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Identification of *DPAGT1* as a new gene in which mutations cause a congenital myasthenic syndrome

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Abstract

Congenital myasthenic syndromes (CMS) are a group of inherited disorders that arise from impaired signal transmission at the neuromuscular synapse. They are characterized by fatigable muscle weakness. This is a heterogenous group of disorders with 15 different genes implicated in the development of the disease. Using whole-exome sequencing we identified *DPAGT1* as a new gene associated with CMS. DPAGT1 catalyses the first step of *N*-linked protein glycosylation. *DPAGT1* patients are characterized by weakness of limb muscles, response to treatment with cholinesterase inhibitors, and the presence of tubular aggregates on muscle biopsy. We showed that *DPAGT1* is required for glycosylation of acetylcholine receptor (AChR) subunits and efficient export of AChR to the cell surface. We suggest that the primary pathogenic mechanism of *DPAGT1*-associated CMS is reduced levels of AChRs at the endplate region. This finding demonstrates that impairment of the *N*-linked glycosylation pathway can lead to the development of CMS.

Keywords

AChR; congenital myasthenic syndrome; *DPAGT1*; glycosylation; neuromuscular junction

Identification of *DPAGT1* as a new gene associated with congenital myasthenic syndrome

Congenital myasthenic syndromes (CMS) are hereditary disorders of neuromuscular transmission.1,2 They are characterized by fatigable muscle weakness that often affects ocular, bulbar, limb, or respiratory muscles. The onset of the disease is usually within infancy or early childhood, but some cases with late onset have been described. The severity of disease varies greatly from mild muscular weakness throughout life to death in early childhood.

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Conflicts of interest
The authors declare no conflicts of interest.

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To date, mutations in 15 different genes have been implicated in the development of CMS.[1,2] These genes encode the presynaptic protein (CHAT), synaptic components (COLQ, AGRN, LAMB2), and postsynaptic proteins (CHRNA1, CHRNB1, CHRNG, CHRND, CHRNE, RAPSN, MUSK, DOK7, GFPT1, PLEC, SCN4A). For the majority of these proteins, their function at the neuromuscular junction has been well established. Some proteins are required for the transmission of the signal from neuron to the muscle, while others are needed for the establishment and maintenance of the synapse structure. However, for some of these proteins, it still remains unclear what their role at the neuromuscular junction (NMJ) is and how mutations in these genes can lead to the development of CMS. For example, *GFPT1* has recently been implicated in the development of CMS.[3] *GFPT1* encodes glutamine-fructose-6-phosphate transaminase 1. This is an enzyme that catalyzes the first and rate-limiting step of the hexosamine pathway. The end product of the hexosamine pathway is uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc)—a nucleotide sugar donor that serves as a substrate for *N*-linked and *O*-linked protein glycosylation, as well as for glycosylation of lipids and proteoglycans. *GFPT1* is ubiquitously expressed and it is not clear how mutations in such an essential gene can specifically lead to a neuromuscular phenotype, without having a more generalized effect.

CMS cases with mutations in the *GFPT1* gene have clinical features that can help distinguish them from the majority of CMS patients.[4] They are characterized by a predominantly limb-girdle pattern of muscle weakness, later onset of the disease, presence of tubular aggregates on muscle biopsies, and response to treatment with cholinesterase inhibitors.

There is a subset of CMS patients with symptoms similar to *GFPT1* patients, but without mutations in *GFPT1*. We have performed whole-exome capture and next-generation sequencing from two of such patients and identified a new gene—*DPAGT1*—in which mutations cause CMS.[5] Screening of a further cohort of CMS patients with varying clinical features allowed us to identify three more patients with mutations in this gene, bringing the total number of patients with *DPAGT1*-associated CMS to five (Table 1). All mutations found in these patients lie in conserved regions of the protein and are likely to be important for protein function (Fig. 1). All five patients have clinical symptoms characteristic of CMS: a decrement on 3-Hz repetitive nerve stimulation and jitter and blocking on single fiber EMG. The most severely affected muscle groups are proximal and distal limb muscles, while the effect on facial, ocular, bulbar, and respiratory muscles is minimal. The age of onset is during childhood, rather than birth or infancy. Similar to *GFPT1* patients, analyzed *DPAGT1* patients have tubular aggregates present on their muscle biopsies. Patients showed a good response to treatment with pyridostigmine (a cholinesterase inhibitor), and two benefited from taking 3,4-diaminopyridine (a drug that increases acetylcholine release from the nerve terminal). Thus, *DPAGT1* patients have clinical features that show similarities to *GFPT1* CMS but should help distinguish them from the rest of CMS patients.

