to yield 2 in 80% yield (225 mg, 0.22 mmol).

\(^1\)H NMR (600 MHz, CDCl\(_3\)): δ = 7.77 (d, \(J=7.6\) Hz, 2H), 7.64 (d, \(J=7.6\) Hz, 2H), 7.42–7.31 (m, 9H), 5.63 (d, \(J=8.9\) Hz, 1H), 5.51 (d, \(J=8.9\) Hz, 1H), 5.43 (dd, \(J=10.0\) Hz, 1H), 5.30 (dd, \(J=15.8, 12.4\) Hz, 2H), 5.20 (t, \(J=10.0\) Hz, 1H), 5.05 (dd, \(J=9.6, 3.1\) Hz, 2H), 4.97 (t, \(J=9.6\) Hz, 1H), 4.78–4.75 (m, 2H), 4.52 (dd, \(J=8.9, 2.1\) Hz, 1H), 4.46–4.34 (m, 3H), 4.27–4.17 (m, 3H), 4.09–3.93 (m, 4H), 2.07–2.06 (m, 9H), 2.03 (s, 6H), 2.02 (s, 3H), 1.91 (s, 3H), 1.31 (d, \(J=6.2\) Hz, 3H)
To a solution of 2 (219 mg, 0.21 mmol) in EtOAc (10 mL) was added Pd-C (10%, 150 mg). The solution was stirred at room temperature under H₂ for 1 h. The reaction mixture was filtered through Celite, washing with EtOAc. The filtrate was concentrated, and then purified by column chromatography (CH₃Cl/MeOH 50:1) to give 3 in 71% yield (141 mg, 0.15 mmol).

¹H NMR (600 MHz, CD₃OD): δ = 7.80 (d, J=7.6 Hz, 2H), 7.71 (dd, J=8.2, 8.9 Hz, 2H), 7.39 (t, J=7.6 Hz, 2H), 7.32 (dd, J=7.6, 6.2 Hz, 2H), 5.50–5.48 (m, 1H), 5.17 (t, J=10.3 Hz, 1H), 5.01 (dd, J=10.3, 3.4 Hz, 1H), 4.98–4.93 (m, 3H), 4.51–4.50 (m, 1H), 4.40 (dd, J=10.3, 6.9 Hz, 1H), 4.34 (dd, J=11.0, 6.9 Hz, 1H), 4.28–4.24 (m, 2H), 4.19–4.14 (m, 3H), 4.07–3.98 (m, 3H), 3.84–3.82 (m, 1H), 2.05 (s, 9H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H), 1.25 (d, J=6.9 Hz, 3H)

GlcNAcβ(1→2)Manα-peptide [Ac-ATPT(GlcNAcβ(1→2)Manα)PVTAIG-NH₂] (4)

Starting from Fmoc-Gly-CLEAR-Amide Resin (0.1 mmol, 0.4 meq/g, 255 mg), peptide chain elongation was carried out manually by the Fmoc strategy. The Fmoc protecting group was removed with 20% (vol/vol) piperidine/NMP, and couplings were performed with a 5-fold excess of Fmoc-protected amino-acid derivative (0.5 mmol) using (benzotriazol-1-yl)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (260 mg, 0.5 mmol) and N,N-diisopropylethylamine (DIEA) (174 µL, 1.0 mmol) in NMP. Coupling time was 1.5 h. Fmoc-Thr[(AcO)₃GlcNAcβ1-2(AcO)₃Manα] 3 (110 mg, 0.12 mmol) was coupled using 0.12 mmol PyBOP (0.25 mmol) and DIEA
(0.5 mmol) in NMP. Coupling time was 18 h. The final N-acetylation was carried out with Ac₂O (100 µL, 1.0 mmol) and DIED (1.0 mmol) for 1 h. The crude glycopeptide resin was treated with 10 mL of TFA:water (95:5) for 2 h at room temperature. The resin was filtered, and the TFA solution was concentrated. The residue was dissolved in water, and the aqueous phase was washed with Et₂O. After lyophilization of the aqueous phase, crude glycopeptide with Ac-protected sugar hydroxyls (101 mg) was obtained. To a solution of an aliquot of the crude glycopeptide (56 mg) in water (5 mL) was added aqueous TBA-OH (0.5 M, 1.3 mL), and the mixture was stirred for 2.5 h at room temperature. HCl/dioxane (4 M, 206 µL) was added, and the mixture was purified by RP-HPLC [buffer A – buffer B 90:10 – 60:40 (linear gradient, 40 min)] to give the GlcNAc-β1,2-Man-peptide 4 (34 mg, 45%). MALDI-TOF-MS, found: m/z [M + Na]⁺: 1355.71, calcd for [M + Na]⁺: 1356.43.

