

**BIOCHEMICAL CORRELATION OF ACTIVITY OF THE  
 $\alpha$ -DYSTROGLYCAN-MODIFYING GLYCOSYLTRANSFERASE  
POMGNT1 WITH MUTATIONS IN MUSCLE-EYE-BRAIN DISEASE**

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**Congenital muscular dystrophies have a broad spectrum of genotypes and phenotypes and there is a need for a better biochemical understanding of this group of diseases in order to aid diagnosis and treatment. Several mutations resulting in these diseases cause reduced *O*-mannosyl glycosylation of glycoproteins, including  $\alpha$ -dystroglycan. The enzyme protein-*O*-mannose *N*-acetylglucosaminyltransferase 1 (POMGnT1; EC 2.4.1.-) catalyzes the transfer of *N*-acetylglucosamine to *O*-linked mannose of  $\alpha$ -dystroglycan, and affect glycosyltransferases or glycosyltransferase-like genes. Here we describe the biochemical characterization of 14 clinical mutants of the glycosyltransferase POMGnT1, which have been linked to Muscle-Eye-Brain disease or similar conditions. Truncated mutant variants of the human enzyme (rPOMGnT1) were expressed in *Escherichia coli* and screened for catalytic activity. We find that three mutants show some activity towards mannosylated peptide substrates mimicking  $\alpha$ -DG; the residues affected by these mutants are predicted by homology modeling to be on the periphery of the POMGnT1 surface. Only in part does the published location of a mutated residue on the periphery of the protein structure correlate with a less severe disease mutant.**

## Introduction

The cell surface receptor  $\alpha$ -dystroglycan ( $\alpha$ -DG) is, alongside  $\beta$ -dystroglycan ( $\beta$ -DG), a central component of the dystrophin-glycoprotein complex, which connects the extracellular matrix to the cytoskeleton.  $\alpha$ -DG is a 650 amino acid protein containing three distinct domains, two globular domains on the N- and the C-terminus, and a large central mucin type domain essential for the binding to proteins in the basal lamina such as laminin, agrin, biglycan, neurexin, and perlecan [1-5]. The proper glycosylation of the mucin type domain of  $\alpha$ -DG with O-linked specific carbohydrates is key for maintenance of membrane stability. The mature human  $\alpha$ -DG glycoprotein has three N-glycosylation sites, up to five predicted glycosaminoglycan attachment sites [6], and more than 50 other serine and threonine residues which can potentially carry O-glycans. Although the predicted protein mass is only 71 kDa, native glycosylated  $\alpha$ -DG migrates as a diffuse band between 120 and 250 kDa on reducing SDS-PAGE [7, 8]. Other than the mucin-like O-glycan structures, another less common O-glycan structure, Neu5Ac( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc( $\beta$ 1-2)Man-O, has been detected on mammalian  $\alpha$ -DG and may play a role in the binding of  $\alpha$ -DG to extracellular protein ligands [9-11]. Originally regarded to be a yeast-specific modification, O-mannosylation in mammals is only present on a limited number of proteins in neuronal and muscular tissue [12]. Although various O-mannose glycopeptides could be identified from total brain tissue [13, 14],  $\alpha$ -DG remains still the only mammalian glycoprotein clearly identified to contain this rare modification (**Figure 1**).

The essential nature of glycosylation for the function of  $\alpha$ -DG has been confirmed by studying muscle tissue of patients with  $\alpha$ -dystroglycanopathies. This heterogenous group of autosomal recessive disorders encompasses a broad spectrum of conditions, with clinical phenotypes ranging from mild limb-girdle muscular dystrophy (LGMD) without cognitive impairment, to Muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWS) with severe brain and eye abnormalities [15, 16]. Immunostaining of muscle samples using the polyclonal GT20ADG antibody raised against hypoglycosylated  $\alpha$ -DG showed that  $\alpha$ -DG expression levels of  $\alpha$ -dystroglycanopathy patients are comparable with these of control samples [10]. In contrast, the same patient samples have reduced or absent immunostaining with I1H6 and VIA4-1 monoclonal antibodies, whose binding requires native glycosylation of  $\alpha$ -DG. The loss of glycosylation is significant, reducing the molecular weight of the mature protein by 50 kDa or more [17].

Molecular testing of patients with clinical signs of congenital muscular dystrophy (CMD) revealed that mutations in six genes encoding for known or putative glycosyltransferases are responsible for hypoglycosylation of  $\alpha$ -DG, supposedly due to the reduced or lost enzymatic function. The mutated forms of the protein-O-mannosyltransferase 1 (*POMT1*), *POMT2*, protein O-mannose N-acetylglucosaminyltransferase 1 (*POMGnT1*), fukutin (*FKTN*), fukutin-related protein (*FKRP*) and *LARGE* genes were originally reported in patients with distinct phenotypes [18-22], and therefore defects of those glycosyltransferases were initially associated with different clinical conditions. In recent years, a wider spectrum of phenotypes with different severities have been reported for mutations in the *POMT1* [23, 24], *POMT2* [25, 26], and the *FKTN* gene [27, 28], whereas mutations affecting *FKRP* [29, 30], *POMGnT1* [31-33] and *LARGE* [34, 35] were described to have less phenotypical variation. However, a study of 81 CMD patients from Italy could only detect 43 homozygous or heterocompound mutations (53%) in those six genes [34], which allows the presumption that even more, as yet unidentified, genes are

involved in the posttranslational modification of  $\alpha$ -DG.

Biochemically, POMT1, POMT2, and POMGnT1 are known to transfer the first two residues (mannose and GlcNAc) of the O-mannosyl modification [22, 36]. Recently, LARGE was shown to be involved in the phosphorylation of O-linked mannose on  $\alpha$ -DG [37], whereas the biochemical functions of fukutin and FKR1P are still unknown. The aim of the present study was to characterize the enzymatic properties of 14 clinical mutations of POMGnT1, which have been linked to MEB, LGMD and similar conditions. Therefore, a simple expression and purification protocol for the POMGnT1 in the prokaryotic host *Escherichia coli* had to be developed; a series of mannopeptides derived from  $\alpha$ -DG were synthesized and tested as acceptor substrate for the recombinant, partially purified wild type enzyme. The optimal substrate was then used for (i) testing recombinantly expressed POMGnT1 mutant enzymes for activity, (ii) determining the kinetic parameters of the active POMGnT1 variants, and (iii) to evaluate the possibility of correlating genotype with the obtained biochemical data.

## Experimental Procedures

### *Subcloning of POMGnT1 from a bacterial cDNA clone*

The cDNA-clone containing the complete POMGnT1 gene was ordered from Geneservice Ltd. (IMAGE #5553465). The plasmid was isolated (Qiagen Miniprep kit) and used as a DNA template for a PCR amplification. The primer pair Fw/EcoRI (acgaattcagtggaagccaatgaagacc) and Rv/XhoI (gactcgagctatgtctgttctgggctc) was used in combination with KOD DNA Polymerase (Roche). The primers were designed to amplify the sequence encoding amino acids 66 to 660, *i.e.*, a form lacking the N-terminal putative transmembrane domain. The PCR fragment was cut with the relevant enzymes and ligated into pET30a which contains a sequence encoding an N-terminal His-tag; clones which displayed no mutation after full sequencing of the insert were used for further experiments.