DPAGT1 encodes dolichyl-phosphate (UDP-*N*-acetylglucosamine) *N*-acetylglucosaminephosphotransferase 1 (EC number 2.7.8.15), which catalyses the first committed step of *N*-linked protein glycosylation.[6] *N*-linked protein glycosylation is an essential form of posttranslational protein modification. The pathway of *N*-linked

glycosylation is conserved in all eukaryotes.7,8 It is a multistep process that involves coordinated functioning of multiple proteins (Fig. 2). It starts with the assembly of the core glycan ($Glc_3Man_9GlcNAc_2$) on the lipid dolichol. The assembly of the core glycan happens in the endoplasmic reticulum (ER) and is carried out by a series of membrane bound enzymes. These enzymes sequentially add monosaccharides to the lipid dolichol. Once the core glycan is assembled, it is transferred from the lipid dolichol onto the asparagine residues of nascent proteins. The core glycan can then undergo a series of trimming and extension events that happen in the ER and Golgi and result in the formation of a complex carbohydrate structure found on mature proteins.

Glycosylation of proteins serves many important biological functions including protein folding and stability, intracellular targeting, cell signaling, intercellular recognition, and others.7,9 Thus, it is not surprising that disruption of the glycosylation pathway leads to the development of serious multisystem disorders. Mutations in different proteins involved in glycan biosynthesis have been described, and the resulting conditions are generally called congenital disorders of glycosylation (CDG). The exact subtype of CDG depends on which particular gene has been disrupted. For example, mutations in PMM2 gene lead to the development of PMM2-CDG (or CDG1A), mutations in the MPI gene will lead to the development of MPI-CDG (or CDG1B), and so on. Most of CDGs are multisystem diseases that simultaneously affect various organs. Manifestations include developmental delay, mental retardation, various neuromuscular defects, hormonal abnormalities, and dysmorphic features.9 However, there are notable exceptions of patients who present with a more organ-restricted phenotype. For example, in CDG1B patients with mutations in MPI (mannose phosphate isomerase) neurological symptoms are usually absent and the most affected organs are the gastrointestinal tract and liver.10 On the other hand, the symptoms of CDG1O patients with mutations in DPM3 (dolichyl-phosphate mannosyltransferase polypeptide 3) can be limited to a muscular dystrophy phenotype.11

DPAGT1 transfers the first sugar—GlcNAc—from the UDP-GlcNAc onto the lipid dolichol. 6 Notably, UDP-GlcNAc is an end product of the hexosamine pathway—the pathway containing GFPT1. Thus, both GFPT1 and DPAGT1 are involved in the same cellular process. The human DPAGT1 is 408 amino acids long and is predicted to span the ER membrane ten times12 (Fig. 1). DPAGT1 is essential for survival, as *Dpagt1* knockout mice die shortly after implantation.13 Four patients with mutations in *DPAGT1* have been previously described and all have been classified as CDG1J patients.14–16 Similar to CMS patients, mutations identified in CDG1J patients disrupt conserved amino acids (Fig. 1). All four patients had severe clinical phenotypes that affect multiple organ systems. The first reported patient had delayed development, severe hypotonia, microcephaly, medically intractable seizures, and mental retardation.15 The second and third patients were siblings from a consanguineous family and had delayed development, hypotonia, intractable seizures, and microcephaly, and both died within the first year of life.16 The final patient had severe fetal hypokinesia, moderate multiple contractures, camptodactyly, hypotonia with no spontaneous movement, and died at 1.5 months of age.14 Interestingly, all four patients had severe hypotonia, suggesting that the neuromuscular function in these patients was seriously compromised.