3. Crystallization and structure determination

Purified proteins were subjected to initial crystallization screening using an automated protein crystallization and monitoring system (4). Subsequent optimization of conditions was carried out by hanging-drop vapor diffusion by mixing equal volumes of protein and reservoir solutions.

Crystals of the apo form of POMGnT1 were obtained in 1.4–1.6 M Na-malonate (pH 6.5) at 277 K, and 1.3–1.5 M NaKPO₄ (pH 6.6–6.8) at 293K, respectively. Before X-ray diffraction experiments, the crystals were soaked in reservoir solution containing an additional 25% xylitol (for form I) or 25% ethylene glycol (for form II). Initial phase determination was performed by the iodide–SAD (single wavelength anomalous dispersion) method using a wavelength of 1.893 Å at beamline PF AR-NE3A (KEK, Japan) at 100 K. Crystals were soaked in 0.5 M NaI within the reservoir solution and incubated for 2–3 h at 20°C. Phases were determined by autoSHARP (5). For crystallization of mannosylated peptide complexes, purified POMGnT1 was mixed with 5 mM MnCl₂, 5 mM UDP, and 10 mM mannosylated peptide [Ac-AAPT(-α-Man)PVAAP-NH₂] and incubated for 2 h on ice. The sample was crystallized under two conditions: 1.3–1.5 M NaKPO₄ (pH 6.6–6.8) / 0.9–1.1 M NaK-tartrate and 0.1 M NaKPO₄ (pH 6.8) / 0.2 M LiSO₄. The resultant crystals were soaked in reservoir solution containing an additional 25% ethylene glycol.
Stem domain (residues 92–250) was mixed with 10 mM pNP-sugars (Sigma-Aldrich), incubated for 2 h on ice, and subjected to crystallization. pNP-β-mannose complex crystals appeared in 0.1 M Tris-HCl (pH 8.5), 16% PEG-10000. Other pNP-α-mannose complex crystals were obtained in 0.1 M Tris-HCl (pH 8.8), 18–22% PEG-6000 or PEG-8000, by streak seeding using a crystal of the pNP-β-mannose complex. For crystallization of GlcNAc-β1,2-mannosylated peptide–bound stem domain, purified stem domain was incubated with 2.5 mM GlcNAc-β1,2-mannosylated peptide [Ac-ATPT(GlcNAc-β1,2-Man)PVTAIG-NH₂] for 1 h on ice prior crystallization. The crystals were obtained in 0.1 M MES-NaOH (pH 6.0), 1.0 M LiCl₂, 0.1 M Na acetate, 24% PEG-6000.

X-ray diffraction data were collected at beamlines BL-1A, BL-17A, AR-NE3A (Photon Factory, KEK, Japan), BL32XU (SPring-8, Japan), and BL15A (NSRRC, Taiwan). All datasets were processed using XDS (6) and scaled using aimless in the CCP4i package (7). Except for the apo form structure of POMGnT1, crystal structures were solved by molecular replacement method using Phaser (8), with the POMGnT1 structure as a search model. Further model-building and refinement were performed with COOT (9) and Phenix.refine (8), respectively. Diffraction and refinement data statistics are summarized in Table S1.