### *Expression and purification of POMGnT1 mutants*

Complementary oligonucleotide primers containing the desired mutation were used to generate POMGnT1 mutant genes (see **Supplementary Table S1**) according to the standard protocol for QuikChange Site-Directed Mutagenesis (Stratagene); plasmid DNA of transformants was sequenced to verify that selected clones contain the desired mutation. Mutant plasmids were transformed into *E. coli* BL21(DE3) competent cells (Invitrogen) and plated on LB agar plates containing 50 µg/mL kanamycin. Alternatively, plasmids were transformed into *E. coli* ArcticExpress™ (DE3) competent cells (Stratagene) and plated on LB agar plates containing 50 µg/mL kanamycin and 20 µg/mL gentamycin. For each transformed mutant, 400 mL of LB medium (Sigma) was inoculated with 2 mL of starter culture and cells were grown to an OD<sub>600</sub> value of 0.6. Induction was carried out with IPTG at a final concentration of 500 µM and cells were typically incubated at 13 °C for 16 hours. The cells were harvested at 4000 g for 15 minutes and the supernatant discarded. The resulting cell pellet (ca. 500 mg) was resuspended in 20 mL lysis buffer (50 mM Tris/HCl, 500 mM NaCl, pH 8.0) and sonicated for 20 minutes. The lysates were centrifuged at 24,000 g for 30 minutes, the supernatant was syringe-filtered (0.45 µm pore size, Sartorius) and the resulting protein solution was loaded onto a 1 mL Ni<sup>2+</sup>-chelation column (Hitrap, GE Healthcare). The column was washed with 20 mL of binding buffer (50 mM Tris/HCl, 10 mM imidazole, 500 mM NaCl, pH 8.0) to remove unbound proteins from the column. The partially purified recombinant protein (rPOMGnT1, see also **Figure 2a**) was eluted with 2 mL of elution buffer (50 mM Tris/HCl, 500 mM imidazole, 500 mM NaCl, pH 8.0), and concentrated to 100 µL using centrifugal concentrators (Sartorius, 30 kDa MWCO).

### *SDS-PAGE and Western blotting*

The partially purified mutant rPOMGnT1 proteins were incubated with SDS-PAGE buffer at 95 °C for 10 minutes, then separated by SDS-PAGE and visualized by Coomassie staining. Protein concentrations of the expressed mutant rPOMGnT1 ranged from 50 to 250 µg/mL and calculated by densitometric comparison with a 70 kDa protein standard (300 µg/mL) using ImageJ analysis software [38]. For Western blotting, protein gels were transferred to nitrocellulose using a semi-dry blotting apparatus. After blocking with 0.5% (w/v) bovine serum albumin, the membranes were incubated with peroxidase conjugated mouse anti-polyHistidine antibody (anti-His; 1:1000, Sigma); after washing, recombinant protein was visualized chromogenically using the SigmaFAST™ 3,3'-diaminobenzidine (DAB) reagent.

### *Assay of Recombinant rPOMGnT1 variants*

The mannopeptide acceptor substrates were derived from the sequence of human  $\alpha$ -dystroglycan, a glycoprotein that plays a role in the formation of a transmembrane link between laminin and dystrophin [39]. Sequences corresponding to Ser/Thr-rich regions of the protein were selected for solid phase peptide synthesis (see **Figure 3a-c**, chemical synthesis described in the **Supplementary Information**).

For testing the enzymatic activity of the wild type rPOMGnT1 variant towards the chemically synthesized acceptor substrates, 5  $\mu$ L of partially purified enzyme solution was added to 5  $\mu$ L of 1 mM acceptor, 2  $\mu$ L of 0.4 M MES, pH 7, 2  $\mu$ L of 0.2 M MnCl<sub>2</sub>, 6  $\mu$ L of 1 mM UDP-GlcNAc and incubated for 16 hours at 37 °C; a volume of 0.2  $\mu$ L from the reaction mix was analyzed using a Voyager-DE STR MALDI-ToF mass spectrometer (Applied Biosystems) with 2,5-dihydroxybenzoic acid as matrix.

The HPLC-based enzymatic assay for testing the activity of the different rPOMGnT1 variants was performed with the synthetic mannopeptide Ac-ATPT•PVTAIG-OH (T• indicates the  $\alpha$ -mannosylated threonine); in contrast to the other tested mannopeptides, this acceptor substrate allowed, after optimizing of the solvent gradient and the type of reverse phase column, baseline separation between substrate and product peak. The different retention times of the mannopeptide substrate and GlcNAc-manno-peptide product (17.7 and 18.5 minutes, respectively) could be achieved using a C8 reversed phase column (Phenomenex Spherclone, 250 x 2 mm, 3  $\mu$ m pore size) with a flowrate of 0.2 mL/minutes. Solvent A was 0.1% trifluoroacetic acid in H<sub>2</sub>O, solvent B 0.1% trifluoroacetic acid in acetonitrile. The program used is as follows: 0–10 minutes, 100% solvent A; 10–40 minutes, linear gradient to 50% solvent and then returning to solvent A (reconditioning for 20 minutes). The elution of the glycopeptides was monitored via UV detection at 214 nm and electrospray MS detection (Agilent System 1100 LC/MSD SL).

For the determinations of the  $K_m$  values of both UDP-GlcNAc and the mannopeptide Ac-ATPT•PVTAIG-OH an enzymatic assay was performed with a series of different substrate concentrations. For determination of the  $K_m$  value of the acceptor substrate for an active rPOMGnT1 mutant, assays were performed for 4 hours and 37 °C using variable concentrations of the mannopeptide (2 mM to 10 mM), and a constant UDP-GlcNAc concentration of 10 mM. After heat-inactivation at 95 °C, the reaction mix was analyzed using the HPLC method described above. All experiments were performed in triplicate, and the kinetic data were calculated by using the relative peak areas corresponding to acceptor substrate and product.

### *Homology Modeling of POMGnT1*

As the crystal structure of POMGnT1 has not yet been solved, a homology model of POMGnT1 was built based on the known crystal structure of rabbit *N*-acetylglucosaminyltransferase I (GnTI) encoded by the MGAT1 gene [40]. The catalytic domains of human POMGnT1 and GnTI show 45% homology over 159 amino acid residues [41]. All modeling was performed using Accelrys Discovery Studio 2.0 with default parameters (cut overhangs, no disulfides, no ligands, no loop refining). In order to identify a homologous structure, a BLAST search of the POMGnT1 amino acid sequence was performed against all sequences in the PDB database [42]. The top hit was found to be 1FOA, which corresponds to the catalytic fragment of GnTI. The sequence of POMGnT1 was then aligned with the sequence of GnTI using the 'Align Multiple Sequences' protocol. The overall sequence identity was found to be 18.8% and the sequence similarity was 33.5%. The homology model was then generated based on the sequence alignment and structure of GnTI using the 'Build Homology Models' protocol. A final alignment was created in which the POMGnT1 sequence was aligned with the



sequence of the homology model.