Thus, disruption of normal neuromuscular function is a feature reported for both CDG1J and CMS patients. In terms of other characteristics, the patients with *DPAGT1*-associated CMS differ from CDG1J patients, since they do not display the non-muscle symptoms typical of CDG1J. It is as yet unclear why mutations in the same gene can lead to a different clinical presentation. It is possible that the mutations in the CMS patients are less damaging than the mutations found in CDG1J patients, so that DPAGT1 retains sufficient activity in the majority of tissues. The neuromuscular junction might be especially sensitive to the disruption of *DPAGT1*, so that even a minor reduction in DPAGT1 activity might be sufficient to cause a phenotype. In the future, it will be interesting to measure catalytic activity of different *DPAGT1* mutants and compare mutations leading to the development of CMS to those leading to the development of CDG1J. These experiments should help elucidate how different mutations in the same gene can lead to the development of different disorders.

The role of *DPAGT1* at the neuromuscular junction

Since *DPAGT1* is required for *N*-linked protein glycosylation, it is likely that disruption of this gene leads to a defect in glycosylation of one or several NMJ components. It is known that multiple components of NMJ are glycosylated. Notable examples include the subunits of the AChR receptor, Agrin and Musk (both of which are essential for clustering of AChR receptors at the endplate region), and laminin.17

To establish the effect that mutations in *DPAGT1* have on the structure of NMJ, we analyzed muscle biopsies from patients with *DPAGT1* mutations. The endplates from these patients displayed two notable abnormalities.5 First, the amount of postsynaptic folding present at the endplate region was reduced fivefold compared to control muscles. The reduction in postsynaptic folds is likely to coincide with a decrease in the number of available voltagegated sodium channels that are concentrated in the depths of the postsynaptic folds. This will increase the threshold depolarization required for triggering the action potential. 18,19 Second, the amount of acetylcholine receptor present at the endplate of *DPAGT1* patients was decreased. Reduced levels of AChR decrease the sensitivity of the postsynaptic membrane to the neurotransmitter acetylcholine. Taken together, these two defects reduce the safety factor for neuromuscular transmission and are sufficient to explain the muscle weakness observed in the patients. Notably, similar abnormalities of the NMJ ultrastructure are characteristically observed in AChR deficiency patients, where the amount of AChR that is present at the NMJ is significantly reduced.20–22 Both AChR deficiency patients and *DPAGT1* CMS patients benefit from treatment with cholinesterase inhibitors.2,5 In combination, these results indicate that for patients with mutations in *DPAGT1*, the likely primary cause of the weakness is a reduction of AChR at the endplate region.

Adult AChR is a pentameric receptor consisting of two alpha, one beta, one delta, and one epsilon subunits. The pentamer is assembled in the ER.23 All subunits of the AChR undergo *N*-linked glycosylation in the ER, which is required for appropriate assembly of the pentamer and for the insertion of the AChR into the plasma membrane.24–26 It is possible that disruption of DPAGT1 function leads to abnormal glycosylation of AChR subunits and therefore prevents insertion of the receptor into the plasma membrane.

Using the DPAGT1-specific inhibitor tunicamycin, we showed that DPAGT1 is indeed required for glycosylation of AChR subunits.5 Addition of tunicamycin to cells transfected with AChR subunits led to a loss in subunit glycosylation, while overexpression of wild-type *DPAGT1* in these cells rescued the inhibition with tunicamycin. In accordance, treatment of cells with tunicamycin resulted in a fivefold reduction in the amount of AChR inserted into the plasma membrane compared to non-treated cells. At the same time, overexpression of wild-type DPAGT1 protein in these cells restored the normal levels of AChR inserted into the plasma membrane. These results demonstrate that DPAGT1 is required for glycosylation of AChR subunits and for export of AChR to the cell surface. This proposed pathogenic mechanism is in keeping with the phenotype observed in the patient muscle biopsies, where the levels of AChR in the endplate region are reduced.

We thus propose that the primary defect in *DPAGT1* CMS patients is loss of AChR from the NMJ region due to deficient glycosylation of AChR subunits. It is likely that the same mechanism explains the neuromuscular weakness observed in *GFPT1*-associated CMS. It is not yet clear whether glycosylation of other NMJ proteins is affected in *DPAGT1* patients, and what effect they might have on the development of the disease.

