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Congenital Disorders of Glycosylation: an Update on Defects Affecting the Biosynthesis of Dolichol-Linked Oligosaccharides

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Abstract

Defects in the biosynthesis of the oligosaccharide precursor for N-glycosylation lead to decreased occupancy of glycosylation sites and thereby to diseases known as Congenital Disorders of Glycosylation (CDG). In the last 20 years, approximately 1000 CDG patients have been identified presenting with multiple organ dysfunctions. This review sets the state of the art by listing all mutations identified in the 15 genes (*PMM2*, *MPI*, *DPAGT1*, *ALG1*, *ALG2*, *ALG3*, *ALG9*, *ALG12*, *ALG6*, *ALG8*, *DOLK*, *DPM1*, *DPM3*, *MPDU1* and *RFT1*) yielding a deficiency of dolichol-linked oligosaccharide biosynthesis. The present analysis shows that most mutations lead to substitutions of strongly conserved amino acid residues across eukaryotes. Furthermore, the comparison between the different forms of CDG affecting dolichol-linked oligosaccharide biosynthesis shows that the severity of the disease does not relate to the position of the mutated gene along this biosynthetic pathway.

Key Words

Glycosylation, endoplasmic reticulum, CDG, dolichol, glycoprotein

Introduction

N-glycosylation is an essential form of post-translational modification in eukaryotes. Several types of N-glycosylation disorders have been described over the last decade, thereby expanding the list of congenital disorders of glycosylation (CDG) (Freeze, 2006). Looking at the N-glycosylation disorders identified so far, it is reasonable to predict that all genes involved in the biosynthesis of N-glycans are likely to be once associated with a form of CDG. Despite the achievement of the last years, the identification of novel N-glycosylation disorders remains challenging, mainly because of their rarity and because of their rather nonspecific clinical pictures (Leroy, 2006). The widespread application of a simple isoelectric focusing (IEF) test allowing the detection of underglycosylated serum transferrin (van Eijk, et al., 1983) has been instrumental in pointing at potential cases of N-glycosylation disorders. The pattern of transferrin glycoforms obtained by IEF usually allows differentiating between defects of N-glycosylation site occupancy and defects of N-glycan trimming and elongation. These two groups of defects have been originally defined as CDG type-I or CDG-I, and CDG type-II or CDG-II, respectively (Aebi, et al., 1999). Defects in other classes of glycosylation and in sugar-nucleotide transporters have also been designated as CDG-II (Lubke, et al., 2001; Topaz, et al., 2004; Martinez-Duncker, et al., 2005). However, the recent description of glycosylation disorders caused by defects of vesicular transport (Wu, et al., 2004; Foulquier, et al., 2006; Foulquier, et al., 2007), which by definition fall in the category of CDG-II, has prompted for a revision of the CDG nomenclature. Along this line, it has been suggested to abandon the differentiation between CDG-I and -II, and to name the glycosylation disorders by using the official abbreviation of the defective gene (Jaeken, et al., 2008).

Since the first description of mutations in the phosphomannomutase-2 (*PMM2*) gene as causing CDG (Matthijs, et al., 1997), 25 additional disorders of N-glycosylation have been identified (Jaeken, et al., 2008). Clinically, most of these disorders lead to psychomotor

retardation with variable neuromuscular involvement and additional features like hormonal abnormalities and coagulopathies (Leroy, 2006). The severity of these symptoms often varies tremendously, ranging from slight mental retardation to multiorgan dysfunctions often associated to infantile lethality. A typical question arising when comparing the clinical manifestations described is the following: does the severity of a CDG case relate to the position of the defect along the N-glycosylation biosynthetic pathway or rather to the degree of inactivation conferred by the mutation underlying the gene defect? This question can be particularly well examined when considering the glycosylation disorders caused by defective assembly of the dolichol-linked oligosaccharides since this biosynthetic pathway is sequential (Figure 1). Moreover, the assembly of dolichol-linked oligosaccharides is strongly conserved among eukaryotes, thereby enabling the application of asparagine-linked glycosylation (ALG) mutant strains of the yeast *Saccharomyces cerevisiae* as tools for investigating the functional impact of human mutations (Westphal, et al., 2001a; Grubenmann, et al., 2004; Haeuptle, et al., 2008).

The aim of this review is to define the state of the art for the known disorders of dolichol-linked oligosaccharide biosynthesis and especially to discuss the effect of identified mutations on the functions of the affected proteins. To date, 15 gene defects have been described in this group (Matthijs, et al., 1997; Niehues, et al., 1998; Imbach, et al., 1999; Körner, et al., 1999; Imbach, et al., 2000b; Schenk, et al., 2001; Chantret, et al., 2002; Chantret, et al., 2003; Thiel, et al., 2003; Wu, et al., 2003; Frank, et al., 2004; Grubenmann, et al., 2004; Kranz, et al., 2007b; Haeuptle, et al., 2008; Lefeber, et al., 2009). Common for all of these defects is the insufficient supply of dolichol-linked oligosaccharide precursor, which leads to decreased occupancy of N-glycosylation sites. The defective glycosylation reactions can be organized in five categories based on enzymatic activity and on subcellular localization (Fig. 1). The first category comprises the cytosolic enzymes PMM2 and mannose phosphate isomerase (MPI)

(Matthijs, et al., 1997; Niehues, et al., 1998). The second category includes the N-acetylglucosaminyl- and mannosyltransferase enzymes involved in the assembly of the dolichol-linked oligosaccharide at the cytosolic side of the endoplasmic reticulum (ER) membrane (Thiel, et al., 2003; Wu, et al., 2003; Grubenmann, et al., 2004). The third and fourth categories include the mannosyltransferase and glucosyltransferase enzymes that elongate the lumenally-oriented dolichol-linked oligosaccharide (Imbach, et al., 1999; Körner, et al., 1999; Chantret, et al., 2002; Chantret, et al., 2003; Frank, et al., 2004), respectively, whereas the last category comprises the proteins that modify dolichol or affect the availability of dolichol-linked carbohydrates to the assembly pathway (Imbach, et al., 2000b; Schenk, et al., 2001; Kranz, et al., 2007b; Haeuptle, et al., 2008; Lefeber, et al., 2009).

Over the last decade, close to 1000 CDG patients were diagnosed with a disorder of dolichol-linked oligosaccharide assembly, thereby unravelling more than 200 mutations in 15 genes (Table 1). The present review provides a comprehensive overview of the pathway by mapping these mutations on model representations of the affected proteins. By integrating the clinical features associated with each mutation, this overview enables a discussion of the importance of individual proteins in the context of N-glycosylation output and human physiology.

Group 1: Cytosolic enzymes

The glycosyltransferase enzymes of the N-glycosylation pathway use nucleotide- or dolichol-activated monosaccharides as donor substrates. Defects in the biosynthesis of these substrates lead to CDG. The formation of GDP-Man occupies a central stage in the process considering the nine Man residues constituting the N-glycan core (Fig. 1). Accordingly, the cytosolic enzymes PMM2 and MPI, which catalyze the conversion of Man-6-P to Man-1-P and of fructose-6-P to Man-6-P, respectively, are essential members of the N-glycosylation pathway. Note worthily, PMM2 and MPI deficiencies affect additional glycosylation pathways, such as O-mannosylation and glycosylphosphatidylinositol-anchor biosynthesis, which also rely on Man-based donor substrates.

PMM2 (PMM2-CDG, CDG-Ia)

More than 800 patients have been identified with mutations in the *PMM2* gene (PMM2-CDG), thereby constituting the largest group of CDG cases (Table 1). Clinically, neurologic symptoms including psychomotor retardation, developmental delay, epilepsy, ataxia, cerebellar hypoplasia and visual impairment are predominant. Furthermore, symptoms of coagulopathy, hypotonia, cardiomyopathy, gastrointestinal and hepatic problems are also frequently observed. Strong dysmorphic features including severe skeletal deformities are found in most cases (Mizugishi, et al., 1999; Westphal, et al., 2001a; Briones, et al., 2002; Tayebi, et al., 2002; Ono, et al., 2003; Coman, et al., 2005; Schollen, et al., 2007; Vermeer, et al., 2007; Wurm, et al., 2007; Truin, et al., 2008; Perez-Duenas, et al., 2009; Thong, et al., 2009; Vega, et al., 2009). A mortality rate of over 20% within the first years of life is frequent in cases presenting with low residual PMM2 activity (Matthijs, et al., 2000). The essential role of PMM2 is supported by finding that disruption of this gene in mice leads to early embryonic lethality (Thiel, et al., 2006).

PMM2-CDG is the most frequent form of CDG in respect to the number of mutations identified to date, which sums up to 103 (Supp. Table S1) (Matthijs, et al., 1997; Matthijs, et al., 1999; Mizugishi, et al., 1999; Matthijs, et al., 2000; Grunewald, et al., 2001; Westphal, et al., 2001a; Briones, et al., 2002; Schollen, et al., 2002; Tayebi, et al., 2002; Callewaert, et al., 2003; Ono, et al., 2003; Coman, et al., 2005; Le Bizec, et al., 2005; Vuillaumier-Barrot, et al., 2006; Schollen, et al., 2007; Vermeer, et al., 2007; Wurm, et al., 2007; Truin, et al., 2008; Perez-Duenas, et al., 2009; Thong, et al., 2009; Vega, et al., 2009). Three of them (p.Q37H, p.E197A and p.A233T) occur only in combination with two other heterozygous mutations and are therefore assumed to be single nucleotide polymorphisms. The mutations are scattered over the *PMM2* gene and yield a broad range of protein defects (Fig. 2; Supp. Table S1). A total of 80 missense mutations affect 68 different amino acid residues. Five nonsense mutations introduce an early stop codon and lead to truncated PMM2 proteins. The seven base deletion and insertion mutations identified to date lead to frame shifts and thus to truncated proteins. A total of ten splicing defects have been described, whereas the causative mutations were not only found at the splicing sites, but also as far as 15 kb in intronic regions (Schollen, et al., 2007; Truin, et al., 2008; Vega, et al., 2009). Finally, one patient displayed the complete loss of exon 8 due to a deletion mediated by an Alu retro-transposition (Schollen, et al., 2007).

The human PMM2 enzyme is 246 amino acids long and shows a very high level of amino acid conservation among eukaryotes (Fig. 2). The crystal structure of its isozyme PMM1 has been solved (Silvaggi, et al., 2006), which by extrapolation contributes to assign the impact of mutations on PMM2 protein function and stability. Interestingly, none of the amino acids constituting the active center have been found to be mutated in CDG. However, several mutations have been identified leading to declined substrate or cofactor binding. The arginines at position p.R150, p.R123 and p.R28 in PMM1 (equivalent to p.R141, p.R123 and

p.R21 in PMM2) were shown to be involved in binding either the phosphate or the C2 hydroxyl group of the substrate (Silvaggi, et al., 2006). Substitution of these amino acids in PMM2 (Fig. 2) results in a severe clinical phenotype and it has been shown that homozygosity for the p.R141H mutation is not compatible with life (Matthijs, et al., 1998). The homodimeric interaction of PMM1 is mediated by a hydrophobic core, supported by surrounding hydrogen bonds and salt bridges (Silvaggi, et al., 2006). The mutations p.L104V, p.F119L, p.I120T in PMM2 might disrupt the analogous hydrophobic core (p.L113, p.F128 and p.I129 in PMM1). Furthermore, the mutations p.E93A and p.N101K (p.E102 and p.N110 in PMM1) are suspected to impair the homodimeric interaction by deletion of a hydrogen bond and a salt bridge (Fig. 2) (Silvaggi, et al., 2006). Other mutations have been shown to destabilize the overall protein fold and thereby leading to reduced catalytic activity (Pirard, et al., 1999).

MPI (MPI-CDG, CDG-Ib)

MPI-CDG can be detected biochemically by measuring MPI activity in patients' fibroblasts or leukocytes (Jaeken, et al., 1998). In doing so, 25 patients have been identified so far, exhibiting 18 different mutations in the *MPI* gene (Jaeken, et al., 1998; Niehues, et al., 1998; Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Schollen, et al., 2000a; Westphal, et al., 2001b; Schollen, et al., 2002; Vuillaumier-Barrot, et al., 2002; Penel-Capelle, et al., 2003; Vuillaumier-Barrot, 2005). The clinical presentation of these patients is unique due to the fact that neurological symptoms are usually absent. Mostly affected are the gastrointestinal tract and the liver with symptoms like diarrhoea, vomiting, gastrointestinal bleeding, protein-losing enteropathy, hepatomegaly and hepatic fibrosis. Additionally, coagulopathy, hypoglycaemia and thrombotic events have been observed in moderate to severe cases including at least six lethal outcomes (Jaeken, et al., 1998; Niehues, et al., 1998;

Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Westphal, et al., 2001b; Vuillaumier-Barrot, et al., 2002; Penel-Capelle, et al., 2003). Interestingly, MPI-CDG can effectively be treated by Man supplementation (Niehues, et al., 1998; Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Westphal, et al., 2001b; Penel-Capelle, et al., 2003). The orally applied Man could be phosphorylated by hexokinases yielding Man-6-P, and thereby enabling the functional bypass of the defective isomerase step (Panneerselvam and Freeze, 1996).

The human MPI enzyme is a soluble cytosolic protein of 423 amino acids (Fig. 3). The crystal structure of *Candida albicans* MPI (Cleasby, et al., 1996) suggests that the protein is a metallo-enzyme containing one zinc atom. Whereas most MPI domains show a rather low conservation status among eukaryotes, the central domain, forming the catalytic cleft, is highly conserved (Cleasby, et al., 1996). The human MPI shares 42% sequence homology with its *C. albicans* ortholog and is therefore assumed to present a similar protein fold.

The 18 known mutations (Supp. Table S2) comprise a group of 15 point mutations, while only two frame-shift causing mutations and one splicing defect have been reported. Seven of these missense mutations map to the predicted catalytic domain, which ranges from cysteine p.C11 to phenylalanine p.F151 and from leucine p.L257 to arginine p.R322 based on the *C. albicans* MPI crystal structure (Cleasby, et al., 1996) (Fig. 3). The mutations of strictly conserved residues, such as p.M51T, p.S102L, p.M138T and p.I140T, lie in closest proximity to the active site and might directly impede the enzymatic activity. The p.R295H mutation converts a strictly conserved arginine, which corresponds to p.R304 in the *C. albicans* enzyme. This positively charged residue is positioned at the border of the active site cleft and is proposed to be responsible for substrate phosphate binding (Cleasby, et al., 1996). The remaining missense mutations alter amino acids of various degrees of conservation, thereby leading most probably to a destabilization of the MPI protein fold (Fig. 3).

Group 2: Cytosolically acting glycosyltransferases

The assembly of the dolichol-linked oligosaccharide required for N-glycosylation is initiated on the cytosolic side of the ER membrane and proceeds up to the formation of the intermediate dolichol-PP-GlcNAc₂Man₅ (Fig. 1). Mutations in genes encoding three involved glycosyltransferases, namely *DPAGT1*, *ALG1* and *ALG2*, have been found to cause CDG.

DPAGT1 (DPAGT1-CDG, CDG-Ij)

Deficiency of the UDP-GlcNAc:dolichol-phosphate GlcNAc-1-P transferase initiating the biosynthetic pathway leads to DPAGT1-CDG (Wu, et al., 2003). With only three patients and three mutations identified, it represents a rare form of CDG (Wu, et al., 2003; Vuillaumier-Barrot, 2005). The clinical manifestations associated to DPAGT1 deficiency are unclear since only one of the three patients has been described clinically. This case presented with a developmental delay, microcephaly and exotropia, mental retardation, severe hypotonia and intractable seizures (Wu, et al., 2003). Unfortunately, the clinical description of the two other DPAGT1-CDG patients, a pair of siblings, is not available (Vuillaumier-Barrot, 2005).

The human DPAGT1 is a hydrophobic protein of 408 amino acids that is predicted to span the ER membrane ten times (Fig. 4). The five loops assumed to protrude to the cytosol are mostly conserved among eukaryotes, which supports their function as part of the active center. One of the two identified missense mutations alters a highly conserved isoleucine at position p.I297, which localizes to the last of the five cytosolic loops (Fig. 4; Supp. Table S3). The other point mutation (p.Y170C), which maps to the fifth transmembrane (TM) domain, was found in combination with a splicing defect originating from an unknown genetic reason (Wu,

et al., 2003). The third identified mutation is another splicing defect, assigned to a mutation within the first intron (c.162-8G>A) (Vuillaumier-Barrot, 2005).

