Congenital disorders of glycosylation: genetic model systems lead the way

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N-linked glycosylation is the most frequent modification of secretory proteins in eukaryotic cells. The highly conserved glycosylation process is initiated in the endoplasmic reticulum (ER), where the $Glc_3Man_9GlcNAc_2$ oligosaccharide is assembled on the lipid carrier dolichylpyrophosphate and then transferred to selected asparagine residues of polypeptide chains. In recent years, several inherited human diseases, congenital disorders of glycosylation (CDG), have been associated with deficiencies in this pathway. The ER-associated glycosylation pathway has been studied in the budding yeast *Saccharomyces cerevisiae*, and this model system has been invaluable in elucidating the molecular basis of novel types of CDG.

N-glycans occur on asparagine residues of mature proteins in almost all eukaryotic cells. The structure of glycans can vary between species, cell type and glycoprotein, and N-glycans fulfill a large number of very different functions in the eukaryotic cell[1,2]. N-glycosylation is an essential process because inhibition of N-glycosylation leads to cell death[3]. Recently, deficiencies in the pathway of N-linked protein glycosylation have been identified as the cause of a family of severe inherited human diseases, called congenital disorders of glycosylation (CDG). These deficiencies are characterized by clinical features such as dysmorphism, psychomotor retardation and stroke-like episodes[4–7].

The process of N-linked protein glycosylation is localized in the endoplasmic reticulum (ER) and the Golgi compartment of eukaryotic cells. Three separate phases can be distinguished: first, the oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolichylpyrophosphate at the ER membrane. Secondly, this oligosaccharide core is transferred co-translationally to dedicated asparagine residues of glycoproteins. In this central reaction of the process, oligosaccharyltransferase (OST) recognizes the acceptor sequence NX[S/T] (where X can be any amino acid except proline) on nascent polypeptides and catalyzes the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ *en bloc* from its lipid carrier to the protein. Finally, the N-linked oligosaccharide is further modified by a series of trimming and elongation reactions beginning in the ER and ending in the late Golgi compartment. In contrast to the ER-localized pathway, trimming reactions and the remodeling of the oligosaccharide in the Golgi are much less conserved among eukaryotic cells and occur in a protein-, cell type- and species-specific manner.

The basic principles of the N-linked glycosylation process have been established by studies using higher eukaryotic cells[8]. However, genetic experiments in the budding yeast *Saccharomyces cerevisiae*, initiated in the laboratory of Phil Robbins, have been instrumental in the identification and characterization of the different glycosyltransferases involved in the sections of the pathway that are highly conserved among eukaryotes[9,10].

In the following review, we will first concentrate on the biosynthesis of sugar donors required for protein glycosylation and then describe the glycosylation pathway in the ER. We will explain how the yeast genetic system was used as a tool to characterize specific types of CDG.

The biosynthesis of activated sugars

Luis Leloir was awarded the Nobel Prize for Chemistry in 1970 for his discovery of sugar nucleotides and their role in the biosynthesis of carbohydrates. Leloir originally described UDP-glucose (Glc) as a substrate for glycogen biosynthesis, but this type of catalysis is paradigmatic for nearly all glycosylation reactions. In fact, nucleotide-bound versions of all monosaccharides, such as UDP-galactose (Gal), GDP-mannose (Man) and CMP-sialic acid (Sia), are required for the synthesis of glycoconjugates. The biosynthetic pathways of the nucleotide-activated sugar monomers are largely interconnected, and so a deficiency of one nucleotide sugar can be paralleled by reduced levels of several others (Fig. 1). After uptake by the cell, most monosaccharides are directly phosphorylated by hexokinases to yield sugar-6-P. Others, like Gal and fucose (Fuc), are transformed to sugar-1-P by specific kinases.

The gene encoding phosphomannomutase (PMM), the enzyme that catalyzes the formation of Man-1-P from Man-6-P, was first identified as the *SEC53* locus in *S. cerevisiae*[11]. Based on this sequence information, the two human homologous genes, *PMM1* and *PMM2*, were identified. Mutations in the *PMM2* locus represent the most frequent cause of CDG worldwide[12,13]. This defect was also the first to be characterized biochemically and thus received the name CDG-Ia (Table 1). PMM2 deficiency leads to a shortage of GDP-Man, which is indispensable for the synthesis of the oligosaccharide core.