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## Results

### *Human rPOMGnT1 can be expressed catalytically active in E. coli*

Although the recombinant expression of POMGnT1 was typically performed either as a full-length protein in mammalian cell culture [22, 41], or in soluble form in insect cells, lacking the first 226 residues [43], our strategy was to assess the expression capabilities of the prokaryotic host *E. coli* using a form lacking the first 65 residues. Initial experiments were performed using transformed BL21(DE3) competent cells, the most common host strain for protein expression. Culture samples were taken after 3, 8 and 16 hours of induction with 500  $\mu$ M IPTG at 16, 25, and 30 °C. The highest expression levels were observed after 8 hours of induction at 16 °C, judged by anti-His Western blot. Those conditions were used for a purification experiment using Ni<sup>2+</sup>-chelation chromatography, and the elution fraction analyzed by Western blot showed a band migrating between 70 and 100 kDa, indicating the soluble expression of the recombinant rPOMGnT1 protein. Although a whole series of activity tests was performed, which systematically tested different mannopeptides, substrate and metal ion concentrations, no catalytic activity could be determined for rPOMGnT1 expressed in BL21(DE3) cells. Therefore, a new attempt was made using ArcticExpress™ (DE3) cells, which are in comparison to other strains re-engineered for protein expression at lower temperatures, thereby potentially facilitating increased yield and activity of the recombinant protein [44].

Expression experiments were performed at 13 °C using 500  $\mu$ M IPTG and cells were harvested 16 hours after induction. Anti-His Western blots of the cell lysate and from the eluate after Ni<sup>2+</sup>-chelation chromatography showed both a band migrating around 80 kDa, indicating soluble expression of rPOMGnT1. In contrast to the expression in BL21(DE3) cells, partially purified rPOMGnT1 from ArcticExpress™ (DE3) cells showed catalytic activity. Activity tests with a final concentration of 250  $\mu$ M of the mannopeptide Ac-ATPT•PVTAIG-OH and a 1.2-fold excess of the donor substrate UDP-GlcNAc showed 20-25 % conversion to product within 4 hours. More than 95% conversion could be achieved after 48 hours of incubation.

### *Wild type rPOMGnT1 was active towards some, but not all synthesized mannopeptides*

In order to establish that peptide sequences in  $\alpha$ -DG are indeed substrates for POMGnT1, a library of mannopeptides was generated which correspond to sequences of the mucin-like stem region of  $\alpha$ -DG (**Figure 3b**). Thus, wild type POMGnT1 was screened against a library of 21 mannopeptides produced by solid phase peptide synthesis (SPPS), which was optimized for the introduction of Fmoc-protected mannosyl serine and mannosyl threonine building blocks (**Figure 3a**). 20 of these peptide sequences were chosen according to the serine/threonine-rich sequence of human  $\alpha$ -DG, including three mannopeptides based on the region surrounding Thr319. In total, three variants corresponding to this latter region were used: (i) Ac-ATPT•PVTAIG-OH, (ii) Ac-ATPT•PVTAIGP-NH<sub>2</sub> and (iii) the modified Ac-AAPT•PVAAP-NH<sub>2</sub>, featuring an exchange of Thr317 for alanine, adopted by Takahashi *et al.* [45] in order to avoid modification by a background O-GlcNAc-transferase activity and by proteases present in brain extracts (**Figure 3c**). All three peptides based on the region around Thr319 acted as substrates in a comparable manner; in particular, only one GlcNAc residue was transferred and no proteolysis was observed. Based on this finding, and also as *E. coli* is known to have no endogenous O-GlcNAc transferase activity, the kinetic studies were performed with the first of these peptides, *i.e.* that without the threonine to alanine and C-terminal modifications.



Surprisingly, rPOMGnT1 only displayed catalytic transfer towards six of the screened mannopeptide substrates. None of the mannopeptides containing mannosyl serine were glycosylated and all the positive substrates contained a proline which was C-terminal of the acceptor mannosyl threonine, although such sequences were also observed in the mannopeptides which were not substrates for POMGnT1. Moreover, small variations in the mannopeptide sequences could affect the substrate specificity of POMGnT1. For example, for mannosylated isomers of the H-*TTTTTRRPTK*-OH peptide, only modifications in position 2, 3 and 4 of the penta-threonine motif, but unexpectedly not positions 1 and 5, were accepted as substrates (**Figure 3c**). Nevertheless, these studies allowed us to select a natural mannopeptide fragment of  $\alpha$ -DG which could be used for further kinetic studies of POMGnT1 mutant activities.

#### *Some rPOMGnT1 variants containing clinically relevant mutations show in vitro activity*

A number of clinically relevant POMGnT1 mutations have been described in the literature, ranging from nonsense/frameshift mutations to about 14 different point mutations which encode full length proteins as well as truncated proteins caused by nonsense mutations within the C-terminal region [22, 31-33, 46-53]. **Table 1** gives an overview of described homozygous and compound heterozygous mutations described up to date in the literature.

When expressed in *E. coli*, the 14 point mutant variants and the wild type rPOMGnT1 showed similar expression levels ranging from approx. 20-100  $\mu$ g of rPOMGnT1 per gram wet cell weight (**Figure 4a**). Activity tests showed that three mutants (Arg265His, Cys269Tyr and Asp556Asn) still had catalytic activity, whereas all other mutations resulted in no activity at all. In comparison with the specific enzymatic activity for wild type rPOMGnT1 (25.5 $\pm$ 5.8 mU/mg), Arg265His and Cys269Tyr showed a 5-fold decrease (5.3 $\pm$ 1.4 and 4.4  $\pm$ 0.3 mU/mg, respectively) and Asp556Asn showed a 2.5-fold decrease (10.8  $\pm$ 1.6 mU/mg) in activity (**Figure 4b-g**). Further kinetic analysis of the wild type and those three active POMGnT1 mutants showed similar  $K_m$  values for UDP-GlcNAc between 2-3 mM. Thus, these mutations do not seem to affect the binding to the donor substrate. However, the  $K_m$  values for the mannopeptide Ac-*ATPT* $\cdot$ *PVTAIG*-OH varied from  $\approx$ 6 mM for the wild type enzyme and Asp556Asn mutant to >25 mM for the mutants Arg265His and Cys269Tyr.

#### *Homology modeling suggests locations of mutations in protein structure*

POMGnT1 was first identified as a homologue of the N-glycan-modifying enzyme GnTI and was indeed initially named in one study as GnTI.2 [22, 41]. Sequence alignment showed that the homology of POMGnT1 to GnTI was much higher in the putative catalytic domain than in N- and C- terminal regions, a finding compatible with a previous study which delineated a minimal catalytic region between residues 299 and 642 [43]. Using this sequence alignment, a three-dimensional model of the putative catalytic subunit of POMGnT1 was constructed based on the crystal structure of GnTI. The distribution of the clinically relevant mutations in the enzyme structure was examined (**Figure 2b-c**), no disease-specific 'hot-spot' on the protein structure was identified - some mutations were close to the putative active site, others some distance away. Nevertheless, Arg265, Cys269 and Asp556 are predicted to be on the periphery of the protein's structure.