The interaction areas of the stem and catalytic domains with the linker region were calculated by ePISA (Proteins, Interfaces, Structures and Assemblies [PISA] at the European Bioinformatics Institute, http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (10). Structural alignments and comparisons between proteins were performed using DaliLite with the pairwise option (11).

4. Frontal affinity chromatography (FAC)

FAC analysis was performed as described previously (12). Briefly, the stem domain of POMGnT1 was immobilized on NHS-activated Sepharose 4 Fast Flow (GE Healthcare) at a concentration of 4 mg/ml, packed into a miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 µl, Shimadzu), and connected to an automated FAC system (FAC-2). A panel of pyridylaminated (PA) and pNP glycans, in addition to Man-α-nonapeptide and GlcNAc-β1,2-Man-α-peptide, was successively
injected into the columns by the auto-sampling system, and elution was detected by fluorescence (excitation, 310 nm; emission, 380 nm) or absorbance at 220 nm. The elution front of each glycan relative to that of an appropriate control, referred to as V-V₀, was then determined.

5. Surface plasmon resonance (SPR) analysis

SPR binding assays were performed at 298 K on a Biacore T-200 (GE Healthcare). HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.005% [v/v] Surfactant P20, pH 7.2) was used as the running buffer at a flow rate of 30 µl/min. Stem domain (residues 92–288) was directly immobilized on a CM5 sensor chip by amine coupling. When pNP sugar derivatives were used as analytes, 2% DMSO was added to the HBS-P buffer to improve solubility. The net response was calculated by subtracting the background response from the binding response. The results were analyzed using the Biacore T-200 Evaluation Software (GE Healthcare).

6. Assay for POMGnT1 activity

Secreted soluble forms of POMGnT1 were expressed in HEK293T cells. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under a 5% CO₂ atmosphere. POMGnT1 expression plasmids were transfected into HEK293T cells using Lipofectamine 3000 (Thermo Fisher Scientific). Media were replaced with 10 ml fresh FBS-free Opti-MEM 2 days after transfection, and the cells were incubated for an additional 24 h. FBS-free culture supernatants were subjected to western blot analysis and assayed for POMGnT1 activity.

Proteins were separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.1% Tween 20, incubated with anti-POMGnT1 C-terminus antibody (1), and subsequently incubated with anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare). Blots were developed using an ECL kit (GE Healthcare) (Fig. S6). Purified POMGnT1 was used as a mass standard to determine the amount of each protein. Optical density measurement of the bands using the ImageJ software yielded a standard curve, which
was used to calculate the concentration of each mutant protein.

POMGnT1 activity was calculated from the amount of $[^3]$H-GlcNAc transferred to a mannosylated peptide, as described previously (1). Briefly, reaction buffer containing 140 mM MES buffer (pH 7.0), 0.5 mM UDP-$[^3]$H GlcNAc (450,000 dpm/nmol), 0.5 mM mannosylated peptide, 10 mM MnCl$_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol and enzyme solution (FBS-free culture supernatants) was incubated at 37°C for 2 h. The mixture was separated by reversed-phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm) (Wako Pure Chemical). Peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

7. Circular dichroism analysis

Circular dichroism was recorded in a 1-mm quartz cell on a J-820 CD spectropolarimeter (JASCO). All data were collected with 0.5 mg/ml purified stem domains (residues 92–250) in 6.7 mM HEPES-NaOH (pH 7.5) and 50 mM NaCl. The thermal denaturation experiments were carried out from 20°C to 80°C with a rate of 1°C·min$^{-1}$. The midpoint temperature of the unfolding transition ($T_m$) was determined from van't Hoff analysis.