Most organisms rely on a dietary supply for the majority of saccharides; connections between the various biosynthetic pathways prevent any dramatic consequences that might arise from a deficient intake of specific monosaccharides. For example, both UDP-Glc and UDP-Gal can be produced by the two enzymes Gal-1-P uridyltransferase and galactose epimerase (Fig. 1). A block at the level of these enzymes leads to an accumulation of Gal-1-P, which causes the symptoms related to galactosemia[14]. Furthermore, the levels of

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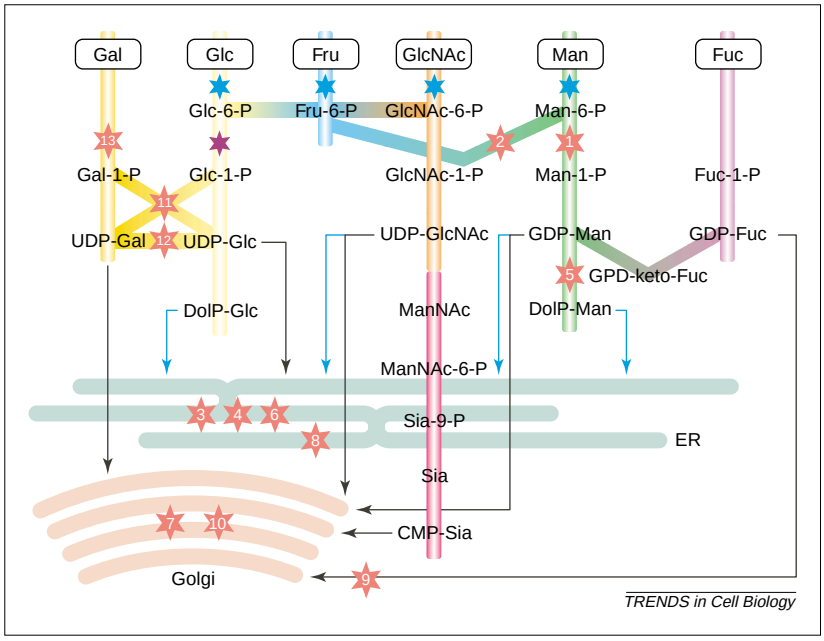

Fig. 1. Biosynthesis pathways of activated sugars used as glycosylation substrates. The pathways of xylose, glucuronic acid and N-acetylgalactosamine are not shown. The metabolism of glucosamine parallels that of N-acetylglucosamine down to (N-acetyl)glucosamine-1-P. Nucleotide-activated sugars and dolichylphosphate-linked sugars are either used at the endoplasmic reticulum (ER) membrane (blue arrows) or actively transported into the organelles (black arrows). The red stars localize glycosylation disorders characterized to date (see Table 1 for identification). Hexokinase deficiency (blue star) is not included as a glycosylation disorder because it primarily affects glycolysis. Phosphoglucomutase deficiency (violet star), although very frequent in humans, is aphenotypic and therefore is not considered as a glycosylation disorder. Abbreviations: DolP-Glc, dolichylphosphoglucose; DolP-Man, dolichylphosphomannose; Fru, fructose; Fuc, fucose; Gal, galactose; GDP-keto-Fuc, GDP-4-keto-6-deoxy-mannose; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; ManNAc, N-acetylmannosamine; Sia, sialic acid.

Man-6-P are controlled by both the direct intake of Man and by the isomerization of fructose (Fru)-6-P to Man-6-P catalyzed by phosphomannose isomerase (PMI). The isomerization of Fru-6-P supplies a significant amount of activated mannose, as the deficiency of PMI leads to insufficient synthesis of N-glycans and hence to a form of CDG called CDG-Ib[15,16]. The sequence information obtained from the homologous yeast locus[17] was essential to identify the human cDNA.

In normal cells, the synthesis of GDP-Fuc is mainly derived from GDP-Man (Fig. 1), which ensures substrate supply for fucosylation reactions. In this pathway, the GDP-D-mannose 4,6-dehydratase enzyme first transforms GDP-Man to the unstable intermediate GDP-4-keto-6-deoxy-Man, which is in turn epimerized and reduced to GDP-Fuc by the FX protein[18]. Notably, this connecting pathway does not work efficiently in the reverse direction – that is, from GDP-Fuc to GDP-Man, thereby precluding a therapeutic use of fucose to bypass defects proximal to GDP-Man formation such as the *PMM2* deficiency.