Tubular aggregates

An interesting feature common to both *DPAGT1* and *GFPT1* CMS patients is the presence of tubular aggregates on muscle biopsy. To date all analyzed *DPAGT1* patients and the majority of *GFPT1* patients have this feature. The exact nature of tubular aggregates is as yet unknown. They are usually characterized as structures composed of membranes and protein, and they are believed to arise from the membranes of sarcoplasmic reticulum, and are thought to be filled with unfolded or misfolded protein.27,28 It is possible that in CMS patients with mutations in *DPAGT1* and *GFPT1* genes, several cellular proteins become abnormally glycosylated, fail to be properly folded, and accumulate in the sarcoplasmic reticulum, leading to the formation of tubular aggregates. It will be of interest to determine the molecular composition of tubular aggregates in the muscles from these CMS patients.

Conclusions and future perspectives

Our recent identification of *DPAGT1* as a novel gene in which mutations cause CMS, together with the recently published papers on *GFPT1*-associated CMS, highlight a new pathway leading to the development of NMJ disorders. Both *DPAGT1* and *GFPT1* are involved in protein glycosylation, which emphasizes that glycosylation plays a crucial role in correct functioning of the NMJ. The reduction of endplate AChR explains why the patients respond well to treatment with cholinesterase inhibitors. It is possible, however, that other proteins apart from AChR subunits might also be abnormally glycosylated in *DPAGT1* and *GFPT1* patients.

The protein glycosylation pathway is a multistep process involving many different proteins. Since mutations in two of the genes have already been shown to lead to the development of CMS, it is likely that disruption of other genes in the pathway might lead to the development of similar phenotypes. Future genetic testing will help identify the genes involved and the

exact clinical features associated with the disruption of these genes. Another interesting avenue for investigation is to understand why the symptoms of *DPAGT1*- and *GFPT1*-associated CMS are limited to NMJ function, and how different mutations in the same gene (*DPAGT1*) can lead to the development of two very different phenotypes found in CMS and CDG1J. In particular, it will be interesting to compare protein stability, catalytic activity, and the degree of impairment of AChR glycosylation caused by these mutations.

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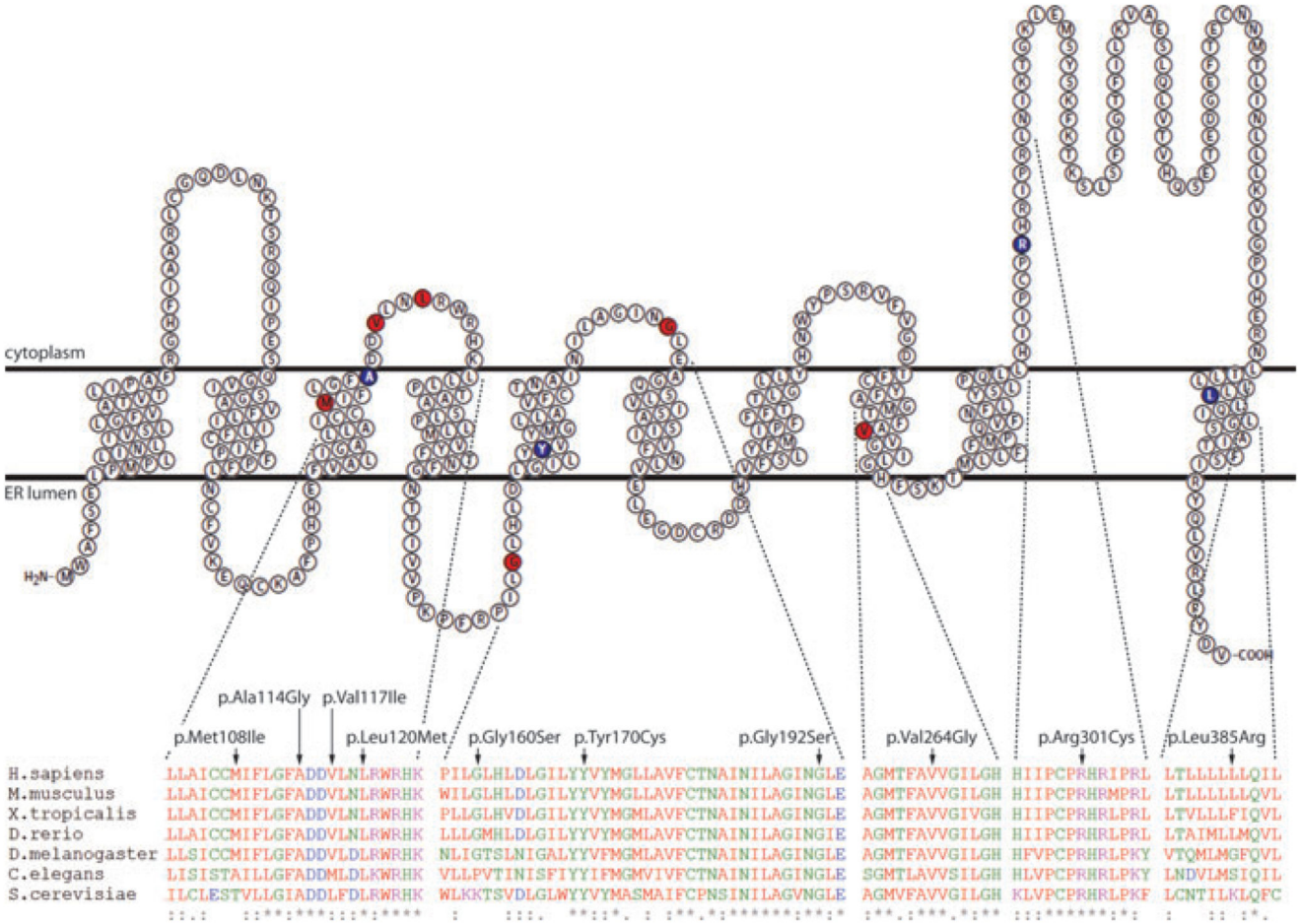