8. Model structure construction

The model of the complex between the stem domain and the ligand GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man-O-peptide was constructed using the docking software Glide (13-16) and Maestro (17). The ligand was modeled using Maestro, and the protonation state of the ligand was estimated using Epik (18). For the receptor, the crystal structure of the stem domain in complex with GalNAc-β1,3-GlcNAc-β-pNP was used. GalNAc-β1,3-GlcNAc-β-pNP was removed from the crystal structure, while the water molecules in the binding pocket were retained. Hydrogen atoms were added to the stem domain and water molecules, and the positions of the hydrogen atoms were optimized for hydrogen bonding. Then, GalNAc-β1,3-GlcNAc-β1,4-(phosphate-6)-Man-O-peptide was docked into the binding site of the stem domain using Glide. In the docking calculation, the docking poses of
GalNAc and GlcNAc were constrained to their original positions in the crystal structure of GalNAc-β1,3-GlcNAc-β−pNP.

9. Assay for rescue of IIH6 epitope synthesis using POMGnT1-deficient cells

The POMGnT1-deficient cells were prepared by CRISPR/Cas9 gene editing using the targeting sequence TGATGGCTCGCCGAGTGTCC. The oligonucleotide was inserted into the Cas9 Smart Nuclease All-in-One vector (System Biosciences) with an additional 8-base sequence at the 5’ terminus. The vector was transfected into HEK293 cells, and then IIH6-negative cells were isolated by fluorescence activated cell sorting (FACS; MoFlo, Beckman Coulter). Each cell clone was verified for IIH6 reactivity by western blotting and DNA sequencing. The mutations in clone POMGnT1 s4o-3 were a 2-bp deletion, a 4-bp deletion, and a 137-bp insertion, all of which caused frameshifts (HEK293 cells are triploid). Anti–glycosylated-α-DG (IIH6) was purchased from Millipore.

Expression plasmids for the transmembrane form of POMGnT1 (wild type (WT), R129A, R179A, and W473A/M477A) were transfected into >90% confluent POMGnT1-deficient cells using Lipofectamine 3000. To avoid overgrowth, the cells were subcultured to reduce their density to 70% at 24 h after transfection. The transfectants were cultured for 4 days, collected, and homogenized in 1 ml of 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, and protease inhibitor cocktail (3 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 mM benzamidine-HCl, and 1 mM PMSF). After centrifugation at 900 g for 10 min, the supernatant was subjected to ultracentrifugation at 100,000 g for 1 h. The precipitate (microsomal membrane fraction) was solubilized with 10 mM Tris-HCl (pH 7.4) containing 1% Triton X-100, 500 mM NaCl, and protease inhibitor cocktail. α-DG was enriched from the solubilized microsomal membrane fraction using wheat germ agglutinin (WGA)-agarose (J-OIL MILLS). IIH6 epitope synthesis was assayed by Western blot analysis using IIH6 antibody and anti-α-DG core protein antibody (3D7) as described previously (19, 20).
SI APPENDIX REFERENCES


**SI APPENDIX FIGURE LEGENDS**

**Fig. S1. Dystrophin glycoprotein complex and POMGnT1**

(A) Dystrophin glycoprotein complex. α-DG links extracellular matrix components such as laminin to β-DG, which binds to dystrophin, and thus to the actin cytoskeleton.
α-DG is heavily glycosylated, and its O-Man glycan is crucial for laminin binding. Mutations in *POMGnT1* cause muscle-eye-brain disease (MEB). (B) The proposed biosynthetic pathway for core M1, core M2, and core M3. Protein O-mannosylation is initiated by POMT1 and POMT2. *POMGnT1* transfers a GlcNAc residue from UDP-GlcNAc to the O-Man residue to form core M1. After GlcNAc-β1,2-Man formation, GnT-IX (Vb) acts on it to form core M2. The biosynthetic pathway of core M3, involving sequential action by GTDC2, B3GALNT2, and SGK196, was proposed by Yoshida-Moriguchi et al. (21). The details of post-phosphoryl glycan modification remain unclear, except for formation of the xylosyl–glucuronyl polysaccharide chain ([β1,3-Xyl-α1,3-GlcA]-n), which is essential for the interaction between α-DG and laminin. FKTN is thought to be involved in post-phosphoryl modification.