UDP-GlcNAc and GDP-Man are directly utilized in the initial steps of the biosynthesis of the lipid-linked oligosaccharide required for N-linked glycosylation (Fig. 1). To serve as sugar donors in the glycosylation reactions that take place in the lumen of the ER, dolichylphosphoglucose (DolP-Glc) and dolichylphosphomannose (DolP-Man) are synthesized at the cytoplasmic side of the ER membrane from dolichylphosphate and UDP-Glc and GDP-Man, respectively. The human dolichylphosphomannose synthase comprises three subunits. The protein encoded by *DPM1* is the largest subunit and represents the catalytic center of the enzyme, whereas *DPM2* and *DPM3* anchor the complex at the cytosolic side of the ER membrane[19]. Mutations in the *DPM1* gene have been identified in CDG-Ie patients[20,21]. The ensuing decreased *DPM1* activity leads to the formation of a truncated dolichylpyrophosphate-linked oligosaccharide and to

Table 1. Congenital disorders of glycosylation[a]

Name	Gene defect	OMIM[b]	Activity	On Fig. 1[c]
CDG-Ia	*PMM2*	212065	Phosphomannomutase (Man-6-P → Man-1-P)	1
CDG-Ib	*PMI*	602579	Phosphomannose isomerase (Fru-6-P → Man-6-P)	2
CDG-Ic	*ALG6*	603147	α1-3 Glucosyltransferase	3
CDG-Id	*ALG3*	601110	α1-3 Mannosyltransferase	4
CDG-Ie	*DPM1*	603503	Dolichyl-phosphate-mannose synthase (GDP-Man → Dol-P-Man)	5
CDG-If	*LEC35*	604041	Unknown	6
CDG-IIa	*MGAT2*	202066	β1-2 *N*-acetylglucosaminyltransferase	7
CDG-IIb	*GLS1*	601336	α1-2 Glucosidase	8
LADII/CDG-IIc	GDP-Fuc transporter	266265	Import of GDP-Fuc into Golgi and export of GMP	9
Ehlers–Danlos syndrome (progeroid form)	*XGPT*	130070	Xylose β1-4 galactosyltransferase	10
Galactosemia I	*GALT*	230400	Gal-1-P uridyltransferase (Gal-1-P + UDP-Glc ↔ UDP-Gal + Glc-1-P)	11
Galactosemia I	*GALE*	230350	Galactose epimerase (UDP-Gal ↔ UDP-Glc)	12
Galactosemia II	*GALK*	604313	Galactokinase (Gal → Gal-1-P)	13

[a]Glycogen storage diseases do not represent biosynthetic (anabolic) disorders and are not included in this list.

[b]Accession numbers for the 'Online Mendelian inheritance in man' (OMIM; www.ncbi.nlm.gov/omim/) database are given.

[c]Refer to Fig. 1 in this article to locate the listed activity in the biosynthesis pathway.