Figure 1.

Predicted membrane topology of the DPAGT1 protein. Residues that are mutated in CMS patients are shown in red. Residues that are mutated in CDG1J patients are shown in blue. Transmembrane structure of the protein was visualized using TEXtopo.29 Multiple sequence alignment was performed using ClustalW.30

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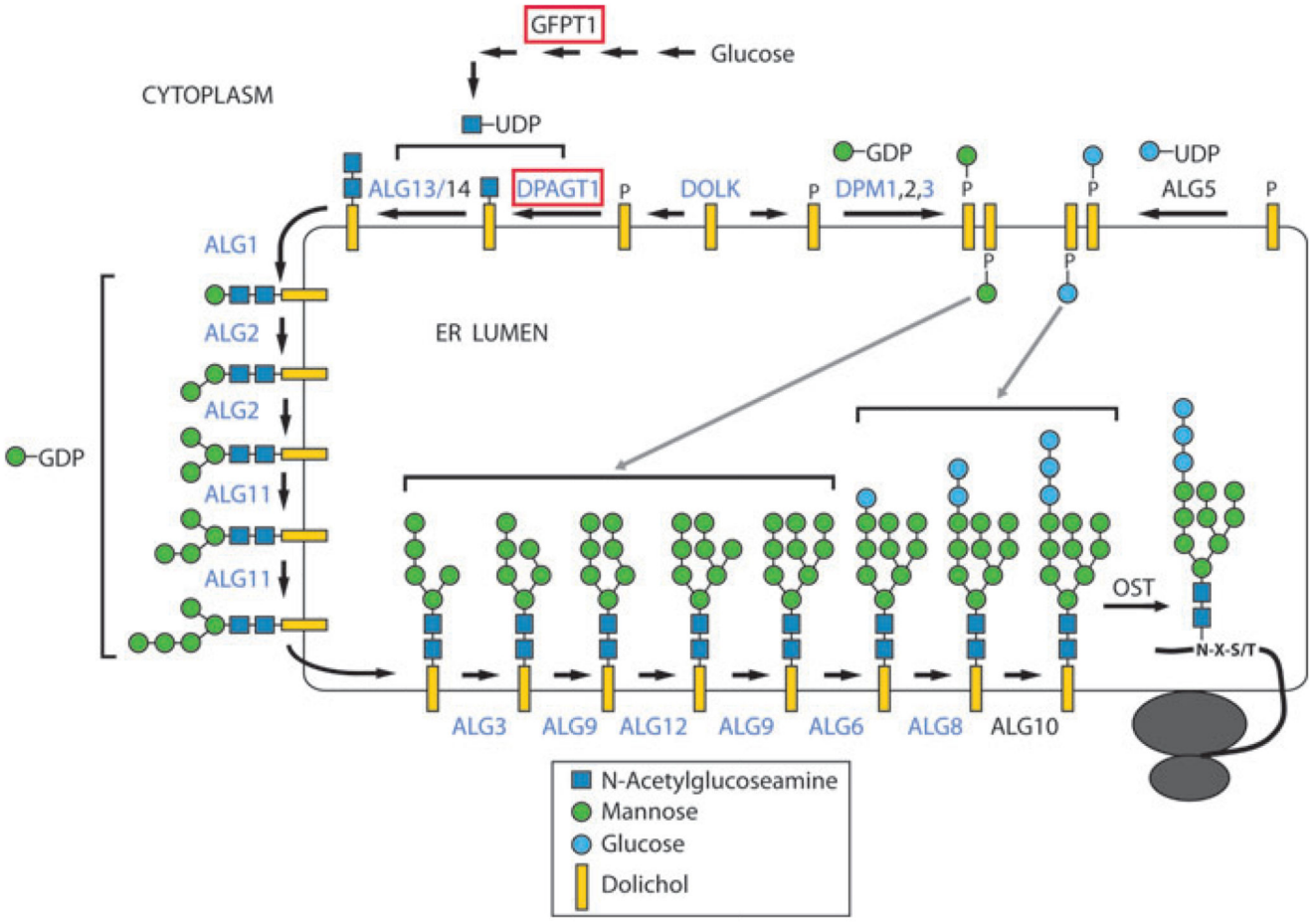