**Fig. S2. Crystal structure and electron density of POMGnT1**

(A) Superimposition of the stem domain of POMGnT1 (green) and FAM3B (blue, PDB ID: 2YOQ). (B) Superimposition of the catalytic domain of POMGnT1 (cyan) and rabbit GnT I (magenta, PDB ID: 2AM3). (C) Electron-density map of mannosylated peptide at the catalytic domain. 2Fo-Fc omit map contoured at 1.0 σ is shown in blue mesh. (D) Detailed structure of the second mannosylated peptide-bound site in the stem domain. The mannosylated peptide is shown as an orange stick model. The color configuration of POMGnT1 is as in (B). 2Fo-Fc omit map contoured at 1.0 σ of the mannosylated peptide is shown as a blue mesh.

**Fig. S3. Sequence alignment of stem domains with FAM family proteins**

Structure-based sequence alignment of the stem domain of POMGnT1 and its homologues, calculated using the program MAFFT (22). White letters on red fields represent identical amino-acid residues; red letters and boxed regions represent homologous residues. Arrows, Ts, and coil symbols above the sequences represent secondary structural elements (β-strand, turn, and α-helix, respectively) in the stem domain, determined and drawn using the program ESPRRIPT 2.2 (ENDscript, http://endscript.ibcp.fr). The aligned proteins sequences are human POMGnT1 (Q8WZA1), rat POMGnT1 (Q1XIN7), mouse FAM3B (also called PANDER)
(Q9D309), rat FAM3C (Q810F4), human FAM3C (Q92520), *Xenopus laevis* FAM3C (Q6GQC1), zebrafish FAM3C (Q7ZYY4), and human FAM3D (Q96BQ1). UniprotKB/Swiss-Prot entries are indicated in parentheses. Arg129, Asp179, and Arg207 are indicated by blue arrows.

Fig. S4. Frontal affinity chromatography analysis of stem domain
Affinity constants (\(K_a\), M\(^{-1}\)) of stem domain for a series of pyridylaminated (PA) glycans (002-947) and pNP-glycans are shown as a bar graph. The following panel shows the glycan structures of a series of PA-glycans.

Fig. S5. Mapping of disease-related mutations on the crystal structure of POMGnT1
Stem domain (residues 97–250), linker region (residues 251–299), and catalytic domain (residues 300–647) are shown in green, yellow, cyan (N-lobe), and blue (C-lobe), respectively. Bound UDP is shown as a stick model. Mn\(^{2+}\) and mannosylated peptide were removed for clarity. Red spheres represent the C\(\alpha\) of disease-related mutation sites reported in the UniProtKB database (UniProt ID: Q8WZA1). The database lists T176P, S198P, E223R, R265H, C269Y, R311H, R367H, W425S, D427H, R442H, C490Y, P493R, and S550N as causing muscular dystrophy–dystroglycanopathy congenital with brain and eye anomalies A3 (MDDGA3); R311Q, C490Y, and R605P as causing muscular dystrophy–dystroglycanopathy congenital with mental retardation B3 (MDDGB3); and D556N as causing muscular dystrophy–dystroglycanopathy limb-girdle C3 (MDDGC3).

Fig. S6. Expression of soluble form proteins of POMGnT1 mutants
The long (L, residues 62–660) and short (S, residues 248–660) constructs were expressed in HEK293T cells, fractionated by SDS–PAGE, transferred to PVDF membranes, and detected with an antibody against the POMGnT1 C-terminus. Expression levels of the constructs were quantitated using ImageJ.

Fig. S7. Glycosyltransferase activities of POMGnT1 in the presence of GlcNAc-\(\beta\)-pNP or GlcNAc-\(\alpha\)-pNP
The rate of GlcNAc transfer to mannosylated peptide was measured using the soluble long form of POMGnT1 (62–660). The concentrations of GlcNAc-β-pNP or GlcNAc-α-pNP in the reaction mixture are indicated on the graph labels. Glycosylation activities are reported relative to the activity level in the absence of pNP reagent, defined as 1.