## Discussion

Since the correlation between mutations of the glycosyltransferase POMGnT1 in patients with Muscle-Eye-Brain disease was discovered almost a decade ago [22], a continuous stream of case studies have described more than 25 independent homozygous mutations distributed throughout the 21 exonic regions of the POMGnT1 locus [32]. Some of the studied phenotypes widened the spectrum of the disease caused by these mutations to the milder form of limb-girdle muscular dystrophy [31], whereas other POMGnT1 mutations result in symptoms overlapping with the more severe form of Walker-Warburg-Syndrome [32, 33]. The 14 POMGnT1 mutations analyzed for catalytic activity in our study are derived from gene analyses described in 11 different previously-published studies (see **Table 1**). All of these studies showed a diverse subset of diagnostic methods for the determination of the phenotypical findings, such as the evaluation of psychomotoric and mental development, muscle biopsies with immunostaining or immunoblotting, magnetic resonance imaging (MRI), creatine kinase (CK) analysis or biochemical assays for each examined patient. To the best of our knowledge, our study gives the only comprehensive biochemical evaluation of all clinically relevant POMGnT1 point mutations known up to date.

One of the open questions was whether the full loss of enzyme activity is required for the phenotype or if reduced activities also have the potential for pathogenic alterations. In good correlation to the observed genotypes, we show that 3 of the 14 tested mutants display between 17-42% residual activities compared to the wild type enzyme whereas the remaining 11 showed no activity. As noted by others, the Asp556Asn mutation (42% activity as compared to the wild type) introduces a putative *N*-glycosylation site into POMGnT1, which cannot be glycosylated in our *E. coli* expression system; the introduction of such a modification into the human enzyme might account for the comparably less severe phenotype observed for this mutation *in vivo* [31]. Previously this Asp556Asn was assayed from crude extracts of patient fibroblasts.

The mutation Cys269Tyr (17% activity) was observed in one allele of a patient who is a compound heterozygote; the other allele displays a frameshift at base 1077. Thus it appears that POMGnT1 containing this mutation is not able to function properly and, despite a reasonable degree of activity *in vitro*, still results in symptoms; it has been proposed by others that Cys269 lies within the so-called stem region and a expression of full-length form of this mutant resulted in more-or-less no activity in transfected cell lysates [33], whereas a soluble form had 10% [43]. In the case of the other patients, the combination of mutant alleles does not allow us to draw a conclusion as to the *in vivo* effect of the specific mutation which is still compatible *in vitro* activity. The Arg265His mutation (21% activity) was observed in a patient together with mutation Arg311Gln (not active in our assay) on the same allele in addition to mutation Arg442Cys (not active) on the other allele. Although the Arg265His mutation resulted in residual activity, one would expect to observe a severe phenotype in this patient, due to the other mutation observed [53].

The distribution of the analyzed POMGnT1 mutations within the enzyme was visualized using a model of POMGnT1. Therefore, the crystal structure of GnT1, a homologous glycosyltransferase also from GT family 13 was used as a template. Interestingly, the three mutants shown here to be active affect side chains (residues 265, 269 and 556) on the periphery of our homology model. Furthermore, the six mutations in the region between Arg311 and Pro493, which result in an apparent total loss of activity, correspond

to residues conserved also in human GnT1; indeed, based on homology Arg311 itself is postulated to form a key contact with the O-2a atom of the  $\alpha$ -phosphate of the UDP-GlcNAc donor substrate [41]. On the other hand, essential residues affected by three mutations of GnT1 described in the literature (Chinese hamster ovary *lec1* and *Arabidopsis thaliana cgl*) are not represented among the POMGnT1 variants [54]. However, other than the peripheral location of the three 'active' mutations, no absolute disease-specific correlation between biochemical data and the position of a mutation on the protein structure could be determined. Thus, it was concluded that neither genetic data nor structural modeling would allow us to correlate point mutations with severity of disease and that a biochemical analysis is required in each case.

Possibly significant, though, is the location of a predicted cystine bridge, based on that revealed in the crystal structure of GnT1, between Cys421 and Cys490 of POMGnT1 [41]. The reduced activity of the rPOMGnT1 Cys490Tyr mutant tested in this study suggests that Cys-Cys bonding of these residues is essential for the catalytic function of POMGnT1. As disulfide bond formation in the cytoplasm of *E. coli* has been described to be a rare event [55], the presence of this disulphide could explain the initial difficulties of expressing catalytically active wild-type rPOMGnT1 in BL21 (DE3) cells. POMGnT1 had previously been expressed in insect and mammalian cell culture by recombinant methods [22, 41], but full-length expression in mammalian cells has the disadvantage of endogenous GlcNAc transferase activity, which leads to high background and reduced sensitivity. In eukaryotic expression systems, the role of cofactor proteins *in vivo* that may play a regulatory role cannot be excluded and, indeed, fukutin may interact intracellularly with POMGnT1 [56]. However, as wild-type POMGnT1 has no putative N-glycosylation sites, it was a good candidate for expression in *E. coli*. When induced at low temperature, the enzyme was well expressed in a soluble form in bacteria and was shown to be fully active with  $K_m$  values similar to those reported for the enzyme expressed in other systems [22, 31]. The use of a higher-throughput soluble expression system also allows us to focus on the effect of the mutations on catalytic function without considering the role of the cytosolic, transmembrane or stem regions in intracellular trafficking.

Previous biochemical data indicate that POMGnT1 transfers GlcNAc to mannosylated sites on  $\alpha$ -DG [41, 45]. These *in vitro* studies with either Man( $\alpha$ 1)-O-benzyl or a synthetic glycopeptide such as NH<sub>2</sub>-CYAT<sup>•</sup>AV-OH (not found in  $\alpha$ -DG) and Ac-AAPT<sup>•</sup>PVAAP-NH<sub>2</sub> (similar to  $\alpha$ -DG region 316–321) were performed; in our study, we greatly extended the range of tested substrates. From the 20 mannopeptide sequences chosen, only six were shown to be POMGnT1 substrates. It seems that there is some selectivity of glycosylation by POMGnT1 and perhaps not all potential glycosylation sites on  $\alpha$ -DG are modified by this enzyme – in agreement with the reports on other O-glycans found in  $\alpha$ -DG [37]. Two recent reports describe either three [57] or five [58] O-mannosylated glycan sites in tryptically digested  $\alpha$ -DG peptides isolated from skeletal muscle. These two papers do not, however, describe the same glycosylation sites; this raises the question as to exactly how many sites are mannosylated *in vivo* as well as whether there are species differences. Certainly, further synthesis of mannopeptides to find potential POMGnT1 glycosylation sites is an essential part of further studies to uncover the substrate specificities of enzymes involved in the O-glycosylation of  $\alpha$ -DG.

Kinetic analysis of the wild type and three active POMGnT1 mutants showed  $K_m$  values for UDP-GlcNAc between 2-3 mM, similar to those reported in the literature using other

methods [31]. Thus, these mutations do not seem to affect the binding to the donor substrate. However, the  $K_m$  values for the mannopeptide Ac-ATPT•PVTAIG-OH varied from  $\approx 6$  mM for the wild type enzyme and Asp556Asn mutant to  $>25$  mM for the mutants Arg265His and Cys269Tyr.  $K_m$  values of the two other acceptor substrates Man( $\alpha$ 1-O)-benzyl and the mannopeptide H-CYA T•AV-OH reported in the literature were determined at  $>15$  mM and  $\approx 12$  mM respectively [41]. Perhaps because of using natural substrate sequences, the present kinetic data compare favorably to those previously reported and show that strategies based on glycopeptides sequences derived from protein substrates can lead to significantly improved chemical probes.