Mannosyltransferase 1 (ALG1-CDG, CDG-Ik)

The phenotype of ALG1-CDG is very severe, given that at least four of seven patients died in childhood. Common symptoms are dysmorphic features, microcephaly, intractable seizures, hypotonia, coagulopathy and visual impairment (de Koning, et al., 1998; Grubenmann, et al., 2004; Kranz, et al., 2004; Schwarz, et al., 2004). Individual patients present with nearly the complete set of CDG symptoms including immunoglobulin G deficiency (Kranz, et al., 2004) and recurrent non-immune hydrops fetalis (de Koning, et al., 1998).

The human *ALG1* gene encodes a β -1,4 mannosyltransferase, which catalyses the addition of the first Man to dolichol-PP-linked chitobiose. The 464 amino acid protein is predicted to have a type-I ER membrane topology with a large cytosolic C-terminal domain harbouring the active site (Fig. 5). To date, four *ALG1* mutations have been described (Supp. Table S4) (de Koning, et al., 1998; Grubenmann, et al., 2004; Kranz, et al., 2004; Schwarz, et al., 2004). However, one of three heterozygous point mutations (p.D429E) mapped in a single CDG patient, most likely represents a single nucleotide polymorphism (Grubenmann, et al., 2004). The residual three point mutations are spread all over the cytosolic domain, where they mainly convert weakly conserved amino acids (Fig. 5).

Mannosyltransferase 2 (ALG2-CDG, CDG-Ii)

The rarest form of CDG described to date is the ALG2 mannosyltransferase deficiency with a single patient identified (Thiel, et al., 2003). This patient is only mildly affected with developmental delay, seizures, poor vision, coagulopathy and delayed myelination.

Accordingly, it is not possible to draw any conclusion on the severity of ALG2-CDG from the description of this single case.

The α -1,3 mannosyltransferase ALG2 enzyme is 416 amino acid-long and is predicted to form a type-I TM protein (Fig. 6). Like the ALG1 mannosyltransferase, its active site is cytosolically oriented and uses GDP-Man as donor substrate. Two heterozygous mutations were identified in the *ALG2* gene of the index patient (Supp. Table S5). The first induces a frame-shift through a single base deletion (c.1040delG). The effect of the second mutation is unclear, since the point mutation, c.393G>T, results in an amino acid exchange (p.K131N), but also seems to alter the stability of the *ALG2* transcript (Thiel, et al., 2003).

Group 3: Luminally acting mannosyltransferases

In the ER lumen, three mannosyltransferases catalyze the stepwise addition of the last four Man units ending with the formation of dolichol-PP-GlcNAc₂Man₉. Those three enzymes are hydrophobic proteins with multiple TM domains and use dolichol-P-Man as donor substrate. All of them have been related to a form of CDG.

Mannosyltransferase 6 (ALG3-CDG, CDG-Id)

The clinical presentation was comparable in most of the eleven ALG3-CDG patients and could be summarized as moderate with mainly neurological symptoms. Accordingly, the patients present with a failure to thrive, psychomotor retardation, epilepsy and microcephaly. Facial dysmorphism, hypotonia and visual impairment are also observed in the majority of the cases (Stibler, et al., 1995; Denecke, et al., 2005; Schollen, et al., 2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008). However, a particular feature of this form of CDG constitutes deformations of hands and feet (Denecke, et al., 2005; Schollen, et al.,

2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008), which are not commonly seen in CDG.

After being flipped into the ER lumen, the dolichol-PP-GlcNAc₂Man₅ intermediate is elongated for one Man unit by the *ALG3* encoded α -1,3 mannosyltransferase 6. It is predicted to span the ER membrane seven times within its 438 amino acid sequence (Fig. 7). The asparagines at positions p.N83 and p.N253 represent potential N-glycosylation sites. The TM domains and the loops protruding to the ER lumen display the highest level of conservation among eukaryotes.

The eleven *ALG3*-CDG cases account for eight point mutations and a single splicing defect (Supp. Table S6) (Stibler, et al., 1995; Körner, et al., 1999; Schollen, et al., 2002; Denecke, et al., 2004; Denecke, et al., 2005; Schollen, et al., 2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008). Five of these missense mutations lead to substitutions of highly conserved amino residues within the lumenally oriented loops (Fig. 7). Considering their degree of conservation and their orientation with respect to the ER membrane, these mutated amino acids are likely to be involved in the formation of the active center.

Mannosyltransferase 7-9 (ALG9-CDG, CDG-II)

Only three cases of *ALG9*-CDG have been characterized to date (Frank, et al., 2004; Weinstein, et al., 2005; Vleugels, et al., 2009b). The corresponding patients presented with typical CDG symptoms such as developmental delay, psychomotor retardation, hypotonia, seizures, hepatomegaly, microcephaly and pericardial effusion. Gastrointestinal problems (Vleugels, et al., 2009b) and bronchial asthma (Frank, et al., 2004) were reported in one case. This form of CDG exhibits no unique feature and can be classified as moderate, although the three cases described to date do not allow drawing a conclusion on the clinical picture.

The two identified mutations (Supp. Table S7) occur homozygously in the *ALG9* gene, which codes for a 611 amino acid α -1,2 mannosyltransferase. The enzyme, which is predicted to include eight TM domains and two N-glycosylation sites, catalyzes the addition of the seventh and the ninth Man of the dolichol-linked oligosaccharide. The mutations p.Y287C and p.E523K lie within conserved loops protruding into the ER lumen (Fig. 8) (Frank, et al., 2004; Weinstein, et al., 2005; Vleugels, et al., 2009b).

Mannosyltransferase 8 (ALG12-CDG, CDG-Ig)

Six of the eight characterized ALG12-CDG patients present a similar set of clinical features, including facial dysmorphism, psychomotor retardation, developmental delay, hypotonia and decreased coagulation factors. Prominent also are respiratory impairment, feeding problems and the absence of seizures, gastrointestinal and hepatic symptoms (Chantret, et al., 2002; Grubenmann, et al., 2002; Thiel, et al., 2002; Di Rocco, et al., 2005; Eklund, et al., 2005a). The low levels of serum immunoglobulin G define a possible indicator for mannosyltransferase 8 deficiency. The overall moderate severity of ALG12-CDG was also supported by the observation that some patients were actually able to walk and showed speech involvement (Eklund, et al., 2005a). However, two ALG12-CDG siblings demonstrate a much more severe disease (Kranz, et al., 2007a). In addition to the symptoms previously mentioned, these two patients presented with skeletal dysplasia, generalized oedema and audiovisual impairment. Both patients died within the first two years of life.

The α -1,6 mannosyltransferase 8, encoded by the human *ALG12* gene, transfers the eighth Man from dolichol-P-Man to the dolichol-linked oligosaccharide. The 488 amino acid protein is predicted to include eleven TM domains (Fig. 9). The enzyme is conserved among eukaryotes, whereas TM domains and the predicted loops oriented to the ER display the

highest level of conservation. Two asparagines at positions p.N250 and p.N463 represent consensus sequences for N-glycosylation.

To date, eight ALG12-CDG cases have been described encompassing eleven mutations, whereof nine are missense, one nonsense and a single base deletion causing a frame shift (Supp. Table S8) (Chantret, et al., 2002; Grubenmann, et al., 2002; Thiel, et al., 2002; Di Rocco, et al., 2005; Eklund, et al., 2005a; Kranz, et al., 2007a). Noteworthy, nearly all mutations are mapped to TM domains or to their borders and most of these mutations lead to substitution of highly conserved amino acids (Fig. 9). The siblings, which presented with the more severe clinical features, were compound heterozygous for the p.G101R and the p.R146Q mutations (Kranz, et al., 2007a). While the first mutation is so far unique, the second was already discovered in another case of ALG12-CDG in combination with the p.T67M mutation (Grubenmann, et al., 2002). According to the severe clinical outcome, the p.G101 and the p.R146 residues seem to be essential for ALG12 functionality. This assumption is supported by finding that the glycine at position p.G101 is highly conserved among eukaryotes, while the arginine at position p.R146 is even strictly conserved in all eukaryotic model organisms examined (Fig. 9).

Group 4: Luminally acting glucosyltransferases

The biosynthesis of the dolichol-linked oligosaccharide is completed by the successive addition of three Glc units (Fig. 1). This task is achieved by three ER membrane glucosyltransferases, encoded by the *ALG6*, the *ALG8* and the *ALG10* gene, which utilize dolichol-P-Glc as donor substrate. While defects in the first two enzymes have been associated to CDG, no mutation in the *ALG10* gene has so far been identified.

Glucosyltransferase 1 (ALG6-CDG, CDG-Ic):

The deficiency of ALG6 glucosyltransferase is the second most frequent form of CDG after PMM2-CDG with 36 cases registered so far and representing 20 distinct mutations (Imbach, et al., 1999; Grunewald, et al., 2000; Hanefeld, et al., 2000; Imbach, et al., 2000a; Westphal, et al., 2000a; Westphal, et al., 2000b; de Lonlay, et al., 2001; Schollen, et al., 2002; Newell, et al., 2003; Westphal, et al., 2003; Sun, et al., 2005b; Vuillaumier-Barrot, 2005; Eklund, et al., 2006). The clinical presentation could be described as mild to moderately severe with psychomotor retardation, developmental delay, seizures, hypotonia, coagulopathy, feeding problems and visual impairment. The occurrence of strong dysmorphic features, gastrointestinal problems or protein-losing enteropathy is rather rare.

The ALG6 α -1,3 glucosyltransferase is 507 amino acid-long and is predicted to span the ER membrane eleven times (Fig. 10). The 20 *ALG6* mutations represent nine missense, one nonsense, four splicing and five deletion mutations (Supp. Table S9). In one case, a portion of the chromosome 1 including the *ALG6* gene is deleted as a *de novo* event (Eklund, et al., 2006). Two of the identified point mutations (p.Y131H and p.F304S) are assumed to be single nucleotide polymorphisms (Vuillaumier-Barrot, et al., 2001; Westphal, et al., 2003). While these single nucleotide polymorphisms do not appear to be pathogenic by themselves, they may lead to reduced N-glycosylation when combined to other mutations along the pathway of dolichol-linked oligosaccharide assembly (Westphal, et al., 2002). Surprisingly, three of the five deletion mutations lead to an in-frame removal of isoleucine at position p.I299 (Hanefeld, et al., 2000; Westphal, et al., 2000b; Sun, et al., 2005b). The deletion of three consecutive base triplets in three independent patients determines a deletion hotspot. The amino acids surrounding p.I299 are encoded to some extent by the DNA repeat c.382TAATAAT, which might facilitate deletions. The majority of the mutations affect amino acids positioned within the eleven TM domains (Fig. 10). This fact suggests that TM domains might not only play an

important role in defining the protein structure, but also in catalysis, presumably through the binding to dolichol-linked substrates. Two missense mutations (p.R113H and p.S170I) change strictly conserved amino residues mapping to ER luminal loops, which might constitute part of the active site (Fig. 10).

Glucosyltransferase 2 (ALG8-CDG, CDG-Ih)

Whereas ALG6 deficiency yields a rather mild form of CDG, mutations in the *ALG8* glucosyltransferase gene lead to a severe form of the disease. Five of nine ALG8 deficient patients died within the first months of life (Schollen, et al., 2004; Eklund, et al., 2005b; Stölting, et al., 2009; Vesela, et al., 2009). These cases were accompanied by multiple symptoms like strong dysmorphic features, hypotonia, gastrointestinal disorders, hepatomegaly, coagulopathy, oedema, cardio-respiratory problems, protein-losing enteropathy and ascites. Remarkably, neurologic involvement was either minimal in most of the patients (Chantret, et al., 2003; Schollen, et al., 2004; Eklund, et al., 2005b; Vesela, et al., 2009). However, a pair of siblings displayed a much milder form of ALG8 deficiency. Besides dysmorphic features, they presented with hypotonia, ataxia, mental retardation (Stölting, et al., 2009).

The ALG8 α -1,3 glucosyltransferase is predicted to be an eleven TM domain ER protein with a size of 526 amino acids (Fig. 11). To date, twelve mutations have been described (Supp. Table S10) (Chantret, et al., 2003; Schollen, et al., 2004; Eklund, et al., 2005b; Stölting, et al., 2009; Vesela, et al., 2009). The p.N222S mutation is likely to represent a single nucleotide polymorphism, since the healthy father of a patient is homozygous for this mutation (Schollen, et al., 2004). The other eleven mutations segregate into four missense mutations, one nonsense mutation, three splicing defects and three frame-shift mutations. The missense mutations p.T47P, p.P69L, p.G275D and p.A282V locate to ER luminal domains and lead to

substitutions of strictly conserved amino (Fig. 11), thereby probably disrupting the enzymatic center. The residual mutations, leading to truncated forms of the ALG8 protein, decrease the catalytic ability of the affected enzyme drastically. This might, at least partly, explain the severe progression of ALG8-CDG.

Group 5: Proteins affecting dolichol-linked carbohydrates

In addition to the cytosolic PMM2 and PMI enzymes and the ER glycosyltransferases, a handful of proteins are also involved in the biosynthesis of the dolichol-linked oligosaccharide required for N-glycosylation. Some of these proteins have an established activity, whereas others represent essential components without clearly assigned functions. By phosphorylating dolichol, the dolichol kinase enzyme enables the transfer of GlcNAc-P to dolichol-P, thereby initiating the biosynthesis of dolichol-linked oligosaccharide (Fig. 1) (Shridas and Waechter, 2006). The products of the *DPM* genes form a trimeric complex that catalyses the synthesis of dolichol-P-Man (Maeda and Kinoshita, 2008). In contrast to the previous enzymes, the function of the MPDU1 and RFT1 proteins can only be predicted. MPDU1 and its hamster ortholog Lec35 have been proposed to be involved in the utilization of the sugar donor substrates dolichol-P-Man and -Glc (Anand, et al., 2001; Schenk, et al., 2001). The RFT1 protein has been demonstrated to be involved in the translocation of the dolichol-linked GlcNAc₂Man₅ intermediate into the ER lumen (Helenius, et al., 2002). Although the proteins included in this group have diverse biological functions, they all yield to a CDG phenotype in case of mutation.

Dolichol kinase (DOLK-CDG, CDG-Im)

Four cases of DOLK-CDG have been reported to date (Kranz, et al., 2007b). The loss of dolichol kinase affects the biosynthesis of dolichol-linked oligosaccharides, therefore it is also classified as a form of CDG. All four reported patients died in early childhood (Kranz, et al., 2007b). This very severe clinical phenotype is marked by hypotonia, skin disorders and the loss of hair. Individual patients also presented with cardiomyopathy, seizures, hypoglycaemia, microcephaly and visual impairment (Kranz, et al., 2007b).

The mainly hydrophobic 538 amino acids of the dolichol kinase are predicted to form a 15 TM domain protein, which might be glycosylated at asparagine p.N500 (Fig. 12). This prediction differs slightly with an earlier model, which assigned 13 membrane spanning domains (Shridas and Waechter, 2006). Nevertheless, the N- and the C-termini, as well as the strongly conserved putative CTP-binding pocket (p.S459–p.E474) respect the experimentally proven orientation of the human enzyme regarding the ER membrane (Fig. 12) (Shridas and Waechter, 2006). The level of sequence conservation of the N-terminal region is relatively low, because the *Caenorhabditis elegans* dolichol kinase, with its 281 amino acids, is much shorter than the average dolichol kinases' size in other organisms. This short enzyme overlaps mainly within the C-terminal part, containing the putative CTP-binding domain (Fig. 12). One of the two homozygously occurring point mutations converts a mainly conserved tyrosine (p.Y441S) in the C-terminal part of the kinase (Supp. Table S11). The other mutation leads to the alteration of a non-conserved cysteine at position p.C99 to a serine, this time in the N-terminal part of the enzyme (Fig. 12). The severe outcome of two trivial point mutations indicates that accurate dolichol kinase function is essential for viability. This statement might not only be due to a defect of N-glycosylation since other functions are assigned to dolichol and dolichol-P (Swiezewska and Danikiewicz, 2005).

Dolichol-P-Man synthase (DPM1-CDG, CDG-Ie and DPM3-CDG, CDG-Io)

Recurrent seizures, hypotonia, developmental delay, dysmorphic features, microcephaly, visual impairment and in some cases ataxia and coagulopathy are the most prominent symptoms found in DPM1-CDG patients (Imbach, et al., 2000b; Kim, et al., 2000; Garcia-Silva, et al., 2004; Dancourt, et al., 2006). The individual cases exhibit certain clinical variations, although all in the range of moderate to severe. The index DPM3-CDG patient described recently (Lefeber, et al., 2009) presented with a very mild phenotype. Except for a mild myopathy, a dilated cardiomyopathy, moderate muscular dystrophy and a single stroke like episode the adult patient is able to lead a virtually normal life.