Fig. S8. Thermal stability analysis of wild type or mutant stem domains
Thermal denaturation experiments were carried out from 20°C to 80°C, with temperature changing at a rate of 1°C·min⁻¹. Black, red, and blue lines show stem domain (92–250) WT, R147A, and D179A, respectively. The midpoint temperatures of the unfolding transition (T_m) were determined from van't Hoff analysis. The Tm values of the stem domain (92–250) WT, R147A, and D179A were 53.6, 42.8, and 53.7°C, respectively.
Defect in POMGnT1 (MEB)

Laminin

αDG

βDG

Sarcolemma

Basal Lamina

Cytoplasm

Actin-cytoskeleton

Dystrophin

No-extend

X

Figure S1

SI APPENDIX FIGURES
Human POMGnT1 stem

**Figure S3**
Figure S4
$N$-glycans

High-mannose-type

Hybrid type

Agalactosylated type

Galactosylated type

Sialylated type

Figure S4 (continued)
Figure S4 (continued)
Figure S5
Figure S6
Figure S7
Figure S8
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<td>BL15A</td>
<td>BL15A</td>
<td>BL-17A</td>
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<td>1.8935</td>
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<td>1.0000</td>
<td>1.1000</td>
<td>1.0000</td>
<td>0.9800</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.9800</td>
</tr>
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<td>Space group</td>
<td>P4₁2,2</td>
<td>P4₁2,2</td>
<td>C222₁</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
<td>P₂₁,2</td>
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<td>Unit cell (Å, °)</td>
<td>a=b=99.9</td>
<td>a=b=99.7</td>
<td>a=168.7</td>
<td>a=125.7</td>
<td>a=70.4</td>
<td>a=70.1</td>
<td>a=68.9</td>
<td>a=69.5</td>
<td>a=70.0</td>
<td>a=111.1</td>
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<td>c=236.1</td>
<td>c=239.6</td>
<td>c=186.45</td>
<td>b=172.0</td>
<td>b=90.4</td>
<td>b=89.9</td>
<td>b=88.2</td>
<td>b=88.7</td>
<td>b=49.7</td>
<td>b=49.7</td>
<td>c=61.1</td>
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<tr>
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<td>c=143.9</td>
<td>c=67.3</td>
<td>c=53.6</td>
<td>c=53.4</td>
<td>c=53.3</td>
<td>c=53.3</td>
<td>c=53.3</td>
<td>c=53.3</td>
<td>c=53.3</td>
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<td>353,544</td>
<td>519,355</td>
<td>1,227,868</td>
<td>742,885</td>
<td>1,031,190</td>
<td>1,091,654</td>
<td>438,166</td>
<td>393,320</td>
<td>141,169</td>
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<tr>
<td>Unique reflection</td>
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<td>26,072</td>
<td>78,422</td>
<td>46,907</td>
<td>104,368</td>
<td>83,257</td>
<td>86,073</td>
<td>62,431</td>
<td>54,584</td>
<td>41,949</td>
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<td>Resolution (Å)</td>
<td>44.68 - 3.00</td>
<td>48.82-2.96</td>
<td>47.20-2.50</td>
<td>46.37 - 2.60</td>
<td>19.4 - 1.21</td>
<td>45.8 - 1.30</td>
<td>42.1 - 1.27</td>
<td>19.2 - 1.42</td>
<td>45.9 - 1.50</td>
<td>46.6 - 1.60</td>
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<tr>
<td>(3.18 - 3.00)</td>
<td>(3.14-2.96)</td>
<td>(2.55-2.50)</td>
<td>(2.69 - 2.60)</td>
<td>(1.23-1.21)</td>
<td>(1.32-1.30)</td>
<td>(1.29 - 1.27)</td>
<td>(1.45 - 1.42)</td>
<td>(1.53-1.50)</td>
<td>(1.63-1.60)</td>