In conclusion, the biochemical data obtained from studying clinical POMGnT1 mutants with synthetic glycopeptides derived from glycoprotein substrate sequences should be an important tool to understand mechanism and severity of disease. This methodology should also be applicable to other congenital disorders of glycosylation (such as those caused by mutations of LARGE [37] which may use similar mannopeptide substrates) and might help us in future to generate structure-activity models of the enzyme, by extending our understanding of the molecular basis of these diseases.

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- 1 Bowe, M. A., Mendis, D. B. and Fallon, J. R. (2000) The small leucine-rich repeat proteoglycan biglycan binds to alpha-dystroglycan and is upregulated in dystrophic muscle. *The Journal of cell biology.* **148**, 801-810
- 2 Gee, S. H., Montanaro, F., Lindenbaum, M. H. and Carbonetto, S. (1994) Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell.* **77**, 675-686
- 3 Peng, H. B., Ali, A. A., Daggett, D. F., Rauvala, H., Hassell, J. R. and Smalheiser, N. R. (1998) The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. *Cell adhesion and communication.* **5**, 475-489
- 4 Smalheiser, N. R. and Schwartz, N. B. (1987) Cranin: a laminin-binding protein of cell membranes. *Proceedings of the National Academy of Sciences of the United States of America.* **84**, 6457-6461
- 5 Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K. and Sudhof, T. C. (2001) A stoichiometric complex of neuexins and dystroglycan in brain. *The Journal of cell biology.* **154**, 435-445
- 6 Patnaik, S. K. and Stanley, P. (2005) Mouse large can modify complex N- and mucin O-glycans on alpha-dystroglycan to induce laminin binding. *The Journal of biological chemistry.* **280**, 20851-20859
- 7 Ervasti, J. M. and Campbell, K. P. (1991) Membrane organization of the dystrophin-glycoprotein complex. *Cell.* **66**, 1121-1131
- 8 Ibraghimov-Beskrovnaya, O., Milatovich, A., Ozcelik, T., Yang, B., Koepnick, K., Francke, U. and Campbell, K. P. (1993) Human dystroglycan: skeletal muscle cDNA, genomic structure, origin of tissue specific isoforms and chromosomal localization. *Human molecular genetics.* **2**, 1651-1657
- 9 Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A. and Endo, T. (1997) Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. *The Journal of biological chemistry.* **272**, 2156-2162
- 10 Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A. and Campbell, K. P. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature.* **418**, 417-422
- 11 Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A. and Endo, T. (1998) Detection of O-mannosyl glycans in rabbit skeletal muscle alpha-dystroglycan. *Biochimica et biophysica acta.* **1425**, 599-606
- 12 Endo, T. (1999) O-mannosyl glycans in mammals. *Biochimica et biophysica acta.* **1473**, 237-246
- 13 Krusius, T., Reinhold, V. N., Margolis, R. K. and Margolis, R. U. (1987) Structural studies on sialylated and sulphated O-glycosidic mannose-linked oligosaccharides in the chondroitin sulphate proteoglycan of brain. *The Biochemical journal.* **245**, 229-234
- 14 Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R. and Dell, A. (1998) Structural analysis of sequences O-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified from sheep brain. *The Journal of biological chemistry.* **273**, 23698-23703
- 15 Endo, T. (2007) Dystroglycan glycosylation and its role in alpha-dystroglycanopathies. *Acta Myol.* **26**, 165-170
- 16 Hewitt, J. E. (2009) Abnormal glycosylation of dystroglycan in human genetic disease. *Biochimica et biophysica acta.* **1792**, 853-861
- 17 Saito, F., Blank, M., Schroder, J., Manya, H., Shimizu, T., Campbell, K. P., Endo, T., Mizutani, M., Kroger, S. and Matsumura, K. (2005) Aberrant glycosylation of alpha-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy. *FEBS letters.* **579**, 2359-2363
- 18 Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H. and Brunner, H. G. (2002) Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *American journal of human genetics.* **71**, 1033-1043
- 19 Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K. and Toda, T. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature.* **394**, 388-392
- 20 Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L., Sewry, C. A., Brown, S. C. and Muntoni, F. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. *Human molecular genetics.* **12**, 2853-2861
- 21 van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabe, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C.

- A., Barth, P. G., Brunner, H. G. and van Bokhoven, H. (2005) POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. *Journal of medical genetics*. **42**, 907-912
- 22 Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T. and Endo, T. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev Cell*. **1**, 717-724
- 23 Akasaka-Manya, K., Manya, H. and Endo, T. (2004) Mutations of the POMT1 gene found in patients with Walker-Warburg syndrome lead to a defect of protein O-mannosylation. *Biochemical and biophysical research communications*. **325**, 75-79
- 24 D'Amico, A., Tessa, A., Bruno, C., Petrini, S., Biancheri, R., Pane, M., Pedemonte, M., Ricci, E., Falace, A., Rossi, A., Mercuri, E., Santorelli, F. M. and Bertini, E. (2006) Expanding the clinical spectrum of POMT1 phenotype. *Neurology*. **66**, 1564-1567; discussion 1461
- 25 Murakami, T., Hayashi, Y. K., Ogawa, M., Noguchi, S., Campbell, K. P., Togawa, M., Inoue, T., Oka, A., Ohno, K., Nonaka, I. and Nishino, I. (2009) A novel POMT2 mutation causes mild congenital muscular dystrophy with normal brain MRI. *Brain & development*. **31**, 465-468
- 26 Yanagisawa, A., Bouchet, C., Van den Bergh, P. Y., Cuisset, J. M., Viollet, L., Leturcq, F., Romero, N. B., Quijano-Roy, S., Fardeau, M., Seta, N. and Guicheney, P. (2007) New POMT2 mutations causing congenital muscular dystrophy: identification of a founder mutation. *Neurology*. **69**, 1254-1260
- 27 Godfrey, C., Escolar, D., Brockington, M., Clement, E. M., Mein, R., Jimenez-Mallebrera, C., Torelli, S., Feng, L., Brown, S. C., Sewry, C. A., Rutherford, M., Shapira, Y., Abbs, S. and Muntoni, F. (2006) Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. *Annals of neurology*. **60**, 603-610
- 28 Murakami, T., Hayashi, Y. K., Noguchi, S., Ogawa, M., Nonaka, I., Tanabe, Y., Ogino, M., Takada, F., Eriguchi, M., Kotooka, N., Campbell, K. P., Osawa, M. and Nishino, I. (2006) Fukutin gene mutations cause dilated cardiomyopathy with minimal muscle weakness. *Annals of neurology*. **60**, 597-602
- 29 Brockington, M., Yuva, Y., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Herrmann, R., Anderson, L. V., Bashir, R., Burgunder, J. M., Fallet, S., Romero, N., Fardeau, M., Straub, V., Storey, G., Pollitt, C., Richard, I., Sewry, C. A., Bushby, K., Voit, T., Blake, D. J. and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Human molecular genetics*. **10**, 2851-2859
- 30 Louhichi, N., Triki, C., Quijano-Roy, S., Richard, P., Makri, S., Meziou, M., Estournet, B., Mrad, S., Romero, N. B., Ayadi, H., Guicheney, P. and Fakhfakh, F. (2004) New FKRP mutations causing congenital muscular dystrophy associated with mental retardation and central nervous system abnormalities. Identification of a founder mutation in Tunisian families. *Neurogenetics*. **5**, 27-34
- 31 Clement, E. M., Godfrey, C., Tan, J., Brockington, M., Torelli, S., Feng, L., Brown, S. C., Jimenez-Mallebrera, C., Sewry, C. A., Longman, C., Mein, R., Abbs, S., Vajsar, J., Schachter, H. and Muntoni, F. (2008) Mild POMGnT1 mutations underlie a novel limb-girdle muscular dystrophy variant. *Arch Neurol*. **65**, 137-141
- 32 Hehr, U., Uyanik, G., Gross, C., Walter, M. C., Bohring, A., Cohen, M., Oehl-Jaschkowitz, B., Bird, L. M., Shamdeen, G. M., Bogdahn, U., Schuierer, G., Topaloglu, H., Aigner, L., Lochmuller, H. and Winkler, J. (2007) Novel POMGnT1 mutations define broader phenotypic spectrum of muscle-eye-brain disease. *Neurogenetics*. **8**, 279-288
- 33 Taniguchi, K., Kobayashi, K., Saito, K., Yamanouchi, H., Ohnuma, A., Hayashi, Y. K., Manya, H., Jin, D. K., Lee, M., Parano, E., Falsaperla, R., Pavone, P., Van Coster, R., Talim, B., Steinbrecher, A., Straub, V., Nishino, I., Topaloglu, H., Voit, T., Endo, T. and Toda, T. (2003) Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. *Human molecular genetics*. **12**, 527-534
- 34 Mercuri, E., Messina, S., Bruno, C., Mora, M., Pegoraro, E., Comi, G. P., D'Amico, A., Aiello, C., Biancheri, R., Berardinelli, A., Boffi, P., Cassandrini, D., Laverda, A., Moggio, M., Morandi, L., Moroni, I., Pane, M., Pezzani, R., Pichiecchio, A., Pini, A., Minetti, C., Mongini, T., Mottarelli, E., Ricci, E., Ruggieri, A., Saredi, S., Scuderi, C., Tessa, A., Toscano, A., Tortorella, G., Trevisan, C. P., Uggetti, C., Vasco, G., Santorelli, F. M. and Bertini, E. (2009) Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. *Neurology*. **72**, 1802-1809
- 35 van Reeuwijk, J., Grewal, P. K., Salih, M. A., Beltran-Valero de Bernabe, D., McLaughlan, J. M., Michielse, C. B., Herrmann, R., Hewitt, J. E., Steinbrecher, A., Seidahmed, M. Z., Shaheed, M. M., Abomelha, A., Brunner, H. G., van Bokhoven, H. and Voit, T. (2007) Intragenic deletion in the LARGE gene causes Walker-Warburg syndrome. *Human genetics*. **121**, 685-690
- 36 Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U. and Endo, T. (2004) Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. *Proceedings of the National Academy of Sciences of the United States of America*. **101**, 500-505