In humans, dolichol-P-Man synthase is an oligomeric enzyme complex assembled by the *DPM1*, *DPM2* and *DPM3* gene products. So far, the 260 amino acid-long catalytic subunit DPM1 and the tethering polypeptide DPM3 have been described as causes of CDG. DPM2 and DPM3, both ER membrane proteins with each two TM domains and a length of 84 and 92 amino acids, respectively, are required to target the cytosolic DPM1 protein to the ER membrane. While DPM3 interacts directly with DPM1, DPM2 stabilizes the complex by binding to DPM3 (Fig. 13) (Maeda and Kinoshita, 2008).

To date, eight DPM1-CDG patients have been described, which represented six distinct mutations (Supp. Table S12) (Imbach, et al., 2000b; Kim, et al., 2000; Garcia-Silva, et al., 2004; Vuillaumier-Barrot, 2005; Dancourt, et al., 2006). Three of the mutations are deletions or splice defects and lead all to a frame-shift and hence to truncated forms of the enzyme. The three point mutations convert single amino acids of various conservation levels at different sites of the DPM1 protein (Fig. 13). In *DPM3*, the homozygous point mutation p.L85S converts a strictly conserved leucine residue within the terminal coiled-coil domain, which is required for tethering the catalytic DPM1 subunit to the ER membrane (Supp. Table S13) (Lefeber, et al., 2009). Notably, mutations in the DPM complex lead not only to a disorder of N-glycosylation, but also O-mannosylation and glycosylphosphatidylinositol-anchor

formation are impaired, since dolichol-P-Man is utilized as donor substrate for these posttranslational modifications as well (Maeda and Kinoshita, 2008).

Man-P-dolichol utilizing defect 1 (MPDU1-CDG, CDG-If)

The clinical outcome of the four described MPDU1-CDG patients is variable. While one patient died in early childhood due to a seizure induced apnea, the others present with mild to moderately severe phenotypes. Shared symptoms are psychomotor retardation, seizures, hypotonia, gastrointestinal problems, visual impairment and, as an atypical hallmark, skin disorders (Kranz, et al., 2001; Schenk, et al., 2001).

The human *MPDU1* gene, whose product is orthologous to the Chinese hamster Lec35 protein, encodes a 247 amino acid ER membrane protein that is predicted to form six TM domains (Fig. 14). Despite its important role in mammalian N-glycosylation, no Lec35 ortholog could be identified in the yeast *S. cerevisiae*. The Man-P-dolichol utilizing defect 1 protein has been proposed to be involved in the lateral distribution of dolichol-P-Man and dolichol-P-Glc within the ER membrane (Schenk, et al., 2001). It is therefore important for the availability of these sugar substrates to the ER mannosyl- and glucosyltransferases. Accordingly, MPDU1 deficiency leads to the accumulation of dolichol-PP-GlcNAc₂Man₅ and dolichol-PP-GlcNAc₂Man₉ intermediates (Kranz, et al., 2001; Schenk, et al., 2001). The four cases of MPDU1-CDG characterized to date revealed five mutations (Supp. Table S14). Four of them are common missense mutations and the fifth is the deletion of a single nucleotide (c.511delC), which leads to a frame-shift (Kranz, et al., 2001; Schenk, et al., 2001). The mutation of the start methionine in one of the patient's allele switches the translation start to the next methionine in a different frame and thus leads to the loss of the MPDU1 protein (Schenk, et al., 2001). The other point mutations affect highly conserved amino acids throughout the protein (Fig. 14).

RFT1 (RFT1-CDG, CDG-In)

Mutations in the *RFT1* gene have only been described recently. They lead to the accumulation of dolichol-PP-GlcNAc₂Man₅ (Haeuptle, et al., 2008; Vleugels, et al., 2009a). The exact function of the RFT1 protein is currently intensively discussed. It is definitely involved in the translocation of the dolichol-linked oligosaccharide GlcNAc₂Man₅ into the ER lumen, but it has still to be demonstrated whether RFT1 is the flippase itself (Helenius, et al., 2002; Frank, et al., 2008). At any rate, mutations in the *RFT1* gene lead to a disorder of N-glycosylation termed RFT1-CDG (Haeuptle, et al., 2008). At least two of the six patients died in childhood, suggesting a severe phenotype. Common clinical features include developmental delay, hypotonia, seizures, feeding problems, dysmorphic features and sensorineural deafness, which could be taken as a characteristic feature. In addition, some patients suffered from coagulopathy, visual impairment and respiratory problems (Haeuptle, et al., 2008; Vleugels, et al., 2009a).

The human RFT1 protein with its 541 amino acids spans the ER membrane eleven times according to prediction algorithms (Fig. 15). The asparagine at position p.N227 might be N-glycosylated. All five identified point mutations convert strongly conserved amino acids (Supp. Table S15) (Haeuptle, et al., 2008; Vleugels, et al., 2009a). Interestingly, one patient is heterozygous for two different point mutations affecting the same nucleotide (c.887T>A/G) and consequently modifying the same amino acid differently (p.I296K/R). All mutated residues are localized to the first three ER lumenally-oriented loops of the RFT1 protein. These loops display, together with the TM domains, an overall high conservation among species and might therefore be important for RFT1 functionality (Fig. 15).

Discussion

The comparison of nearly 1000 cases of deficient dolichol-linked oligosaccharide biosynthesis (Table 1) conveys interesting facts regarding the clinical severity of the disease in relation to the position of the genetic defect along the biosynthetic pathway. The biosynthesis of dolichol-linked oligosaccharides is sequential (Kornfeld and Kornfeld, 1985) (Fig. 1), meaning that a block along the pathway will result in the accumulation of an incomplete oligosaccharide. The oligosaccharyltransferase (OST) complex transfers preferentially the complete oligosaccharide to asparagine residues on acceptor proteins, but incomplete oligosaccharides can to some extent be recognized by OST and transferred to proteins, yet to a low efficiency (Körner, et al., 1999; Cipollo, et al., 2001). It is expected that this efficiency decreases with the degree of incompleteness of the dolichol-linked oligosaccharide. Accordingly, deficiency of ALG3 and ALG9 should be more severe than deficiency of ALG6 and ALG8. Furthermore, deficiency of cytosolically active enzymes like ALG1 and ALG2 should be even more severe, because the accumulating dolichol-linked oligosaccharides remain unavailable to the lumenally-oriented OST complex. This gradation in the level of N-glycosylation output based on the position of the genetic defect is clearly visible in yeast glycosylation mutants (Huffaker and Robbins, 1982; Kukuruzinska and Robbins, 1987; Jackson, et al., 1993; Stagljar, et al., 1994; Burda and Aebi, 1998). However, such a position effect is not clear-cut when examining human CDG cases. This model would predict that the terminal glucosylation defects seen in ALG6 and ALG8 deficiency would be milder than the mannosylation defects seen in ALG3, ALG9 and ALG12 deficiency, for example. However, the association of ALG8 deficiency with a very severe disease (Schollen, et al., 2004) indicates that even dolichol-linked oligosaccharides lacking the terminal two Glc residues accounts for a profound disorder of N-glycosylation. The severity of ALG8-CDG suggests that protein underglycosylation may not be the only defect underlying the disease.

Since the terminal Glc residues of N-glycans are involved in the quality control of glycoprotein folding (Ellgaard and Helenius, 2003), it is possible that glucosylated dolichol-linked oligosaccharides participate to the regulation of this process, too, as for example through the regulation of glucosidase-II activity (Deprez, et al., 2005). Consequently, alteration of glycoprotein folding might also account for the severity of ALG8-CDG.

The absence of ALG10 defects among CDG cases described to date is surprising. The ALG10 enzyme catalyzes the addition of the third Glc (Burda and Aebi, 1998) of the dolichol-linked oligosaccharide (Fig. 1). Assuming that all defects of dolichol-linked oligosaccharide biosynthesis lead to a disorder of N-glycosylation, ALG10 defects would be expected to present with clinical features typical of CDG. Hence, it is puzzling that no case of ALG10 deficiency has been documented yet. On the other hand, this absence of ALG10 deficiency may be explained if the defect indeed does not significantly impair the process of N-glycosylation, thereby remaining clinically undetectable. At the other side of the scale, it could be that *ALG10* is essential for embryonic development and that even a minor decrease of ALG10 activity may not be compatible with life. To further address this question, it is important to continue screening for new forms of CDG.

The identification of additional mutations and gene defects, especially in the glycosylation genes that have not been associated to CDG yet (Fig. 1), will provide additional evidence on the relationship between the extent of N-glycosylation, the position along the biosynthetic pathway and the severity of the clinical picture. Interestingly, a similar absence of position effect is also observed in the forms of congenital muscular dystrophies that are caused by defects of O-mannosylation. In fact, the clinical severity of the disorders is related to the nature of the mutation rather than by the position of the mutated gene along the O-mannosylation pathway (Godfrey, et al., 2007).

Clinical relevance

The analysis of mutations identified to date shows that 150 of the total 203 genetic alterations (Table 1) are missense mutations, whereas 112, i.e. 75%, of these mutations affect highly conserved amino acid residues among eukaryotic proteins. Since conserved amino acids are usually parts of functional motifs (Kinch and Grishin, 2002), it can be assumed that most CDG mutations alter the functions of the affected proteins. Accordingly, mutations of little conserved amino acids would yield minor enzymatic deficiencies that may be accompanied by mild symptoms or even remain asymptomatic. Along this line, it can be predicted that proteins showing the highest degree of sequence conservation among eukaryotes are likely to be more often associated with a disease phenotype. In case of dolichol-linked oligosaccharide biosynthesis, the *PMM2* protein is by far the most conserved protein of the pathway (Fig. 2). Supporting this fact, half of the known mutations (103 out of 203) affect the *PMM2* gene (Table 1). However, other proteins of the pathway also show a high degree of sequence conservation among eukaryotes, as for example the *MPI* and *DPAGT1* proteins (Fig. 3 and 4). Yet, only three mutations have been found in the *DPAGT1* gene so far, indicating that sequence conservation alone does not account for the incidence of a genetic disease.

By examining the occurrence of *PMM2* mutations, Schollen *et al.* (Schollen, et al., 2000b) noticed that the p.R141H mutation is very prevalent in the European population with a carrier frequency of about 1/70. Since this mutation is never found at the homozygous state in CDG patients (Matthijs, et al., 1998) due to its inactivating properties on *PMM2* activity, it can only be assumed that a selection pressure accounts for the maintenance of this mutation in human populations. However, the nature of this selection pressure is unknown and one can only speculate as to whether a reduction of N-glycosylation is related to reducing the susceptibility to pathogens binding N-glycans as receptors. Such arguments are beyond the scope of this

review, but they certainly provide exciting points of reflection when discussing the biological and evolutionary relevance of glycosylation disorders.

Future Prospects

The analysis of mutations in genes involved in dolichol-linked oligosaccharide biosynthesis shows that they mainly affect conserved amino acid residues, thereby impairing protein function. Since a complete loss of protein function is usually not compatible with life, as seen in mice lacking PMM2, MPI and DPAGT1 activity (Marek, et al., 1999; DeRossi, et al., 2006; Thiel, et al., 2006), the mutations encountered in CDG certainly enable a significant level of N-glycosylation output. At the threshold of normal N-glycosylation, the transferrin IEF test may be not sensitive enough to detect N-glycosylation defects caused by mild mutations. The study of these mutations may represent the next challenge in CDG research, since mild disorders of glycosylation are often associated to mild neurological presentations such as slight mental retardation (Giurgea, et al., 2005). The development of new sensitive tests will certainly contribute to determine the true incidence of CDG and to better understand the physiological impact of N-glycosylation.

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Figure legends

Figure 1. Pathway of dolichol-linked oligosaccharide biosynthesis. After phosphorylation (P) of the lipid carrier dolichol (red bar), two GlcNAc (blue box), nine Man (green circle) and three Glc (blue circle) units are successively added by various glycosyltransferases. Nucleotide activated monosaccharides serve as donor substrates for the cytosolically oriented enzymes. After being translocated into the endoplasmic reticulum (ER) lumen, the intermediate dolichol-PP-GlcNAc₂Man₅ is further extended by lumenally acting mannosyl- and glucosyltransferases using dolichol-P activated Man and Glc as sugar building blocks. The complete structure dolichol-PP-GlcNAc₂Man₉Glc₃ is transferred to selected asparagines on newly synthesized glycolproteins by the oligosaccharyltransferase (OST) complex. The gene symbols are indicated next to the catalyzed reactions. The 15 genes associated with CDG are marked in red.

Figure 2. Schematic model of PMM2. PMM2 protein sequences were aligned using the ClustalW program (Thompson, et al., 1994). The *Homo sapiens* sequence (NP_000294.1) was compared to the rodent *Mus musculus* (NP_058577.1), to the zebra fish *Danio rerio* (NP_956378.1), to the fruit fly *Drosophila melanogaster* (NP_648589.1), to the nematode *Caenorhabditis elegans* (NP_502698.2) and to the budding yeast *Saccharomyces cerevisiae* (NP_116609.1). Sequences were obtained from the Genbank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). Black dots represent strictly conserved amino acids and dark gray, light gray, white, those amino acids with a conservation of 83%, 67% and less than 67%, respectively. Mis- and nonsense mutations are marked next to the affect amino acids. The dark red, light red, orange and yellow dots mark the mutated amino acids that are conserved at 100%, 83%, 67% and less than 67%, respectively. Splicing defects are marked with a bar and explicitly entitled in the case of assigned exon skipping. Further or unknown implications on the protein level of the affected enzyme are termed with splicing. Deletion or insertion

mutations causing a frame-shift are marked with a bar as well and entitled as frame-shift, whereas the position of the resulting premature stop codon is given in brackets.

Figure 3. Schematic model of MPI. Mutations and the conservation of the MPI amino acids among the human (NP_002426.1), mouse (NP_080113.1), zebra fish (NP_001028282.1), fruit fly (NP_649940.1), nematode (NP_499174.3) and budding yeast (NP_010918.1) proteins were mapped as in Figure 2.

Figure 4. Schematic model of DPAGT1. Mutations and the conservation of the DPAGT1 amino acids among the human (NP_001373.2), mouse (NP_031901.2), zebra fish (NP_001082880.1), fruit fly (NP_609608.1), nematode (NP_507859.2) and budding yeast (NP_009802.1) proteins were mapped as in Figure 2. The membrane topology prediction of the DPAGT1 enzyme was performed running the TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) (Krogh, et al., 2001), the DAS (<http://www.sbc.su.se/~miklos/DAS/>) (Cserzo, et al., 1997) and the SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) (Hirokawa, et al., 1998) algorithms.

Figure 5. Schematic model of mannosyltransferase 1 (ALG1). Mutations and the conservation of the ALG1 amino acids among the human (NP_061982.3), mouse (NP_663337.2), zebra fish (NP_956161.1), fruit fly (NP_650662.1), nematode (AAC77507.2) and budding yeast (NP_009668.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 1 was performed like for the DPAGT1 enzyme (Fig. 4).

Figure 6. Schematic model of mannosyltransferase 2 (ALG2). Mutations and the conservation of the ALG2 amino acids among the human (NP_149078.1), mouse

(NP_064382.3), zebra fish (NP_001098406.1), fruit fly (NP_647772.1), nematode (NP_495010.2) and budding yeast (NP_011450.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 2 was performed like for the DPAGT1 enzyme (Fig. 4).

Figure 7. Schematic model of mannosyltransferase 6 (ALG3). Mutations and the conservation of the ALG3 amino acids among the human (NP_005778.1), mouse (NP_666051.2), zebra fish (NP_001018532.1), fruit fly (NP_523829.2), nematode (NP_496950.2) and budding yeast (NP_009471.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 6 was performed like for the DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N83 and p.N253 are shown schematically.

Figure 8. Schematic model of mannosyltransferase 7-9 (ALG9). Mutations and the conservation of the ALG9 amino acids among the human (NP_001071158.1), mouse (NP_598742.1), zebra fish (CAN88585.1), fruit fly (NP_651353.1), nematode (NP_496282.2) and budding yeast (NP_014180.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 7-9 was performed like for the DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N77 and p.N593 are shown schematically.

Figure 9. Schematic model of mannosyltransferase 8 (ALG12). Mutations and the conservation of the ALG12 amino acids among the human (NP_077010.1), mouse (EDL04396.1), zebra fish (NP_001092219.1), fruit fly (NP_649939.1), nematode (NP_505071.1) and budding yeast (NP_014427.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 8 was performed like for the

DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N250 and p.N463 are shown schematically.

Figure 10. Schematic model of glucosyltransferase 1 (ALG6). Mutations and the conservation of the ALG6 amino acids among the human (NP_037471.2), mouse (NP_001074733.1), fruit fly (NP_609393.1), nematode (NP_495685.1) and budding yeast (NP_014644.1) proteins were mapped as in Figure 2. The protein sequence of *Tetraodon nigroviridis* (CAG11585.1) was used instead of the zebra fish protein, since only a truncated *D. rerio* isoform could be retrieved from the genome database. Membrane topology prediction of the glucosyltransferase 1 was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N59 is shown schematically.