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<td>Completeness (%)</td>
<td>99.9 (100)</td>
<td>99.8 (98.6)</td>
<td>99.7 (97.5)</td>
<td>100.0 (100.0)</td>
<td>99.4 (93.8)</td>
<td>99.9 (97.5)</td>
<td>100 (100)</td>
<td>99.4 (90.8)</td>
<td>100 (99.9)</td>
<td>98.0 (95.8)</td>
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<td>Rmerge (%)</td>
<td>6.7 (65.7)</td>
<td>12.5 (132.9)</td>
<td>11.2 (120)</td>
<td>33.8 (108.5)</td>
<td>3.5 (52.1)</td>
<td>8.5 (151.4)</td>
<td>9.0 (184.0)</td>
<td>7.7 (98.0)</td>
<td>7.5 (90.1)</td>
<td>9.1 (71.7)</td>
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<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>30.8 (4.6)</td>
<td>17.7 (2.0)</td>
<td>15.3 (1.5)</td>
<td>20.5 (2.1)</td>
<td>24.8 (3.0)</td>
<td>18.9 (1.4)</td>
<td>14.4 (1.3)</td>
<td>12.7 (1.0)</td>
<td>14.7 (2.1)</td>
<td>8.7 (1.5)</td>
</tr>
<tr>
<td>CC (1/2)</td>
<td>0.999</td>
<td>0.999</td>
<td>0.997</td>
<td>0.998</td>
<td>1.000</td>
<td>1.000</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.995</td>
</tr>
<tr>
<td>(0.942)</td>
<td>(0.707)</td>
<td>(0.521)</td>
<td>(0.567)</td>
<td>(0.841)</td>
<td>(0.500)</td>
<td>(0.497)</td>
<td>(0.538)</td>
<td>(0.714)</td>
<td>(0.584)</td>
<td></td>
</tr>
<tr>
<td>Multiplicity</td>
<td>14.2 (14.8)</td>
<td>13.6 (12.4)</td>
<td>6.6 (6.3)</td>
<td>26.2 (11.8)</td>
<td>7.2 (6.5)</td>
<td>12.4 (4.8)</td>
<td>12.7 (12.4)</td>
<td>7.0 (5.2)</td>
<td>7.2 (7.3)</td>
<td>3.4 (3.1)</td>
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Table S1. Data collection and refinement statistics
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<th></th>
<th>Working Set</th>
<th>20.5</th>
<th>23.7</th>
<th>19.3</th>
<th>17.6</th>
<th>15.0</th>
<th>16.6</th>
<th>17.8</th>
<th>17.5</th>
<th>21.6</th>
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<tr>
<td>Refinement statistics</td>
<td>Free R value</td>
<td>24.1</td>
<td>25.7</td>
<td>23.5</td>
<td>18.9</td>
<td>17.7</td>
<td>17.9</td>
<td>20.3</td>
<td>20.8</td>
<td>24.6</td>
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<tr>
<td></td>
<td>Bond (Å)</td>
<td>0.005</td>
<td>0.007</td>
<td>0.008</td>
<td>0.006</td>
<td>0.007</td>
<td>0.008</td>
<td>0.006</td>
<td>0.016</td>
<td>0.007</td>
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<td></td>
<td>Angle (°)</td>
<td>0.947</td>
<td>0.994</td>
<td>0.951</td>
<td>1.162</td>
<td>1.149</td>
<td>1.217</td>
<td>1.058</td>
<td>1.628</td>
<td>1.121</td>
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<tr>
<td>Molprobity Statistics</td>
<td>Favored (%)</td>
<td>93.6</td>
<td>96.1</td>
<td>96.7</td>
<td>98.0</td>
<td>98.0</td>
<td>97.4</td>
<td>97.1</td>
<td>98.0</td>
<td>99.7</td>
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<tr>
<td></td>
<td>Allowed (%)</td>
<td>6.0</td>
<td>3.8</td>
<td>3.2</td>
<td>1.6</td>
<td>2.0</td>
<td>2.6</td>
<td>3.0</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Outlier (%)</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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The values in the highest resolution shell are shown in parentheses.
Table S2. The comparison of interaction atoms between GnT I and POMGnT1 at the active site.