- 37 Yoshida-Moriguchi, T., Yu, L. P., Stalnakar, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B. A., Schachter, H., Wells, L. and Campbell, K. P. (2010) O-Mannosyl Phosphorylation of Alpha-Dystroglycan Is Required for Laminin Binding. *Science (New York, N.Y.)* **327**, 88-92
- 38 Abramoff, M. D., Magalhaes, P. J. and Ram, S. J. (2004) Image processing with ImageJ. *Biophotonics International*, 36-42
- 39 Henry, M. D. and Campbell, K. P. (1999) Dystroglycan inside and out. *Current opinion in cell biology*. **11**, 602-607
- 40 Gordon, R. D., Sivarajah, P., Satkunarajah, M., Ma, D., Tarling, C. A., Vizitium, D., Withers, S. G. and Rini, J. M. (2006) X-ray crystal structures of rabbit N-acetylglucosaminyltransferase I (GnT I) in complex with donor substrate analogues. *Journal of molecular biology*. **360**, 67-79
- 41 Zhang, W., Betel, D. and Schachter, H. (2002) Cloning and expression of a novel UDP-GlcNAc:alpha-D-mannoside beta1,2-N-acetylglucosaminyltransferase homologous to UDP-GlcNAc:alpha-3-D-mannoside beta1,2-N-acetylglucosaminyltransferase I. *The Biochemical journal*. **361**, 153-162
- 42 Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic acids research*. **28**, 235-242
- 43 Akasaka-Manyá, K., Manyá, H., Kobayashi, K., Toda, T. and Endo, T. (2004) Structure-function analysis of human protein O-linked mannoside beta1,2-N-acetylglucosaminyltransferase 1, POMGnT1. *Biochemical and biophysical research communications*. **320**, 39-44
- 44 Ferrer, M., Chernikova, T. N., Yakimov, M. M., Golyshin, P. N. and Timmis, K. N. (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. *Nature biotechnology*. **21**, 1266-1267
- 45 Takahashi, S., Sasaki, T., Manyá, H., Chiba, Y., Yoshida, A., Mizuno, M., Ishida, H., Ito, F., Inazu, T., Kotani, N., Takasaki, S., Takeuchi, M. and Endo, T. (2001) A new beta-1,2-N-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian O-mannosyl glycans. *Glycobiology*. **11**, 37-45
- 46 Balci, B., Morris-Rosendahl, D. J., Celebi, A., Talim, B., Topaloglu, H. and Dincer, P. (2007) Prenatal diagnosis of muscle-eye-brain disease. *Prenat Diagn*. **27**, 51-54
- 47 Biancheri, R., Bertini, E., Falace, A., Pedemonte, M., Rossi, A., D'Amico, A., Scapolan, S., Bergamino, L., Petrini, S., Cassandrini, D., Broda, P., Manfredi, M., Zara, F., Santorelli, F. M., Minetti, C. and Bruno, C. (2006) POMGnT1 mutations in congenital muscular dystrophy: genotype-phenotype correlation and expanded clinical spectrum. *Arch Neurol*. **63**, 1491-1495
- 48 Diesen, C., Saarinen, A., Pihko, H., Rosenlew, C., Cormand, B., Dobyns, W. B., Dieguez, J., Valanne, L., Joensuu, T. and Lehesjoki, A. E. (2004) POMGnT1 mutation and phenotypic spectrum in muscle-eye-brain disease. *Journal of medical genetics*. **41**, e115
- 49 Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B., Straub, V., Robb, S., Quinlivan, R., Feng, L., Jimenez-Mallebrera, C., Mercuri, E., Manzur, A. Y., Kinali, M., Torelli, S., Brown, S. C., Sewry, C. A., Bushby, K., Topaloglu, H., North, K., Abbs, S. and Muntoni, F. (2007) Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. *Brain*. **130**, 2725-2735
- 50 Pascual-Castroviejo, I., Pascual-Pascual, S. I., Gutierrez-Molina, M., Saarinen, A., Joensuu, T. H., Bayes, M. and Cormand, B. (2005) [Muscle-eye-brain disease. Presentation of one case with genetic study.]. *Neurologia (Barcelona, Spain)*. **20**, 261-266
- 51 Shenoy, A. M., Markowitz, J. A., Bonnemann, C. G., Krishnamoorthy, K., Bossler, A. D. and Tseng, B. S. Muscle-Eye-Brain disease. *Journal of clinical neuromuscular disease*. **11**, 124-126
- 52 Topaloglu, H., Cila, A., Tasdemir, A. H. and Saatci, I. (1995) Congenital muscular dystrophy with eye and brain involvement. The Turkish experience in two cases. *Brain & development*. **17**, 271-275
- 53 Vervoort, V. S., Holden, K. R., Ukadike, K. C., Collins, J. S., Saul, R. A. and Srivastava, A. K. (2004) POMGnT1 gene alterations in a family with neurological abnormalities. *Annals of neurology*. **56**, 143-148
- 54 Strasser, R., Stadlmann, J., Svoboda, B., Altmann, F., Glossl, J. and Mach, L. (2005) Molecular basis of N-acetylglucosaminyltransferase I deficiency in *Arabidopsis thaliana* plants lacking complex N-glycans. *The Biochemical journal*. **387**, 385-391
- 55 Derman, A. I., Prinz, W. A., Belin, D. and Beckwith, J. (1993) Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science (New York, N.Y.)* **262**, 1744-1747
- 56 Xiong, H., Kobayashi, K., Tachikawa, M., Manyá, H., Takeda, S., Chiyonobu, T., Fujikake, N., Wang, F., Nishimoto, A., Morris, G. E., Nagai, Y., Kanagawa, M., Endo, T. and Toda, T. (2006) Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan. *Biochemical and biophysical research communications*. **350**, 935-941
- 57 Nilsson, J., Nilsson, J., Larson, G. and Grahn, A. Characterization of site-specific O-glycan structures within the mucin-like domain of alpha-dystroglycan from human skeletal muscle. *Glycobiology*. **20**, 1160-1169