Figure 11. Schematic model of glucosyltransferase 2 (ALG8). Mutations and the conservation of the ALG8 amino acids among the human (NP_076984.2), mouse (NP_950200.2), zebra fish (NP_001017647.1), fruit fly (NP_572355.1), nematode (NP_001021940.1) and budding yeast (NP_014710.1) proteins were mapped as in Figure 2. Membrane topology prediction of the glucosyltransferase 2 was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N96 is shown schematically.

Figure 12. Schematic model of dolichol kinase (DOLK). Mutations and the conservation of the DOLK amino acids among the human (NP_055723.1), mouse (NP_808316.1), zebra fish (NP_001103954.1), fruit fly (NP_611139.1), nematode (NP_001022925.1) and budding yeast (NP_013726.1) proteins were mapped as in Figure 2. The *C. elegans* dolichol kinase is much shorter than the other eukaryotic orthologs, leading to an overall reduced conservation grade within the N-terminal part. Membrane topology prediction of the dolichol kinase was

performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N500 is shown schematically.

Figure 13. Schematic model of dolichol-P-Man synthase (DPM1/2/3). Mutations and the conservation of the DPM1 amino acids among the human (NP_003850.1), mouse (NP_034202.1), zebra fish (NP_001003596.1), fruit fly (NP_609980.1), nematode (NP_499931.2) and budding yeast (NP_015509.1) proteins were mapped as in Figure 2. Conservation of the DPM2 amino acids among the human (NP_003854.1), mouse (NP_034203.1) and zebra fish (NP_001116318.1) proteins and of the DPM3 amino acids among the human (NP_714963.1), mouse (NP_081043.1), zebra fish (NP_957103.2), fruit fly (NP_001034051.1) and nematode (NP_502366.1) proteins and the DPM3 mutation were also mapped as in Figure 2. Given that the *S. cerevisiae* dolichol-P-Man synthase is a monomeric enzyme (Maeda and Kinoshita, 2008), the DPM2 (NP_595676.1) and DPM3 (NP_596640.1) protein sequences from *Schizosaccharomyces pombe* were used for the particular alignments. Additionally, the aberrant DPM2 sequences of *D. melanogaster* and *C. elegans* were displaced by the DPM2 proteins of *Drosophila ananassae* (EDV40477.1) and *Dictyostelium discoideum* (XP_644349.1), respectively. Membrane topology prediction of the dolichol-P-Man synthase subunits 2 and 3 was performed like for the DPAGT1 enzyme (Fig. 4). The organization of the entire complex was adapted from Maeda *et al.* (Maeda and Kinoshita, 2008).

Figure 14. Schematic model of Man-P-dolichol utilizing defect 1 (MPDU1). Mutations and the conservation of the MPDU1 amino acids among the human (NP_004861.2), mouse (NP_036030.2), zebra fish (NP_001002130.1), fruit fly (NP_608889.1) and nematode (NP_505155.1) proteins were mapped as in Figure 2. Since MPDU1 is neither present in *S. cerevisiae* nor in *S. pombe*, the orthologous protein of *Aspergillus niger* (XP_001401680.1)

was used for the alignment. Membrane topology prediction of the Man-P-dolichol utilizing defect 1 protein was performed like for the DPAGT1 enzyme (Fig. 4).

Figure 15. Schematic model of RFT1. Mutations and the conservation of the RFT1 amino acids among the human (NP_443091.1), mouse (NP_808483.2), zebra fish (XP_688354.3), fruit fly (NP_572246.1), nematode (NP_001023610.1) and budding yeast (NP_009533.1) proteins were mapped as in Figure 2. Membrane topology prediction of the RFT1 protein was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N227 is shown schematically.

Abbreviations

ALG, asparagine-linked glycosylation; CDG, congenital disorders of glycosylation; ER, endoplasmic reticulum; IEF, isoelectric focusing; OST, oligosaccharyltransferase; MPI, mannose phosphate isomerase; PMM, phosphomannomutase; TM, transmembrane

Table 1. Gene defects leading to deficient assembly of dolichol-linked oligosaccharides

Gene	OMIM ^a	Enzyme	Disorder ^b	Disorder ^c	OMIM ^a	Mutations	Patients
<i>PMM2</i>	601785	Phosphomannomutase 2	CDG-Ia	PMM2-CDG	212065	103	> 800
<i>MPI</i>	154550	Mannose phosphate isomerase	CDG-Ib	MPI-CDG	602579	18	>25
<i>DPAGT1</i>	191350	GlcNAc-1-P transferase	CDG-Ij	DPAGT1-CDG	608093	3	3
<i>ALG1</i>	605907	Mannosyltransferase 1	CDG-Ik	ALG1-CDG	608540	4	7
<i>ALG2</i>	607905	Mannosyltransferase 2	CDG-li	ALG2-CDG	607906	2	1
<i>ALG3</i>	608750	Mannosyltransferase 6	CDG-Id	ALG3-CDG	601110	9	11
<i>ALG9</i>	606941	Mannosyltransferase 7-9	CDG-II	ALG9-CDG	608776	2	3
<i>ALG12</i>	607144	Mannosyltransferase 8	CDG-Ig	ALG12-CDG	607143	11	8
<i>ALG6</i>	604566	Glucosyltransferase 1	CDG-Ic	ALG6-CDG	603147	20	>36
<i>ALG8</i>	608103	Glucosyltransferase 2	CDG-Ih	ALG8-CDG	608104	12	9
<i>DOLK</i>	610746	Dolichol kinase	CDG-Im	DOLK-CDG	610768	2	4
<i>DPM1</i>	603503	Dolichol-P mannosyltransferase 1	CDG-Ie	DPM1-CDG	608799	6	8
<i>DPM3</i>	605951	Dolichol-P mannosyltransferase 3	CDG-Io	DPM3-CDG	612937	1	1
<i>MPDU1</i>	604041	Man-P-dolichol utilization defect 1	CDG-If	MPDU1-CDG	609180	5	5
<i>RFT1</i>	611908	RFT1 homolog (<i>S. cerevisiae</i>)	CDG-In	RFT1-CDG	612015	5	6

^a <http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM>

^b According to the recommended nomenclature of 1999 (Aebi, et al., 1999)

^c According to the recommended nomenclature of 2008 (Jaeken, et al., 2008)

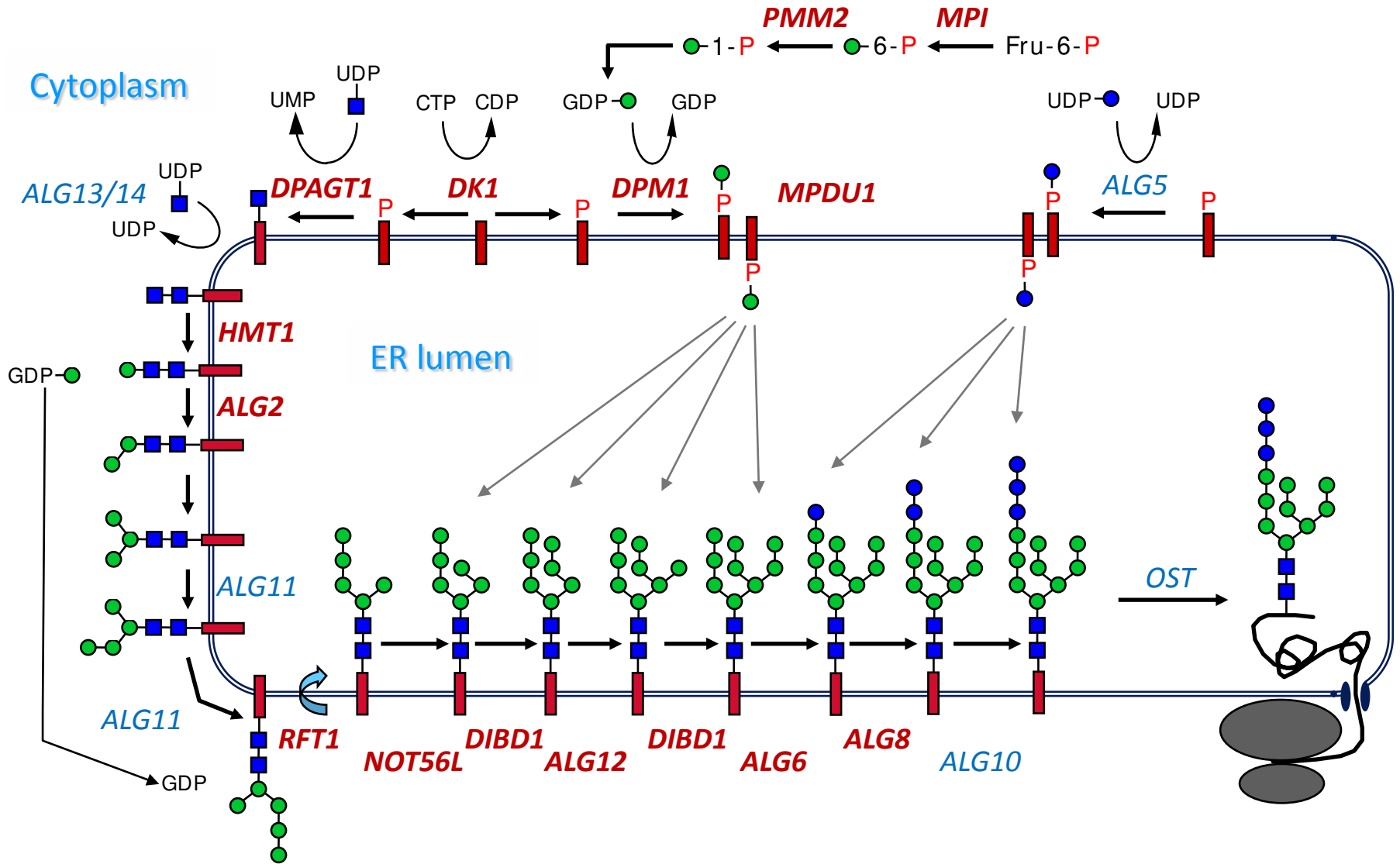


Figure 2 (PMM2)

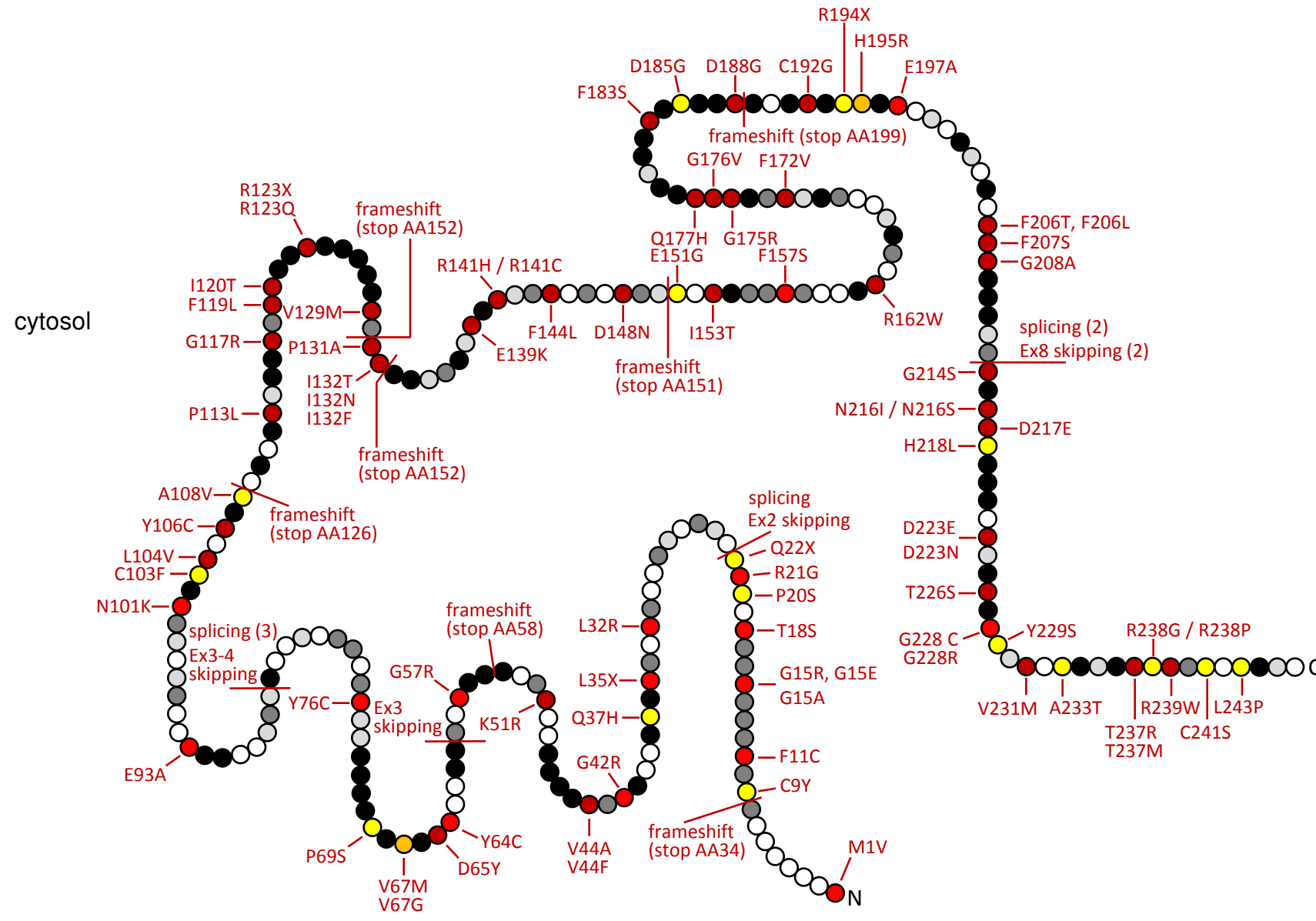


Figure 3 (MPI)

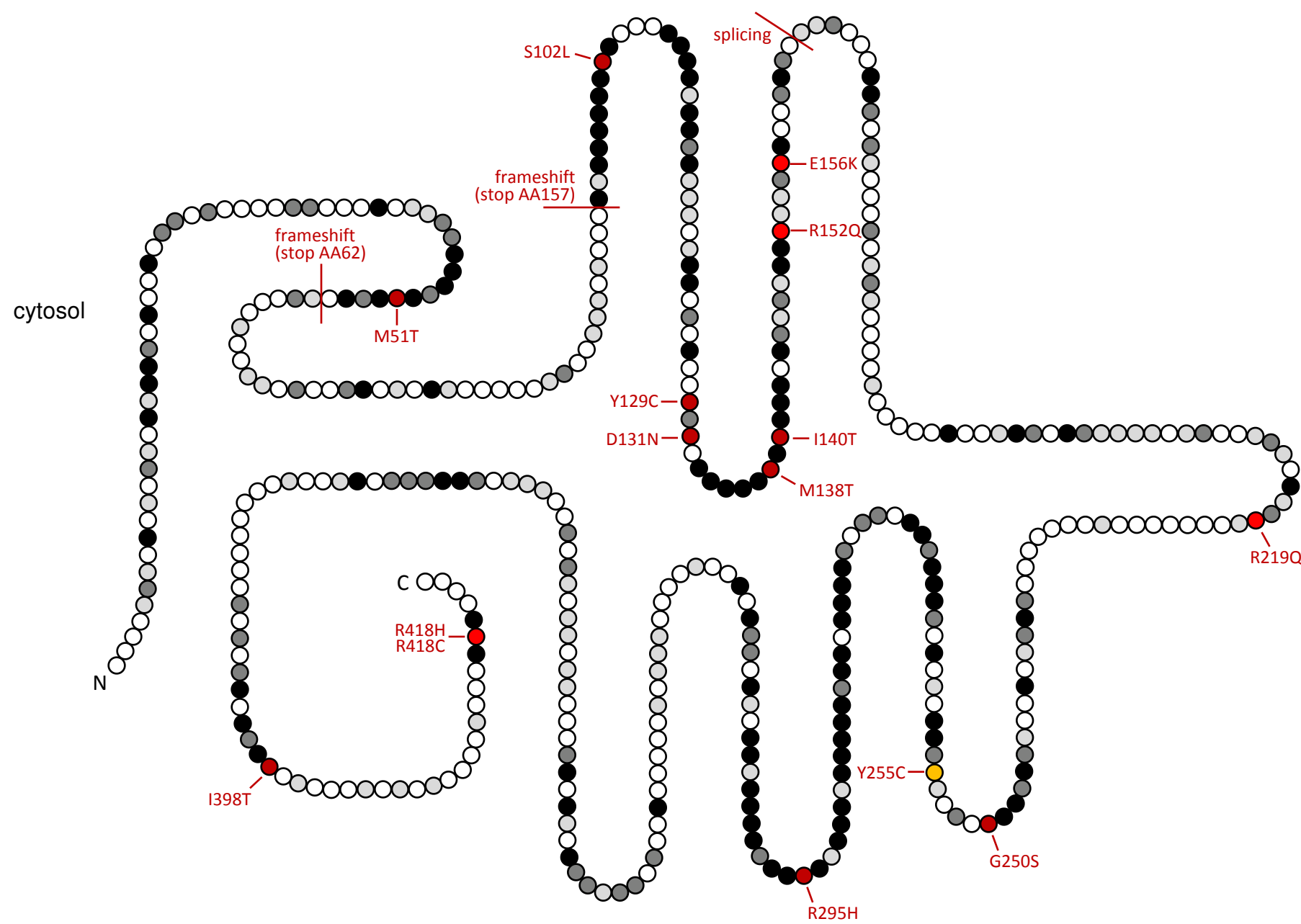


Figure 4 (DPAGT1)