Figure 2.

Schematic representation of the *N*-linked protein glycosylation pathway. *N*-linked protein glycosylation takes place in the ER. It starts with the assembly of the core glycan on the lipid dolichol. Different saccharides are added to the lipid sequentially by different enzymes. The first stage of oligosaccharide assembly takes place on the cytoplasmic face of the ER, where a series of glycosyltransferases uses a cytoplasmic pool of soluble nucleotide sugar donors as substrates for dolichol glycosylation. These nucleotide-monosaccharides are synthesized by multiple cytosolic enzymes, one of which is GFPT1 (involved in the synthesis of UDP-GlcNAc). The first sugar—*N*-acetylglucoseamine (GlcNAc)—is added to dolichol by the enzyme DPAGT1. The second GlcNAc is transferred by the Alg13/Alg14 complex, where Alg13 is a catalytic subunit and Alg14 is an anchoring subunit that targets Alg13 to the ER membrane. Addition of the first mannose is carried out by Alg1, while Alg2 and Alg11 sequentially add four more mannose residues. Next, the resulting Dol-P-P-$GlcNAc_2$ Man_5 intermediate is flipped so that the oligosaccharide is facing inside the ER lumen. This step is carried out by an as yet unknown mechanism. Addition of subsequent sugar moieties is carried out inside the ER lumen. Here, the monosaccharide donor substrates are dolichyl-phosphate–linked monosaccharides Dol-P-mannose and Dol-P-glucose, which are synthesized on the cytoplasmic face of the ER by Dpm1/2/3 and Alg5 enzymes, respectively. Extension of Dol-P-oligosaccharide is carried out by Alg3, Alg9, and

Alg12, which add four mannose residues. The final three glucose residues are added by Alg6, Alg8, and Alg10, completing the assembly of the core glycan. The core glycan is then transferred to asparagine residues of nascent proteins by the multimeric oligosaccharyl transferase complex (OST). The core glycan can then be modified inside the ER and Golgi to yield the final complex saccharide structure found on the mature proteins. Mutations in many of the enzymes of the *N*-linked protein glycosylation pathway have been associated with diseases. In this figure, genes in which mutations are known to lead to the development of CDG disorders are shown in blue. Genes in which mutations cause development of CMS are enclosed in red boxes.

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Table 1

***DPAGT1* mutations found in CMS patients**

	Mutation, DNA	Mutation, protein
Case 1	c.[324G>C]; [349G>A]	p.[Met108Ile]; [Val117Ile]
Case 2	c.[349G>A]; [699dup]	p.[Val117Ile]; [Thr234Hisfs*116]
Case 3	c.[478G>A(;)574G>A]	p.[Gly160Ser(;)Gly192Ser]
Cases 4 and 5	c.[358C>A]; [791T>G]	p.[Leu120Met]; [Val264Gly]