58 Stalnaker, S. H., Hashmi, S., Lim, J. M., Aoki, K., Porterfield, M., Gutierrez-Sanchez, G., Wheeler, J., Ervasti, J. M., Bergmann, C., Tiemeyer, M. and Wells, L. Site mapping and characterization of O-glycan structures on alpha-dystroglycan isolated from rabbit skeletal muscle. *The Journal of biological chemistry*. **285**, 24882-24891

59 Soldin, S. J., Murthy, J. N., Agarwalla, P. K., Ojeifo, O. and Chea, J. (1999) Pediatric reference ranges for creatine kinase, CKMB, Troponin I, iron, and cortisol. *Clinical biochemistry*. **32**, 77-80

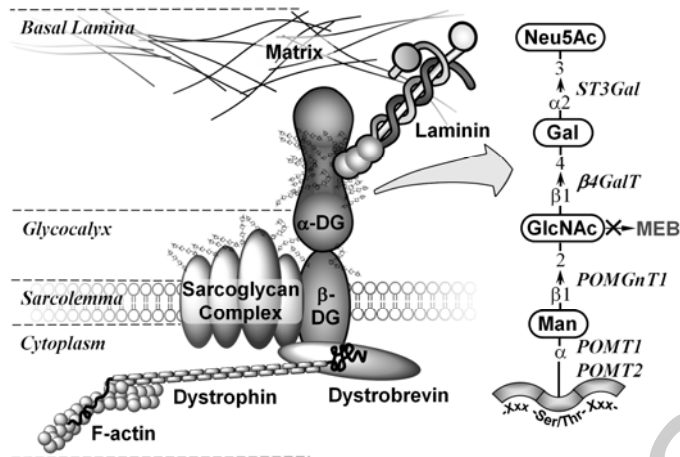
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**Table 1: Description of clinically relevant POMGnT1 mutations.** The following abbreviations were used: *m* male; *f* female; *CK* Creatine kinase measured and given in U/L (normal values in the range of 24–240 for children from 1 to 18 years of age [59]); *nd* information are not available for these patients.

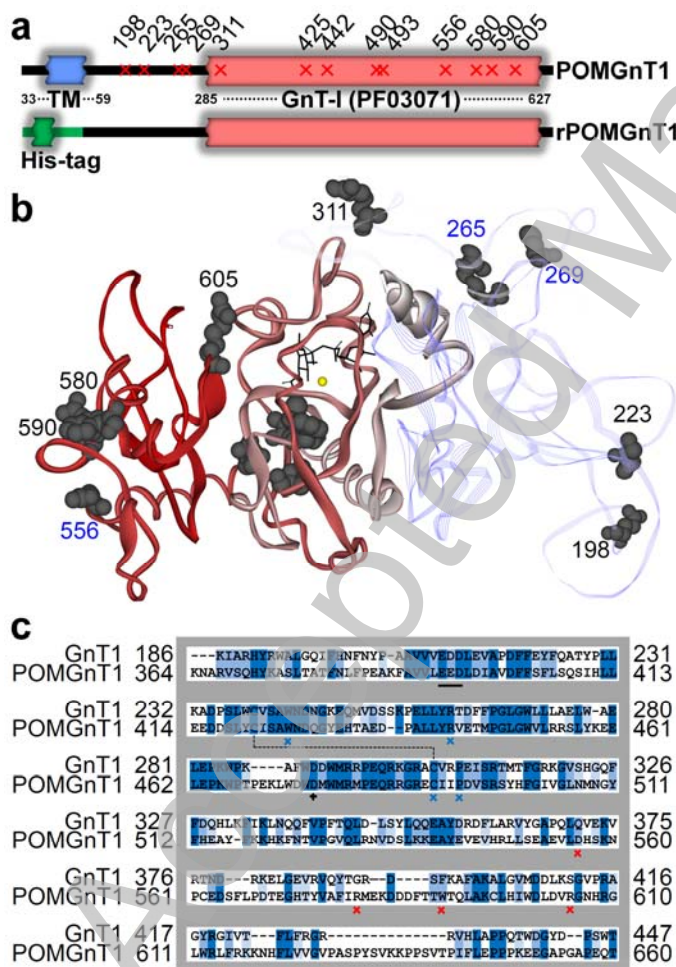
Mutation	Age (sex)	1st allele	2nd allele	CK	Clinical diagnosis	Biopsy	Ref.
1 + 9	7	Ser198Arg	Cys490Tyr	1000	MEB	yes	[47]
2	12 (m)	Glu223Lys	Phe149 frameshift 167 stop	2365	WWS or MEB	nd	[33]
2	8 (f)	Glu223Lys	Mutation in intron 17	1844	MEB or FCMD	nd	[33]
3	7 (f)	Cys269Tyr	Val328 frameshift 338 stop	852	MEB or severe FCMD	nd	[33]
4/5 + 7	11 month (m)	Arg265His Arg311Gln	Arg442Cys	495	Postnatal diagnosis of hydrocephalus	nd	[53]
4/5 + 7	9 (f)	Arg265His Arg311Gln	Arg442Cys	2327	MEB	nd	[53]
4/5 + 13	20 month (f)	Arg265His Arg311Gln	Arg605His	>3000	MEB	yes	[51]
6	17 (m)	Trp425Ser	Mutation in intron 21	nd	MEB	yes	[48, 50]
7	15 months (m)	Arg442Cys	Arg442Cys	2697	MEB	nd	[32]
7	3 months (m)	Arg442Cys	Arg442Cys	1798	MEB	nd	[32]
7	14 (f)	Arg442Cys	Arg442Cys	724	MEB	nd	[32]
7 + 8	2 (m)	Arg442Cys	Arg442His	1000	MEB	nd	[32]
9	nd	Cys490Tyr	Mutation in intron 7	1000	MEB or FCMD	yes	[49]
9	nd	Cys490Tyr	Cys490Tyr	nd	MEB	nd	[48]
10	nd (m)	Pro493Arg	Val262 frameshift 633 stop	nd	MEB	yes	[22]
9 + 11	nd	Trp590 stop	Cys490Tyr	nd	MEB	nd	[48]
12 + 13	5 (f)	Arg580 stop	Arg605His	1953	MEB	yes	[32]
13	16 (f)	Arg605His	Arg605His	9311	MEB	yes	[46, 52]
14	21 (f)	Asp556Asn	Asp556Asn	12000	MEB	yes	[31, 49]