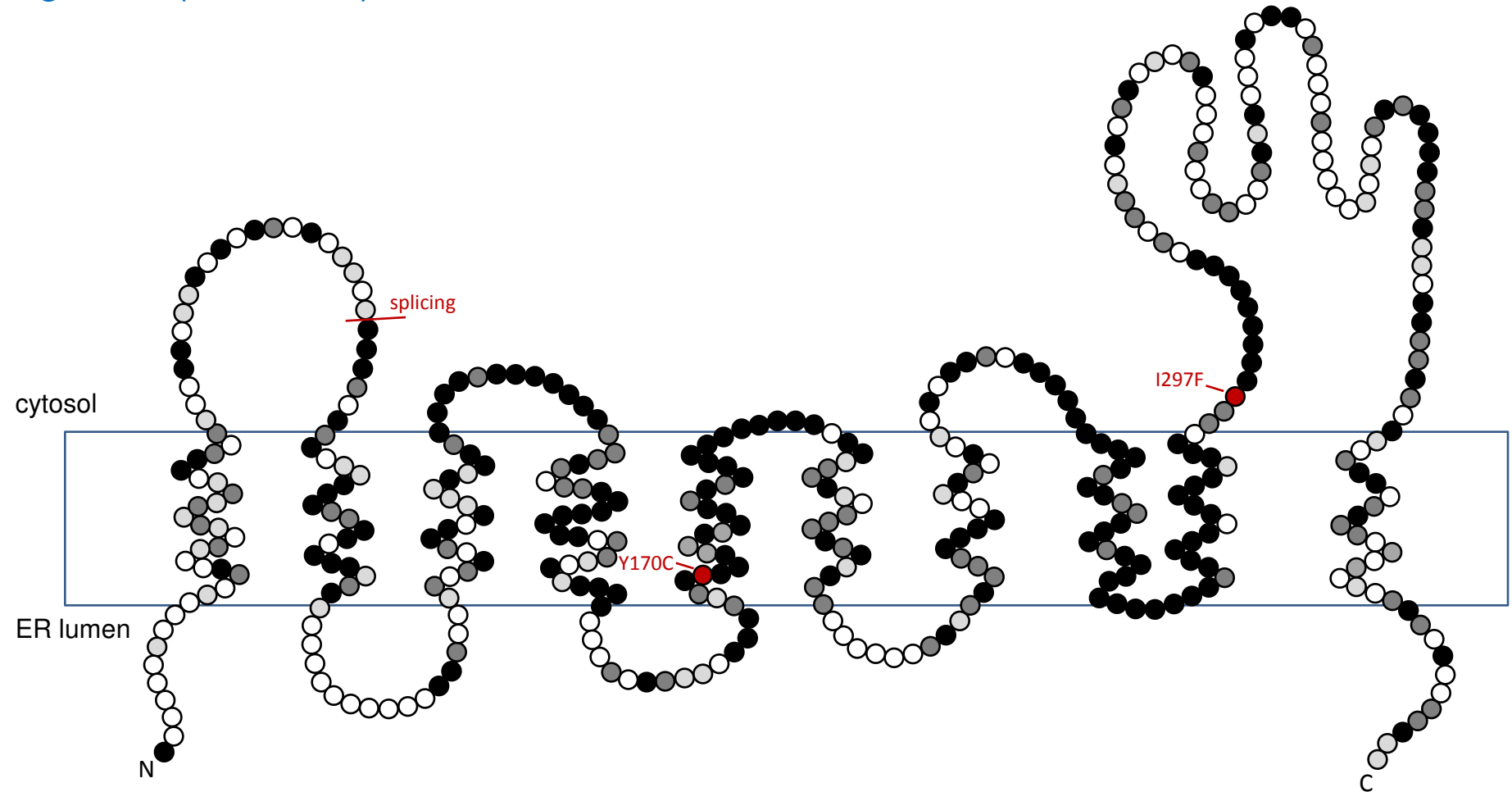


Figure 5 (HMT1)

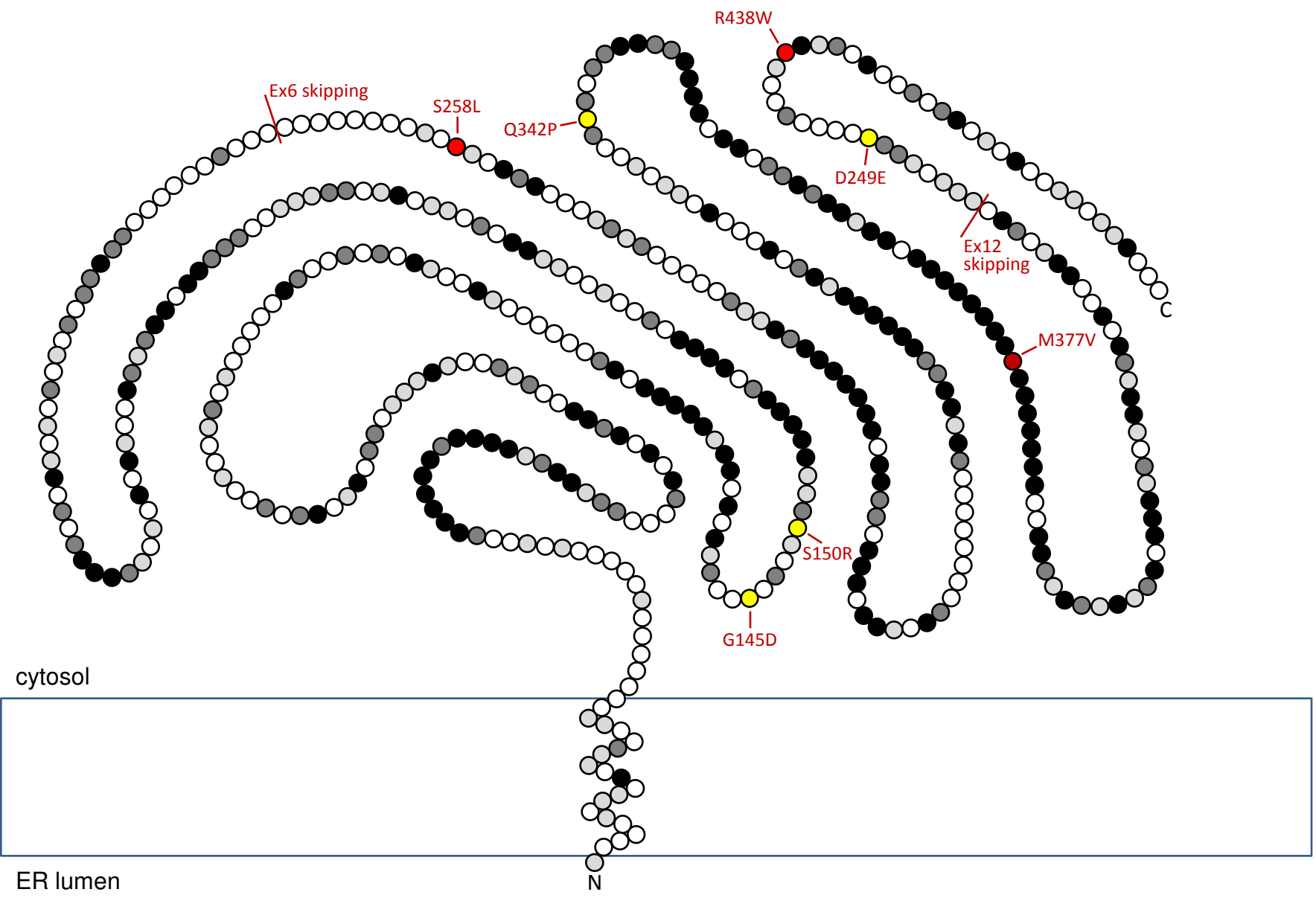
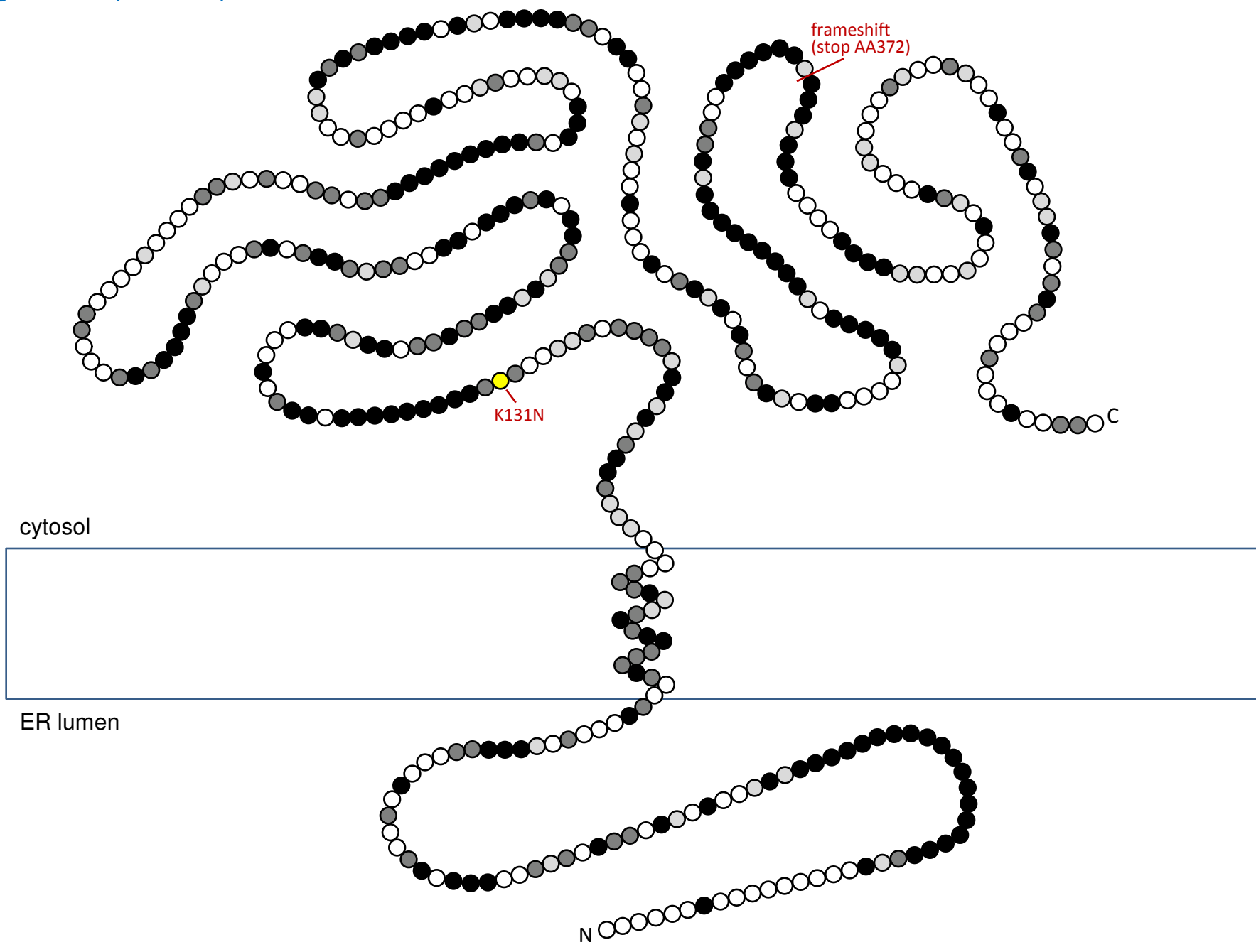
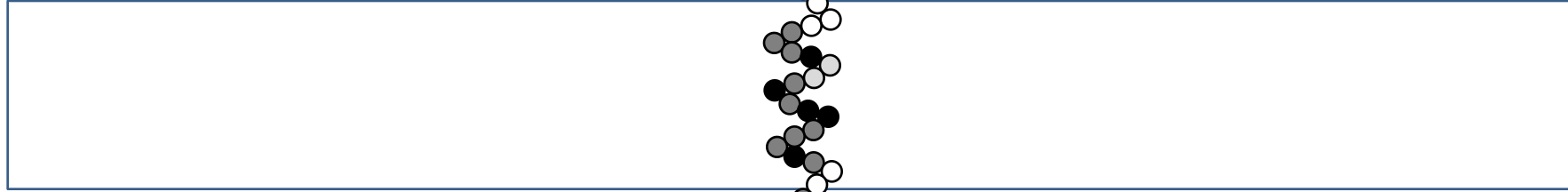


Figure 6 (ALG2)



cytosol



ER lumen

N

C

K131N

frameshift
(stop AA372)

Figure 7 (NOT56L)

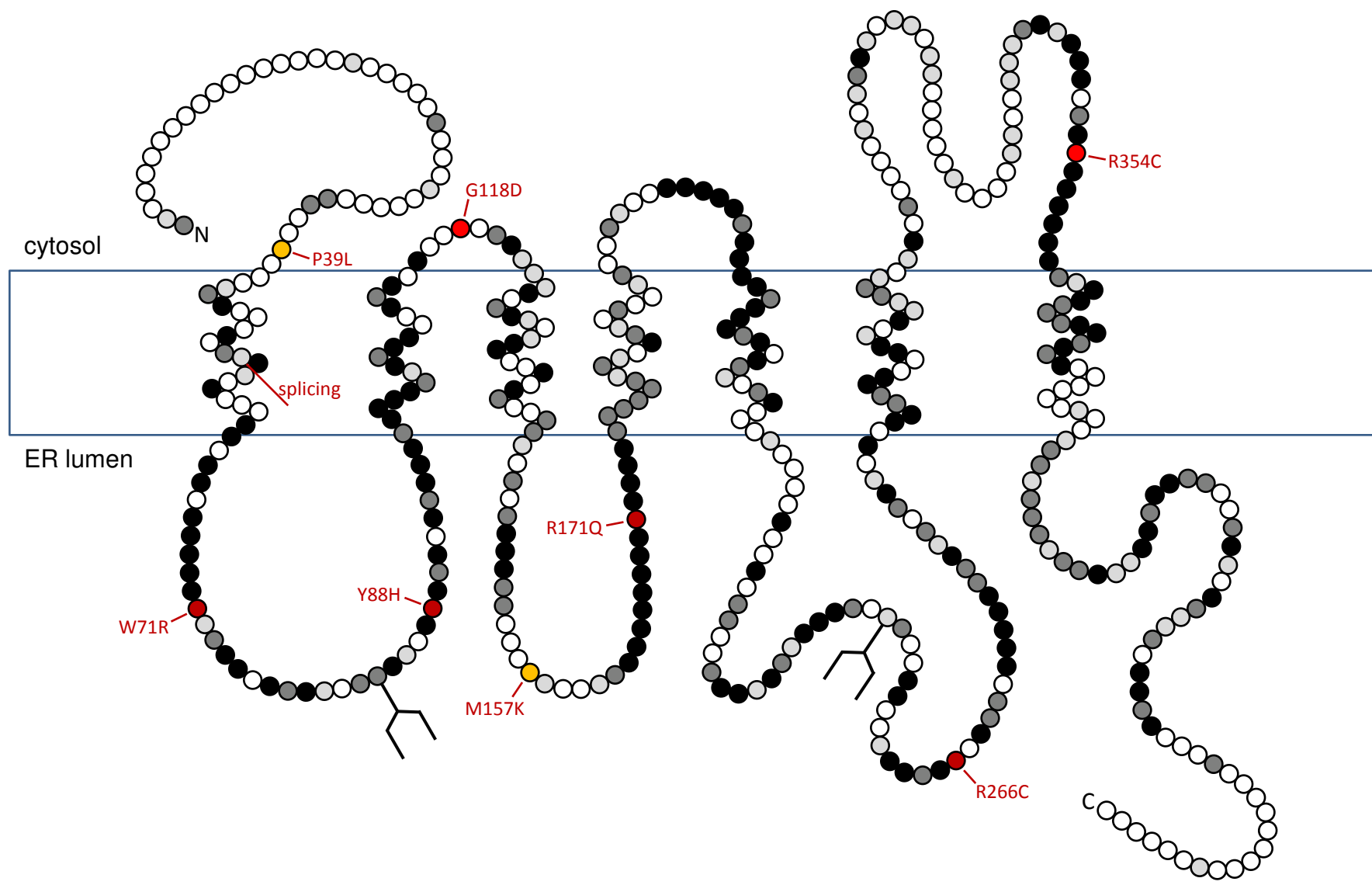


Figure 8 (DIBD1)