<table>
<thead>
<tr>
<th>Interacting atoms</th>
<th>GnT I</th>
<th>Distance (Å)</th>
<th>POMGnT1</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil N3</td>
<td>D144 Oδ2</td>
<td>2.8</td>
<td>D338 Oδ2</td>
<td>2.9</td>
</tr>
<tr>
<td>Uracil O2</td>
<td>H190 Nδ1</td>
<td>2.7</td>
<td>H371 Nδ1</td>
<td>3.1</td>
</tr>
<tr>
<td>Ribose O2’</td>
<td>D212 Oδ1</td>
<td>3.1</td>
<td>E394 Oε2</td>
<td>3.4</td>
</tr>
<tr>
<td>Ribose O2’</td>
<td>H2O 40</td>
<td>2.9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ribose O3’</td>
<td>D212 Oδ1</td>
<td>3.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>α-phosphate O1α</td>
<td>V321 N</td>
<td>2.9</td>
<td>L506 N</td>
<td>2.9</td>
</tr>
<tr>
<td>α-phosphate O1α</td>
<td>H2O 72</td>
<td>2.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>α-phosphate O2α</td>
<td>R117 NH2</td>
<td>2.8</td>
<td>R311 NH2</td>
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<tr>
<td>α-phosphate O2α</td>
<td>Mn²⁺</td>
<td>2.1</td>
<td>Mn²⁺</td>
<td>2.1</td>
</tr>
<tr>
<td>β-phosphate O1β</td>
<td>S322 Oγ</td>
<td>2.5</td>
<td>N507 N</td>
<td>3.0</td>
</tr>
<tr>
<td>β-phosphate O2β</td>
<td>Mn²⁺</td>
<td>2.1</td>
<td>Mn²⁺</td>
<td>2.7</td>
</tr>
<tr>
<td>β-phosphate O2β</td>
<td>GlcNAc N2</td>
<td>2.9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>D213 Oδ2</td>
<td>2.1</td>
<td>D395 Oδ2</td>
<td>2.1</td>
</tr>
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<td>Mn²⁺</td>
<td>H2O 38</td>
<td>2.4</td>
<td>H500 Nε2</td>
<td>2.4</td>
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<tr>
<td>Mn²⁺</td>
<td>H2O 87</td>
<td>2.4</td>
<td>-</td>
<td></td>
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<tr>
<td>Mn²⁺</td>
<td>H2O 116</td>
<td>2.1</td>
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</table>

Distances between the UDP (or UDP moiety of UDP-GlcNAc), the Mn²⁺, bound waters and the protein atoms involved in their binding are listed. The list of distances between the GnT I, UDP moiety of UDP-GlcNAc and Mn²⁺ are based on the ref. (23). In POMGnT1 crystal structure, water molecules around the active site could not be modeled because of low resolution.
### Table S3  Binding affinity between the stem domain and sugar derivatives by frontal affinity chromatography (FAC)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ (mM)</th>
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<td>Gal-α-pNP</td>
<td>N.D.</td>
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<tr>
<td>Gal-β-pNP</td>
<td>N.D.</td>
</tr>
<tr>
<td>GalNAc-α-pNP</td>
<td>N.D.</td>
</tr>
<tr>
<td>GalNAc-β-pNP</td>
<td>N.D.</td>
</tr>
<tr>
<td>Man-α-pNP</td>
<td>N.D.</td>
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<tr>
<td>Man-β-pNP</td>
<td>0.36</td>
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<tr>
<td>Glc-α-pNP</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glc-β-pNP</td>
<td>0.19</td>
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<tr>
<td>GlcNAc-α-pNP</td>
<td>N.D.</td>
</tr>
<tr>
<td>GlcNAc-β-pNP</td>
<td>0.23</td>
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<tr>
<td>Man-α-nonapeptide</td>
<td>N.D.</td>
</tr>
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<td>GlcNAc-β1,2-Man-α-peptide</td>
<td>0.30</td>
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</table>

*N.D. means “not detected.” pNP means $p$-nitrophenyl.