**Figure 1:**  $\alpha$ -DG plays a key role in the formation of the dystrophin glycoprotein complex. Laminin in the basal lamina is binding to O-mannose linked carbohydrates of  $\alpha$ -DG. In muscle cells,  $\alpha$ -DG binds non-covalently to  $\beta$ -dystroglycan in the sarcolemma ( $\beta$ -DG), which is linked to the F-actin cytoskeleton and signalling proteins (dystrobrevin) via dystrophin. Loss of POMGnT1 function results in the truncation of the saccharide structures down to a single O-linked mannose; clinically this loss correlates with muscle-eye-brain disease (MEB) and similar conditions. The sugar phosphate modification of the mannose residue, potentially affected by mutation of *LARGE*, is not shown.



**Figure 2: Native POMGnT1 in comparison to the recombinant form and to GnT1.**

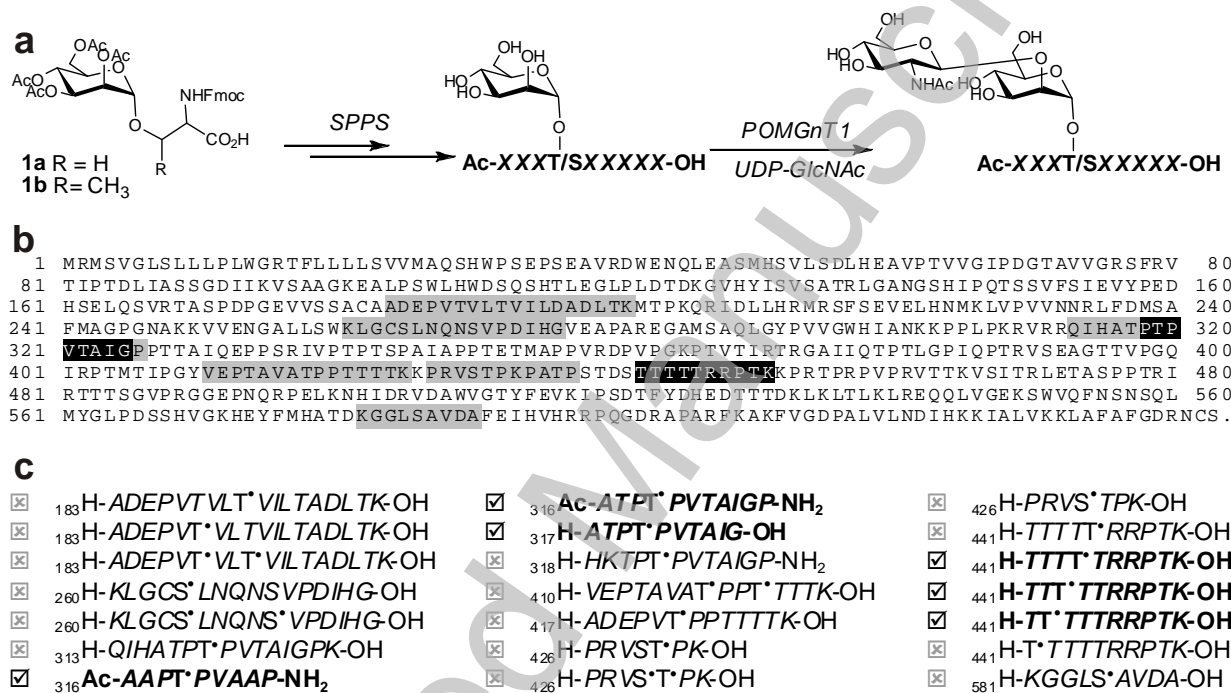
(a) Schematic of native POMGnT1 shows an N-terminal transmembrane domain (TM) and a C-terminal GnT-I domain (Pfam PF03071). The position of mutations introduced are marked with a slanted cross. For expressing the recombinant protein rPOMGnT1 the transmembrane domain was substituted with a His-tag. (b) Homology model of POMGnT1 based on the known crystal structure of GnT1 (1FOA). The modeled C-terminal GnT-I domain is modeled as a red solid ribbon, whereas the non-homologous N-terminal part is displayed as a transparent ribbon loop. Inactive mutants are numbered in black, and mutants with catalytic activity blue. UDP-GlcNAc is shown in stick form. (c) Sequence alignment of POMGnT1 with 1FOA using BLOSUM (default parameters = multiple alignment gap penalty = 10.0, Gap extension penalty = 0.05, Open delay divergent = 40.0) ; comparison of putative catalytic subunits POMGnT1 (R367-T660) and GnT1 (K82-T343) gives a sequence identity (dark blue shading) of 33.2% and a sequence similarity (light blue shading) of 50.3%. Single amino acid changes found in clinical mutants are marked with blue (conserved in GnT1) and red (not conserved in GnT1) crosses; the catalytic amino acid D476 is marked with a black cross; the modified DXD-motif (E393-D395) is underlined with a black bar.



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**Figure 3: Chemical synthesis of mannopeptides derived from  $\alpha$ -dystroglycan.** (a) Solid phase peptide synthesis (SPPS) using Fmoc-protected mannosyl serine (1a) and mannosyl threonine (1b) building blocks for the evaluation of enzyme substrates of POMGnT1. (b) Synthesized peptides corresponding to portions of the amino acid sequence of  $\alpha$ -dystroglycan are highlighted (grey - no enzymatic activity observed, black - enzymatic activity observed). (c) The glycoforms of the synthesized mannopeptide are shown with the glycosylamino acids indicated with superscript dot; those which acted as POMGnT1 acceptor substrates are shown with a tick, while those which did not function are shown with a cross.





**Figure 4: Clinically relevant POMGnT1 mutations of MEB patients and activity studies of the recombinant enzyme.** (a) Western blot analysis of His-tagged rPOMGnT1 mutants expressed in *Escherichia coli*. Molecular weight standards are shown on the left. (b) Assay of active rPOMGnT1 mutants by liquid chromatography. The mannopeptide substrate Ac-ATPT•PVTAIG-OH was incubated for 4 hours with partially purified POMGnT1 wild type and mutant enzymes in the presence of UDP-GlcNAc. The peaks eluting at 17.7 and 18.5 minutes were verified by mass spectrometry to contain the reaction product and substrate, respectively. Characterization of the acceptor substrate Ac-ATPT•PVTAIG-NH<sub>2</sub> (c) and the product after enzymatic reaction of rPOMGnT1 wild-type (d) and mutants (e-g) by MALDI-ToF mass spectrometry.