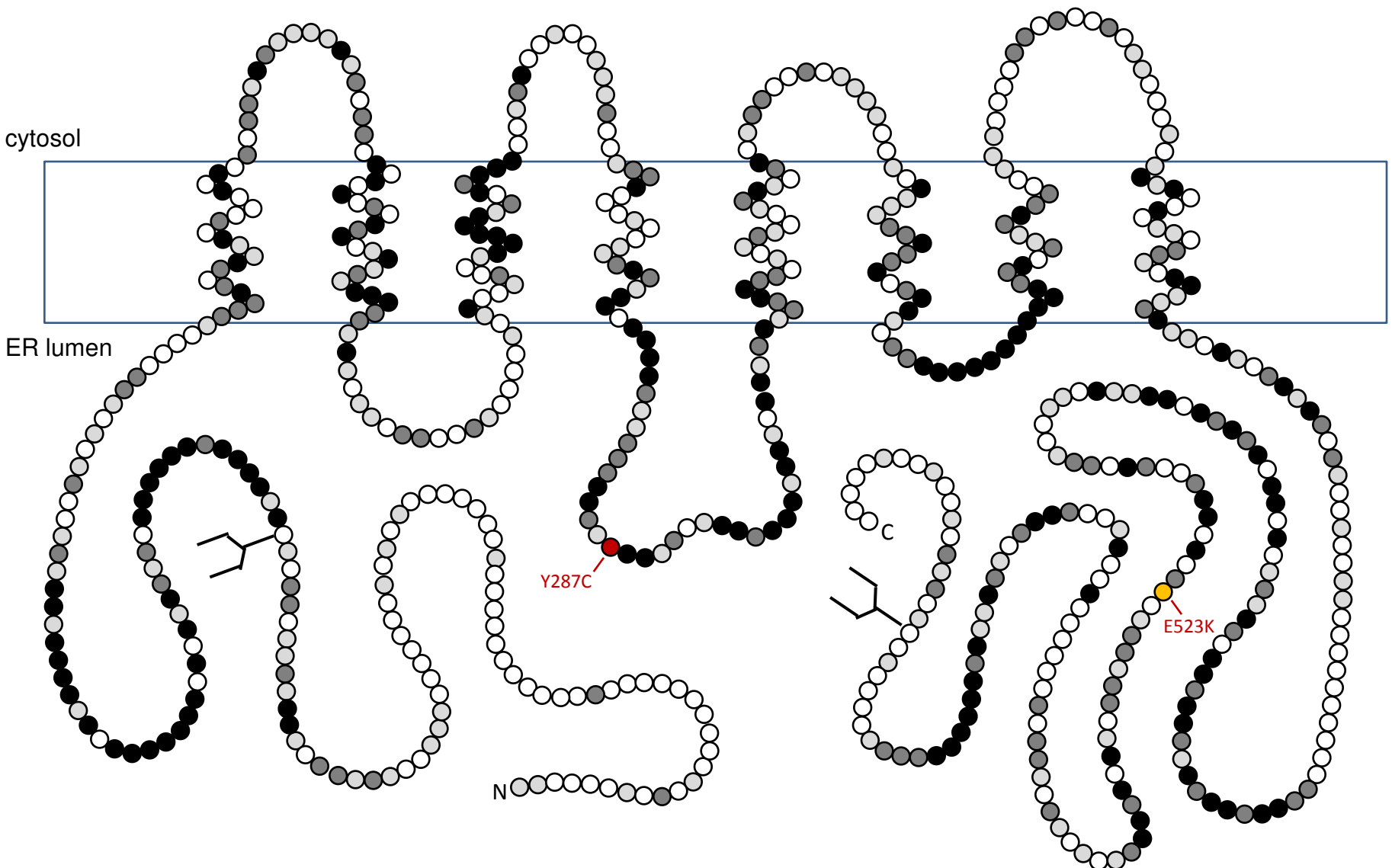


Figure 9 (ALG12)

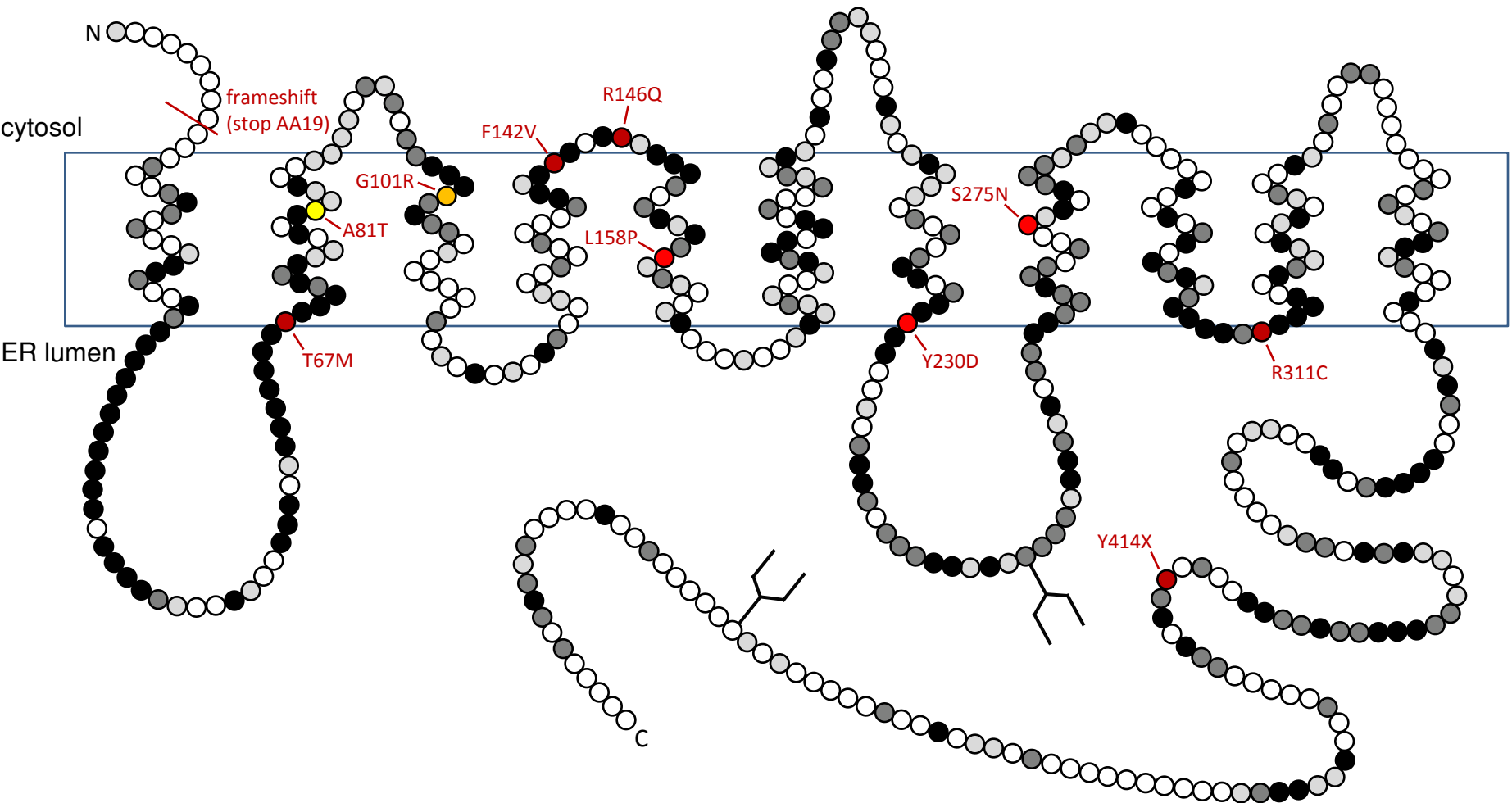


Figure 10 (ALG6)

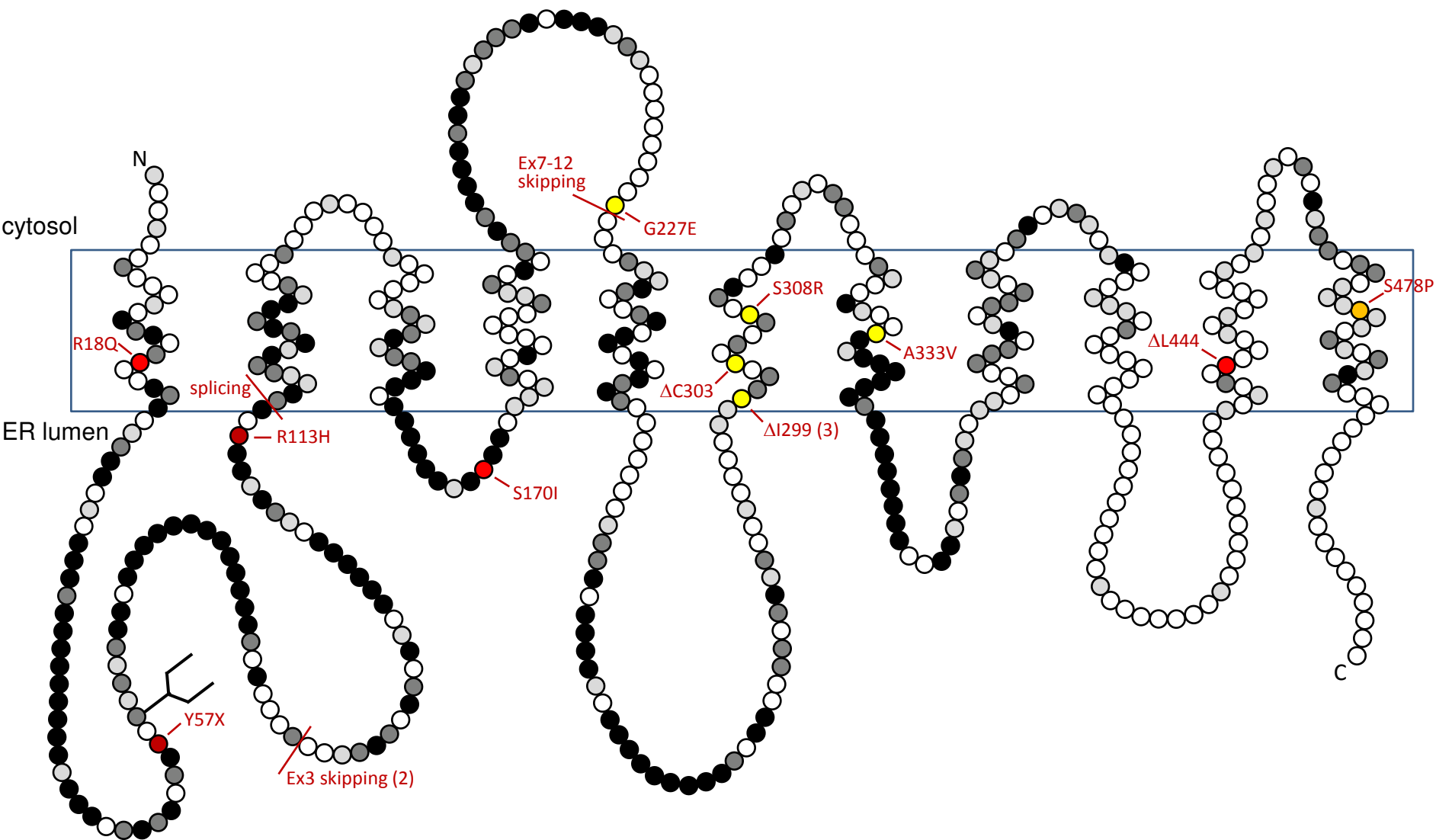


Figure 11 (ALG8)

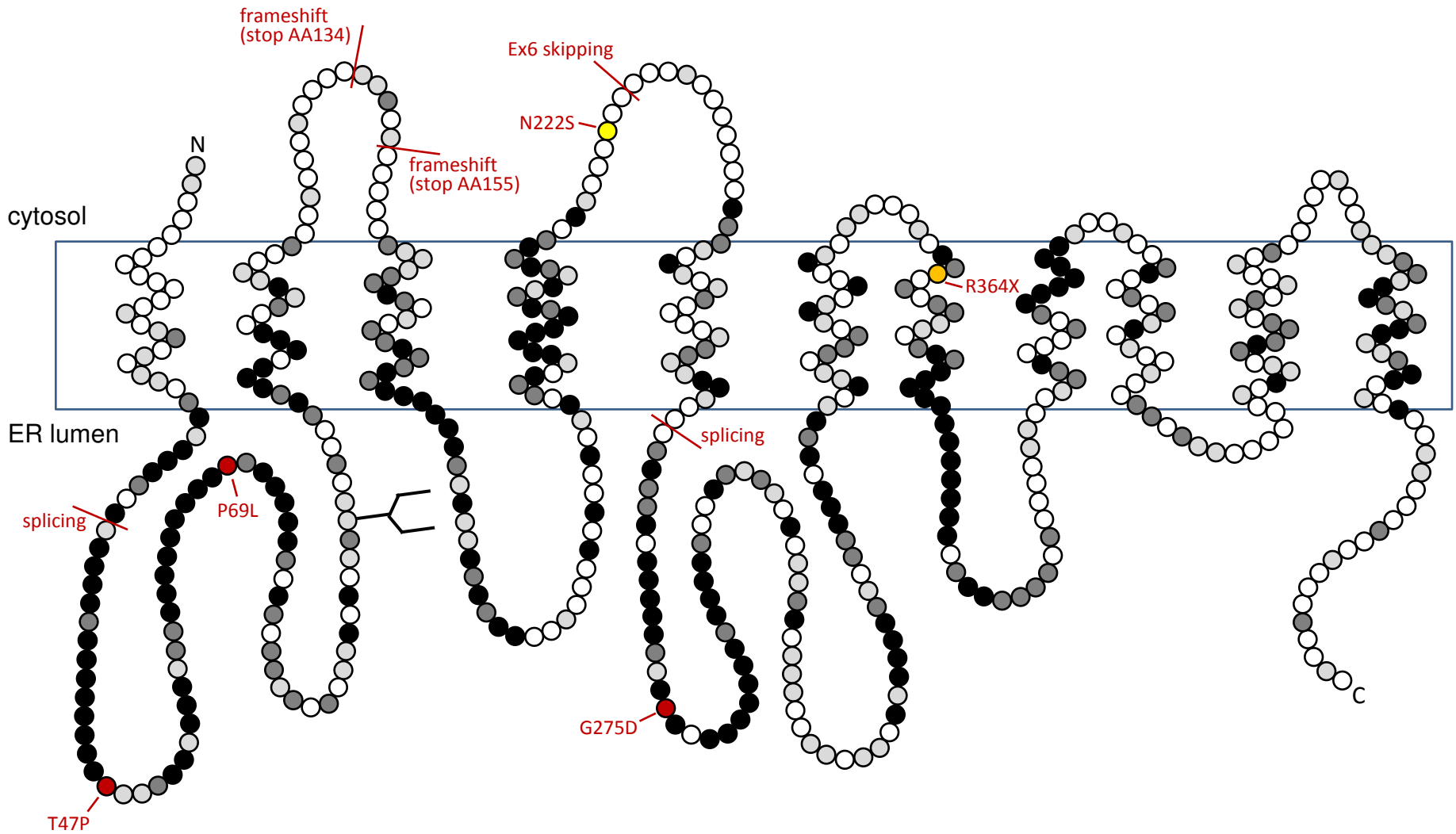


Figure 12 (DK1)

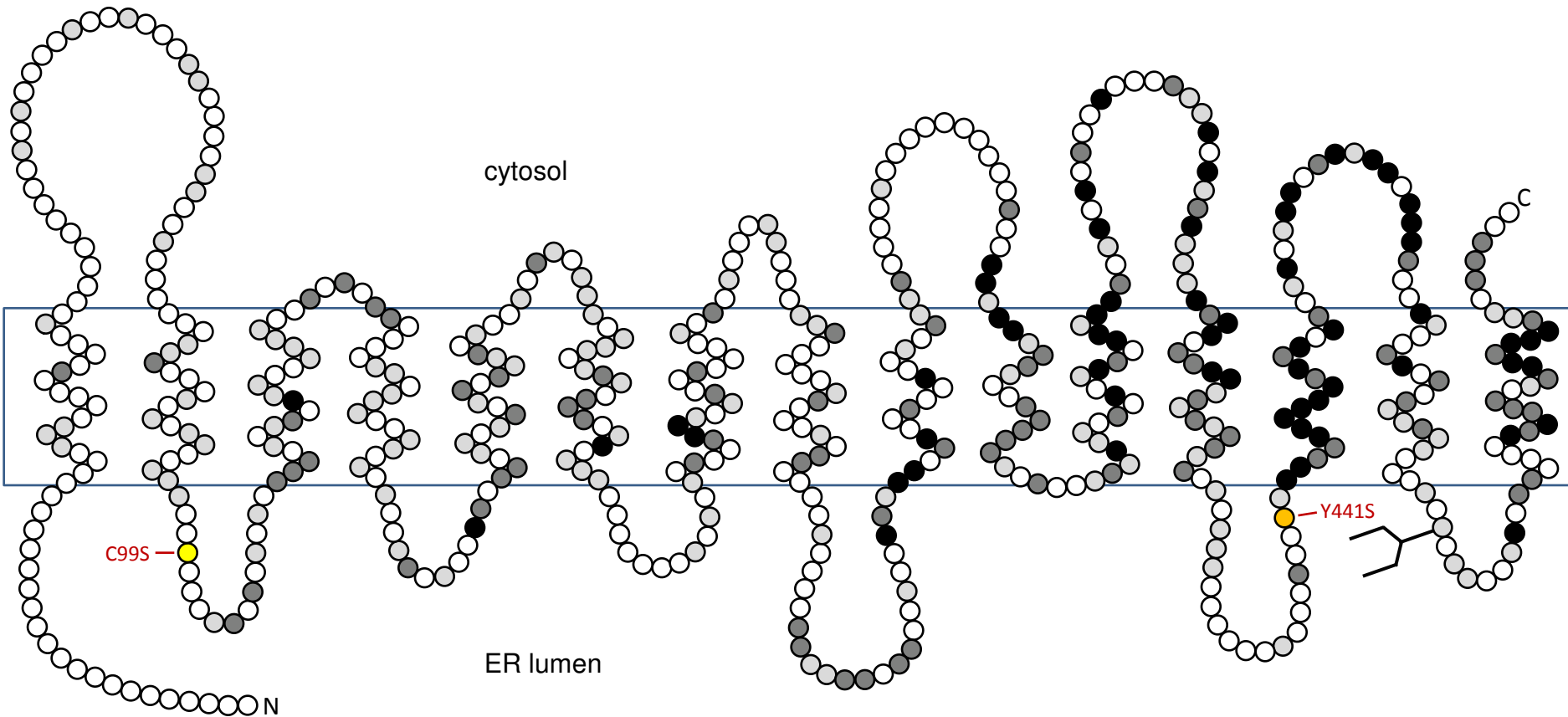


Figure 13 (DPM1-2-3)

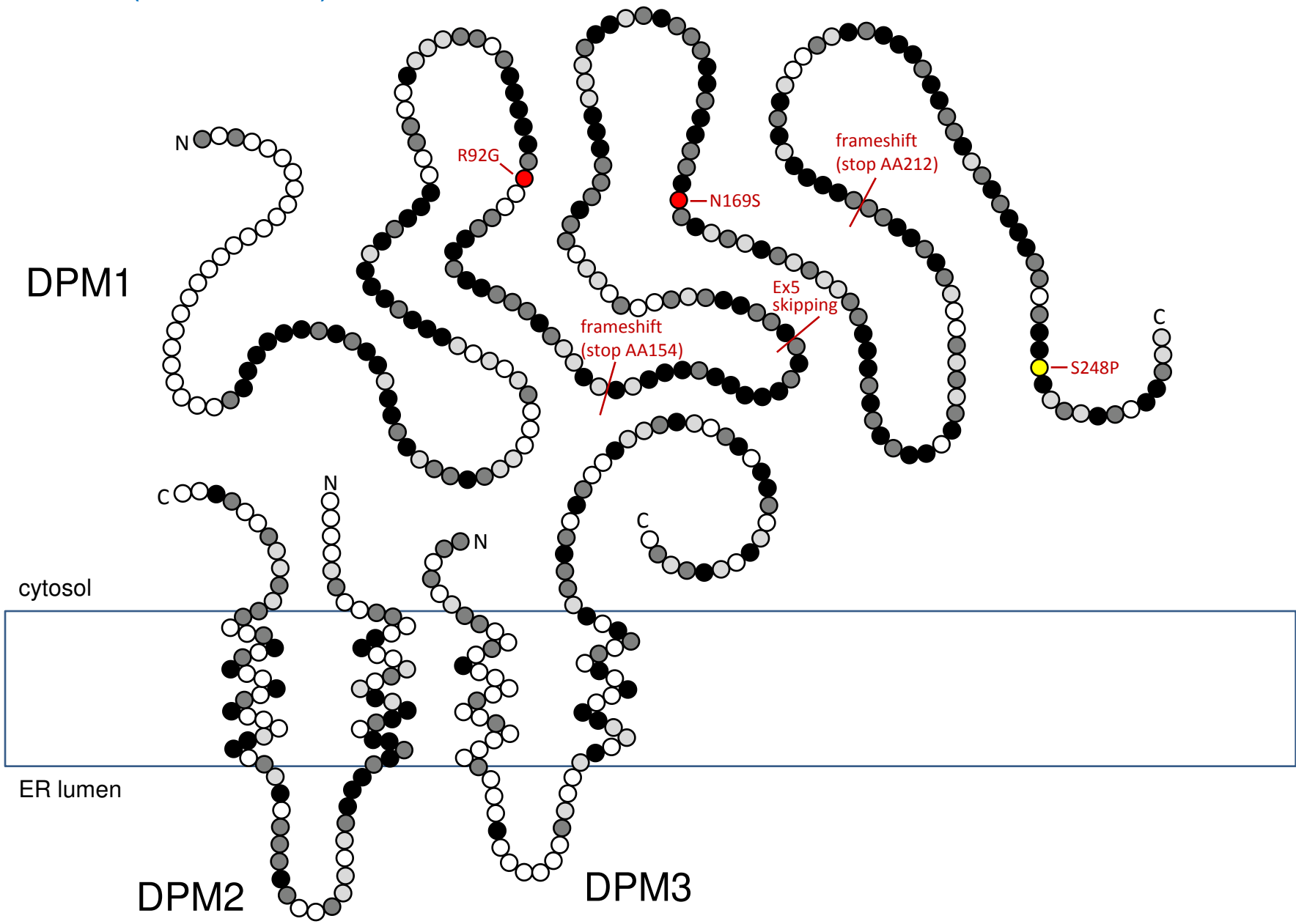


Figure 14 (MPDU1)

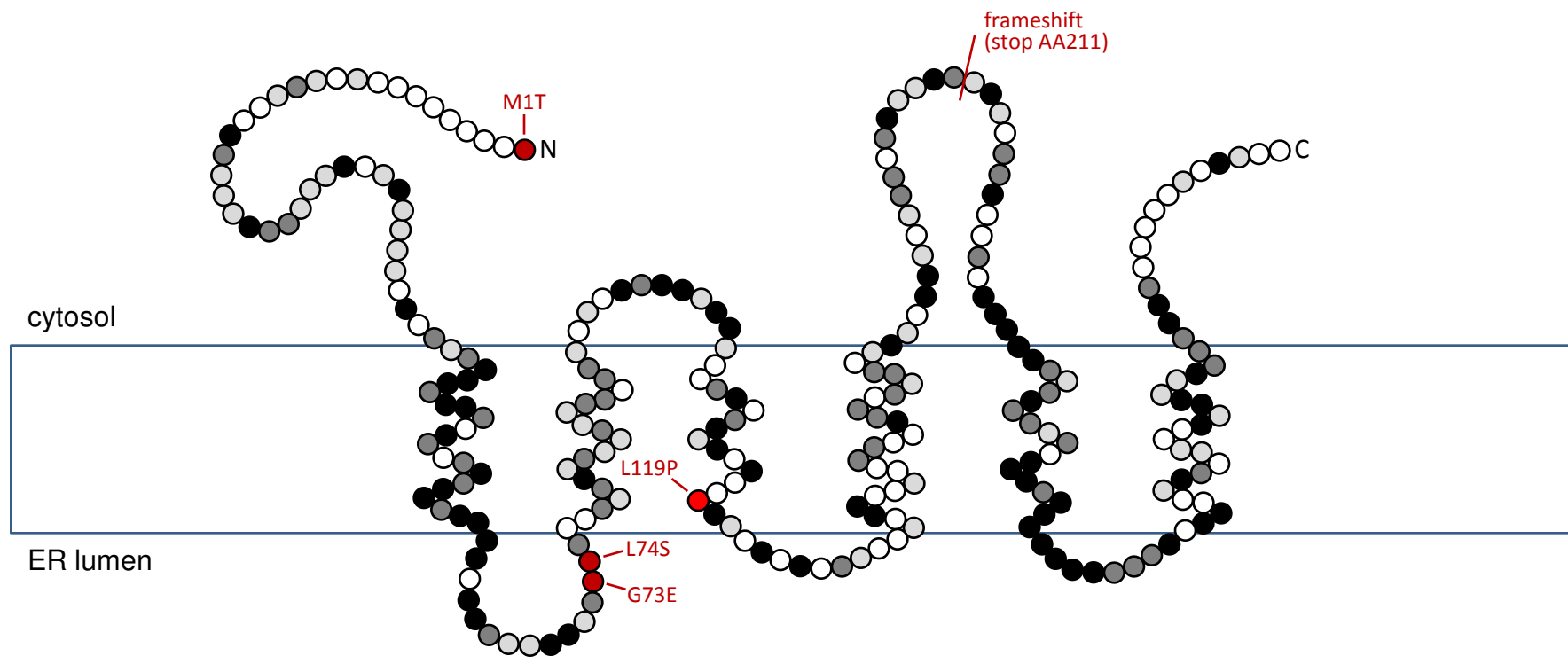
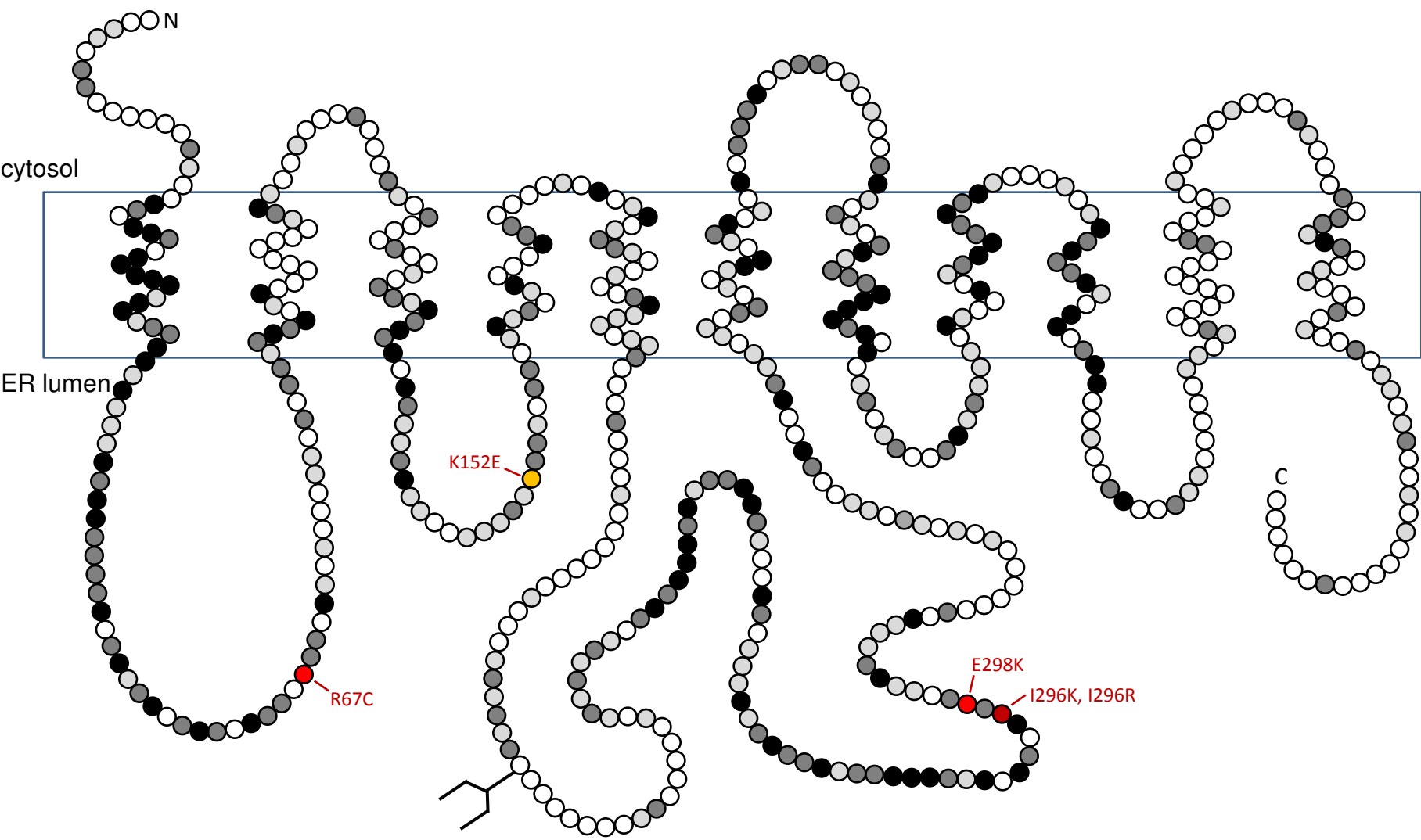


Figure 15 (RFT1)



Supporting table S1: Mutations in the *PMM2* gene (NM_000303.2). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.1A>G	p.M1V	(Perez-Duenas, et al., 2009)
Exon 1	c.24delC	Frameshift	(Matthijs, et al., 2000)
Exon 1	c.26G>A	p.C9Y	(Matthijs, et al., 2000)
Exon 1	c.32TC>GT	p.F11C	(Matthijs, et al., 2000)
Exon 1	c.43G>C	p.G15R	(Schollen, et al., 2002)
Exon 1	c.44G>C	p.G15A	This study ^a
Exon 1	c.44G>A	p.G15E	(Callewaert, et al., 2003)
Exon 1	c.53C>G	p.T18S	(Le Bizec, et al., 2005)
Exon 1	c.58C>T	p.P20S	(Le Bizec, et al., 2005)
Exon 1	c.61C>G	p.R21G	This study ^a
Exon 1	c.64C>T	p.Q22X	This study ^a
Intron 1	c.66+1G>T	Splice variant	(Le Bizec, et al., 2005)
Intron 1	c.67-1G>A	Abnormal splicing of exon 2	(Westphal, et al., 2001a)
Exon 2	c.95TA>GC	p.L32R	(Matthijs, et al., 2000)
Exon 2	c.104T>A	p.L35X	(Truin, et al., 2008)
Exon 2	c.111G>T	p.Q37H (SNP)	(Le Bizec, et al., 2005)
Exon 2	c.124G>A	p.G42R	This study ^a
Exon 2	c.131T>C	p.V44A	(Matthijs, et al., 1999)

Exon 2	c.140C>A	p.S47X	(Le Bizec, et al., 2005)
Exon 2	c.152A>G	p.K51R	(Vermeer, et al., 2007)
Exon 2	c.161-162insG	Frameshift	(Wurm, et al., 2007)
Exon 2	c.169G>C	p.G57R	This study ^a
Intron 2	c.179-25A>G	Skipping exon 3	(Vuillaumier-Barrot, et al., 2006)
Exon 3	c.191A>G	p.Y64C	(Briones, et al., 2002)
Exon 3	c.193G>T	p.D65Y	(Matthijs, et al., 1999)
Exon 3	c.199G>A	p.V67M	(Matthijs, et al., 2000)
Exon 3	c.200T>G	p.V67G	(Coman, et al., 2005)
Exon 3	c.205C>T	p.P69S	(Matthijs, et al., 1999)
Exon 3	c.227A>G	p.Y76C	(Matthijs, et al., 2000)
Exon 3	c.255G>A	p.Q85Q (splice variant)	(Le Bizec, et al., 2005)
Intron 3	c.255+1G>A	Splice variant	(Le Bizec, et al., 2005)
Intron 3	c.255+2T>C	Splice variant	(Matthijs, et al., 1999)
Intron 3	c.256-1G>C	Skipping exons 3 and 4	(Vega, et al., 2009)
Exon 4	c.278A>C	p.E93A	(Briones, et al., 2002)
Exon 4	c.303C>G	p.N101K	(Matthijs, et al., 1999)
Exon 4	c.308G>T	p.C103F	(Matthijs, et al., 2000)
Exon 4	c.310C>G	p.L104V	(Westphal, et al., 2001a)
Exon 4	c.317A>G	p.Y106C	(Matthijs, et al., 1997)
Exon 4	c.323C>T	p.A108V	(Matthijs, et al., 1997)

Exon 4	c.324delG	Frameshift	(Matthijs, et al., 1999)
Exon 4	c.338C>T	p.P113L	(Matthijs, et al., 1997)
Exon 5	c.349G>C	p.G117R	(Matthijs, et al., 2000)
Exon 5	c.357C>A	p.F119L	(Matthijs, et al., 1997)
Exon 5	c.359T>C	p.I120T	(Matthijs, et al., 2000)
Exon 5	c.367C>T	p.R123X	(Matthijs, et al., 2000)
Exon 5	c.368G>A	p.R123Q	(Matthijs, et al., 1999)
Exon 5	c.385G>A	p.V129M	(Matthijs, et al., 1997)
Exon 5	c.389delC	Frameshift	(Matthijs, et al., 1999)
Exon 5	c.391C>G	p.P131A	(Matthijs, et al., 1997)
Exon 5	c.394A>T	p.I132F	(Le Bizec, et al., 2005)
Exon 5	c.395T>C	p.I132T	(Matthijs, et al., 1999)
Exon 5	c.395T>A	p.I132N	(Matthijs, et al., 2000)
Exon 5	c.398delG	Frameshift	(Matthijs, et al., 2000)
Exon 5	c.415G>A	p.E139K	(Matthijs, et al., 2000)
Exon 5	c.421C>T	p.R141C	(Le Bizec, et al., 2005)
Exon 5	c.422G>A	p.R141H	(Matthijs, et al., 1997)
Exon 5	c.430T>C	p.F144L	(Mizugishi, et al., 1999)
Exon 5	c.442G>A	p.D148N	(Matthijs, et al., 2000)
Exon 6	c.451-454delGAAA	Frameshift	(Schollen, et al., 2002)
Exon 6	c.452A>G	p.E151G	(Matthijs, et al., 1999)

Exon 6	c.458T>C	p.I153T	(Matthijs, et al., 2000)
Exon 6	c.470T>C	p.F157S	(Matthijs, et al., 1999)
Exon 6	c.484C>T	p.R162W	(Matthijs, et al., 1997)
Exon 6	c.514T>G	p.F172V	(Matthijs, et al., 2000)
Exon 6	c.523G>C	p.G175R	(Matthijs, et al., 1999)
Exon 7	c.527G>T	p.G176V	(Le Bizec, et al., 2005)
Exon 7	c.531G>C	p.Q177H	(Le Bizec, et al., 2005)
Exon 7	c.548T>C	p.F183S	(Matthijs, et al., 2000)
Exon 7	c.554A>G	p.D185G	(Matthijs, et al., 2000)
Exon 7	c.563A>G	p.D188G	(Matthijs, et al., 1999)
Exon 7	c.565-571delAGAGAT insGTGGATTCC	Frameshift	(Tayebi, et al., 2002)
Exon 7	c.574T>G	p.C192G	(Matthijs, et al., 2000)
Exon 7	c.580C>T	p.R194X	(Ono, et al., 2003)
Exon 7	c.584A>G	p.H195R	(Matthijs, et al., 1999)
Exon 7	c.590A>C	p.E197A (SNP)	(Matthijs, et al., 2000)
Exon 7	c.617T>C	p.F206S	(Matthijs, et al., 2000)
Exon 7	c.618C>A	p.F206L	(Thong, et al., 2009)
Exon 7	c.620T>C	p.F207S	(Briones, et al., 2002)
Exon 7	c.623G>C	p.G208A	(Matthijs, et al., 1999)
Intron 7	c.640-15479C>T	In frame insertion of 41 amino acids between exons 7 and 8	(Schollen, et al., 2007)

Intron 7	c.640-23A>G	Loss of exon 8	(Vuillaumier-Barrot, et al., 2006)
Intron 7	c.640-9T>G	Splice variant	(Vega, et al., 2009)
Exon 8	c.640G>A	p.G214S	(Le Bizec, et al., 2005)
Exon 8	c.647A>T	p.N216I	(Matthijs, et al., 1997)
Exon 8	c.G647A>G	p.N216S	(Matthijs, et al., 2000)
Exon 8	c.651C>A	p.D217E	(Matthijs, et al., 2000)
Exon 8	c.653A>T	p.H218L	(Matthijs, et al., 1999)
Exon 8	c.667G>A	p.D223N	This study ^a
Exon 8	c.669C>G	p.D223E	(Matthijs, et al., 2000)
Exon 8	c.677C>G	p.T226S	(Matthijs, et al., 2000)
Exon 8	c.682G>T	p.G228C	(Matthijs, et al., 1999)
Exon 8	c.682G>C	p.G228R	(Matthijs, et al., 2000)
Exon 8	c.686A>C	p.Y229S	(Matthijs, et al., 1999)
Exon 8	c.691G>A	p.V231M	(Matthijs, et al., 1997)
Exon 8	c.697G>A	p.A233T (SNP)	(Matthijs, et al., 1999)
Exon 8	c.710C>G	p.T237R	(Matthijs, et al., 1999)
Exon 8	c.710C>T	p.T237M	(Matthijs, et al., 1997)
Exon 8	c.712C>G	p.R238G	(Matthijs, et al., 2000)
Exon 8	c.713G>C	p.R238P	(Matthijs, et al., 1999)
Exon 8	c.715A>T	p.R239W	(Grunewald, et al., 2001)
Exon 8	c.722G>C	p.C241S	(Matthijs, et al., 1999)

Exon 8	c.728T>C	p.L243P	This study ^a
Exon 8	Alu retrotransposition mediated deletion	Loss of exon 8	(Schollen, et al., 2007)

^a Mutations identified through the Euroglycanet network (Matthijs, 2005)

Supporting table S2: Mutations in the *MPI* gene (NM_002435.1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 3	c.152T>C	p.M51T	(Schollen, et al., 2000)
Exon 3	c.166-167insC	Frameshift	(Schollen, et al., 2000)
Exon 3	c.282delG	Frameshift	(Vuillaumier-Barrot, 2005)
Exon 3	c.305C>T	p.S102L	(Jaeken, et al., 1998)
Exon 4	c.386A>G	p.Y129C	(Schollen, et al., 2002)
Exon 4	c.391G>A	p.D131N	(Schollen, et al., 2000)
Exon 4	c.413T>C	p.M138T	(Jaeken, et al., 1998)
Exon 4	c.419T>C	p.I140T	(Westphal, et al., 2001b)
Exon 4	c.455G>A	p.R152Q	(Schollen, et al., 2000)
Exon 4	c.466G>A	p.E156K	(Vuillaumier-Barrot, 2005)
Intron 4	c.488-1G>C	Splice variant	(Schollen, et al., 2000)
Exon 5	c.656G>A	p.R219Q	(Niehues, et al., 1998)
Exon 6	c.748G>A	p.G250S	(Schollen, et al., 2000)
Exon 6	c.764A>G	p.Y255C	(de Lonlay, et al., 1999)
Exon 7	c.884G>A	p.R295H	(Vuillaumier-Barrot, et al., 2002)
Exon 8	c.1193T>C	p.I398T	(de Lonlay, et al., 1999)
Exon 8	c.1252C>T	p.R418C	This study ^a
Exon 8	c.1253G>A	p.R418H	(Babovic-Vuksanovic, et al., 1999)

^a Mutations identified through Euroglycanet network (Matthijs, 2005)

Supporting table S3: Mutations in the *DPAGTI* gene (NM_001382.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Intron 1	c.162-8G>A	Splice variant	(Vuillaumier-Barrot, 2005)
Exon 4	c.509A>G	p.Y170C	(Wu, et al., 2003)
Exon 6	c.889A>T	p.I297F	(Vuillaumier-Barrot, 2005)

Supporting table S4: Mutations in the *ALG1* gene (NM_019109.4). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 4	c.450C>G	p.S150R	(Grubenmann, et al., 2004)
Exon 7	c.773C>T	p.S258L	(Schwarz, et al., 2004)
Exon 10	c.1025A>C	p.Q342P	(Kranz, et al., 2004)
Exon 13	c.1287T>A	p.D429E (SNP)	(Grubenmann, et al., 2004)

Supporting table S5: Mutations in the *ALG2* gene (NM_033087.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 2	c.393G>T	p.K131N	(Thiel, et al., 2003)
Exon 2	c.1040delG	Frameshift	(Thiel, et al., 2003)

Supporting table S6: Mutations in the *ALG3* gene (NM_005787.5). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.116C>T	p.P39L	(Rimella-Le-Huu, et al., 2008)
Exon 1	c.165C>T	Splice variant	(Denecke, et al., 2004)
Exon 2	c.211T>C	p.W71R	(Schollen, et al., 2002)
Exon 2	c.262T>C	p.Y88H	This study ^a
Exon 3	c.353G>A	p.G118D	(Körner, et al., 1998)
Exon 4	c.470T>A	p.M157K	(Kranz, et al., 2007c)
Exon 4	c.512G>A	p.R171Q	(Sun, et al., 2005)
Exon 6	c.796C>T	p.R266C	(Schollen, et al., 2005)
Exon 8	c.1060C>T	p.R354C	This study ^a

^a Mutations identified through Euroglycanet network (Matthijs, 2005)

Supporting table S7: Mutations in the *ALG9* gene (NM_001077690.1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 8	c.860A>G	p.Y287C	(Weinstein, et al., 2005)
Exon 13	c.1567G>A	p.E523K	(Frank, et al., 2004)

Supporting table S8: Mutations in the *ALG12* gene (NM_024105.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 2	c.29delG	Frameshift	(Eklund, et al., 2005)
Exon 3	c.200C>T	p.T67M	(Grubenmann, et al., 2002)
Exon 3	c.241G>A	p.A81T	(Di Rocco, et al., 2005)
Exon 4	c.301G>A	p.G101R	(Kranz, et al., 2007a)
Exon 4	c.424T>G	p.F142V	(Chantret, et al., 2002)
Exon 4	c.437G>A	p.R146Q	(Grubenmann, et al., 2002)
Exon 5	c.473T>C	p.L158P	(Thiel, et al., 2002)
Exon 6	c.688T>G	p.Y230D	(Eklund, et al., 2005)
Exon 7	c.824G>A	p.S275N	(Eklund, et al., 2005)
Exon 7	c.931C>T	p.R311C	(Eklund, et al., 2005)
Exon 10	c.1242C>G	p.Y414X	(Thiel, et al., 2002)

Supporting table S9: Mutations in the *ALG6* gene (NM_013339.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.53G>A	p.R18Q	This study ^a
Exon 3	c.171T>A	p.Y57X	(Vuillaumier-Barrot, 2005)
Intron 3	c.257+2-3insT	Skipping Exon 3	(Newell, et al., 2003)
Intron 3	c.257+5G>A	Skipping exon 3	(Imbach, et al., 2000a)
Exon 4	c.338G>A	p.R113H	(Eklund, et al., 2006)
Intron 4	c.347-13G>C	Splice variant	(Vuillaumier-Barrot, 2005)
Exon 5	c.391T>C	p.Y131H (SNP)	(Westphal, et al., 2000a)
Exon 7	c.509G>T	p.S170I	(de Lonlay, et al., 2001)
Exon 7	c.680G>A	p.G227E	(Schollen, et al., 2002)
Intron 7	c.680+2T>G	Skipping exons 7 – 12 and parts of exon 13	(Sun, et al., 2005)
Exon 9	c.895-897delATA	p.I299del	(Westphal, et al., 2000b)
Exon 9	c.896-898delTAA	p.I299del	(Hanefeld, et al., 2000)
Exon 9	c.897-899delAAT	p.I299del	(Sun, et al., 2005)
Exon 10	c.908-910delGTT	p.C303del	This study ^a
Exon 10	c.911T>C	p.F304S (SNP)	(Vuillaumier-Barrot, et al., 2001)
Exon 10	c.924C>A	p.S308R	(Westphal, et al., 2000a)
Exon 11	c.998C>T	p.A333V	(Imbach, et al., 1999)

Exon 14	c.1330-1332delCTT	p.L444del	(de Lonlay, et al., 2001)
Exon 14	c.1432T>C	p.S478P	(Imbach, et al., 2000a)
-	Del(1)(p31.2p32.3)	No protein	(Eklund, et al., 2006)

^a Mutations identified through Euroglycanet network (Matthijs, 2005)

Supporting table S10: Mutations in the *ALG8* gene (NM_024079.4). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Intron 1	c.96-2A>G	Splice variant	(Schollen, et al., 2004)
Exon 2	c.139A>C	p.T47P	(Schollen, et al., 2004)
Exon 3	c.206C>T	p.P69L	This study ^a
Exon 4	c.396-397insA	Frameshift	(Chantret, et al., 2003)
Exon 4	c.413delC	Frameshift	(Chantret, et al., 2003)
Exon 6	c.665A>G	p.N222S (SNP)	(Schollen, et al., 2004)
Intron 6	c.672+4A>G	Skipping exon 6	(Schollen, et al., 2004)
Intron 7	c.778-3C>A	Splice variant	This study ^a
Exon 8	c.824G>A	p.G275D	(Schollen, et al., 2004)
Exon 8	c.845C>T	p.A282V	(Stölting, et al., 2009)
Exon 10	c.1090C>T	p.R364X	(Vesela, et al., 2009)
Exon 13	c.1434delC	Frameshift	(Stölting, et al., 2009)

^a Mutations identified through Euroglycanet network (Matthijs, 2005)

Supporting table S11: Mutations in the *DOLK* gene (NM_014908.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.295T>A	p.C99S	(Kranz, et al., 2007b)
Exon 1	c.1322A>C	p.Y441S	(Kranz, et al., 2007b)

Supporting table S12: Mutations in the *DPM1* gene (NM_003859.1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 3	c.274C>G	p.R92G	(Imbach, et al., 2000b)
Exon 4	c.331-343del GGAAACTACATCA	Frameshift	(Kim, et al., 2000)
Intron 4	c.373-5T>A	Skipping exon 5	(Dancourt, et al., 2006)
Exon 7	c.506A>G	p.N169S	(Vuillaumier-Barrot, 2005)
Exon 8	c.628delC	Frameshift	(Imbach, et al., 2000b)
Exon 9	c.742T>C	p.S248P	(Garcia-Silva, et al., 2004)

Supporting table S13: Mutations in the *DPM3* gene (NM_153741.1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.254T>C	p.L85S	(Lefeber, et al., 2009)

Supporting table S14: Mutations in the *MPDUI* gene (NM_004870.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 1	c.2T>C	p.M1T	(Schenk, et al., 2001)
Exon 3	c.218G>A	p.G73E	(Schenk, et al., 2001)
Exon 3	c.221T>C	p.L74S	(Kranz, et al., 2001)
Exon 4	c.356T>C	p.L119P	(Schenk, et al., 2001)
Exon 6	c.511delC	Frameshift	(Schenk, et al., 2001)

Supporting table S15: Mutations in the *RFT1* gene (NM_052859.3). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Exon/Intron	Base change	Amino acid change	Reference
Exon 3	c.199C>T	p.R67C	(Haeuptle, et al., 2008)
Exon 4	c.454A>G	p.K152E	(Vleugels, et al., 2009)
Exon 9	c.887T>A	p.I296K	This study ^a
Exon 9	c.887T>G	p.I296R	This study ^a
Exon 9	c.892G>A	p.E298K	(Vleugels, et al., 2009)

^a Mutations identified through Euroglycanet network (Matthijs, 2005)

Supporting Methods:

Mutations presented for the first time in this study were detected by sequencing cDNA and genomic DNA isolated from fibroblasts. Total RNA and genomic DNA were prepared from approximately 2×10^7 fibroblasts using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. mRNA was reverse transcribed using Omniscript reverse transcriptase (QUIAGEN). The resulting cDNA samples were amplified by PCR utilizing forward and reverse primers flanking the protein coding regions. After removal of unincorporated nucleotides using QIAquick columns (QUIAGEN), the purified PCR products were directly sequenced. Identified mutations were confirmed at the genomic level by sequencing the corresponding exons. Putative single nucleotide polymorphisms were excluded by analyzing at least 230 control alleles.

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