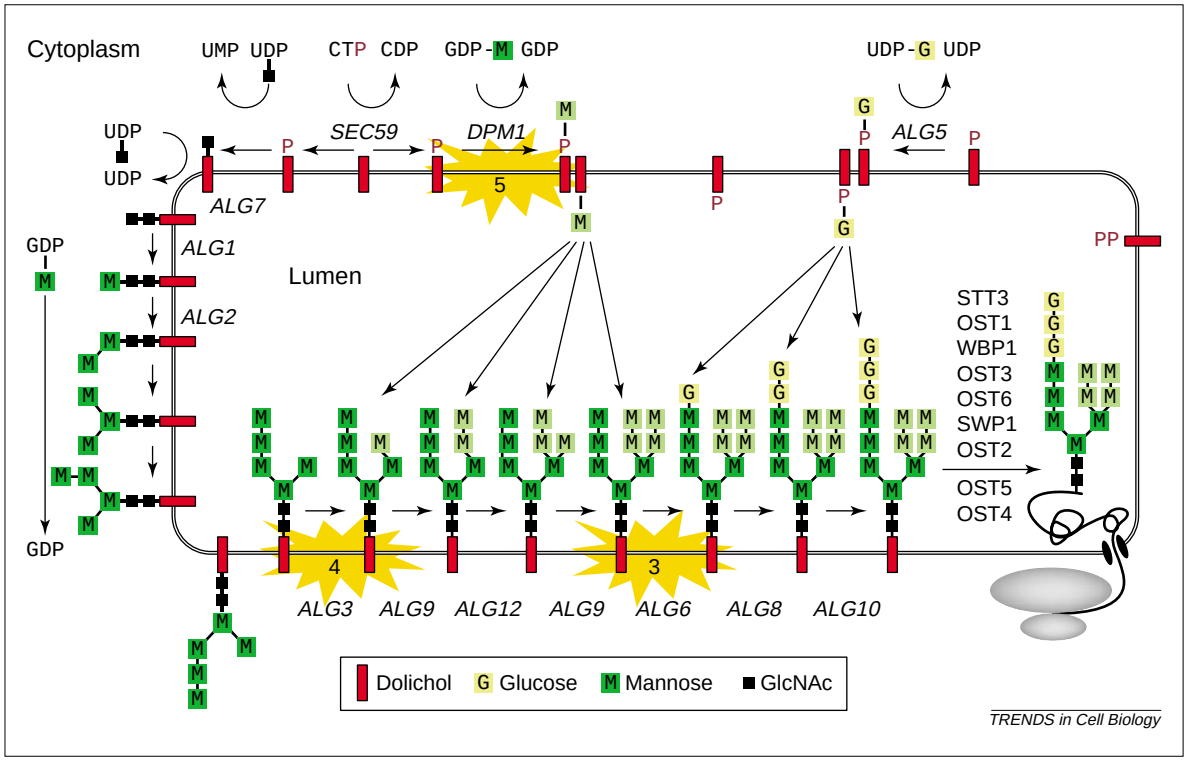

Fig. 2. The biosynthesis of lipid-linked oligosaccharide and its transfer to protein in the process of N-linked protein glycosylation in the endoplasmic reticulum (ER) in the yeast *Saccharomyces cerevisiae*. A schematic view of the ER membrane is shown, where the lipid dolichol (red bar) is phosphorylated (P) and then serves as a lipid carrier in the synthesis of the $Glc_3Man_9GlcNAc_2$ oligosaccharide. At the stage of $Man_5GlcNAc_2$, the lipid-linked oligosaccharide is flipped into the lumen of the ER and further extended by dolichylphosphomannose (DolP-Man) and dolichylphosphoglucose (DolP-Glc)-dependent glycosyltransferases. The oligosaccharyltransferase complex transfers the oligosaccharide to selected asparagine residues of translocating polypeptide chains. Genetic analysis has identified nine loci required for full oligosaccharyltransferase activity in yeast. The yeast loci encoding the different enzymes involved in the process are indicated beside the individual reactions. The biosynthetic steps affected in human CDG-Ic (*ALG6*), CDG-Id (*ALG3*) and CDG-Ie (*DPM1*) are highlighted with yellow stars; numbers refer to Table 1.

lower levels of glycosylphosphatidylinositol-anchored proteins in patient cells.

Nucleotide sugars are transported into the ER and Golgi by dedicated transporters. The cause of a CDG has been identified at the level of such a nucleotide sugar transporter: a defect of the GDP-Fuc transporter[22] is responsible for the fucosylation disorder characterizing a form of immunodeficiency previously known as leukocyte adhesion deficiency type-II (LADII)[23].

The biosynthesis of the lipid-linked oligosaccharide at the ER membrane

Nucleotide-activated sugars – UDP-GlcNAc and GDP-Man – are used as substrates in the initial phase of the lipid-linked oligosaccharide (Fig. 2). The biosynthesis is initiated by the tunicamycin-sensitive transfer of GlcNAc-1-P from UDP-GlcNAc to dolichylphosphate. Specific glycosyltransferases, not all of them identified to date at a molecular level, extend the lipid-linked oligosaccharide until the $Man_5GlcNAc_2$ intermediate is completed. This oligosaccharide is then 'flipped' to the lumenal side of the ER membrane and an additional four mannose and three glucose residues are added to the oligosaccharide by specific glycosyltransferases; DolP-Man and DolP-Glc serve as donors in these glycosyltransferase reactions. It is important to note that, owing to the substrate specificity of each of the branching transferases encoded by *ALG3*, *ALG12* and *ALG6*, a temporal order in the assembly pathway in the lumen of the ER is achieved: the di-mannose branches are assembled before the oligosaccharide is completed by the addition of the three glucose residues. Therefore, the addition of the terminal α1-2 glucose residue signals completion of the lipid-linked oligosaccharide, and this glucose residue is a key element in the recognition of the oligosaccharide substrate by the OST complex[24].

Because of this ordered assembly of the lipid-linked oligosaccharide at the ER membrane, deficiencies in the process result in the accumulation of specific biosynthetic intermediates. Thus, the deficiency of the DolP-Man-dependent ALG3 mannosyltransferase encountered in CDG-Id[25] leads to the accumulation of the $Man_5GlcNAc_2$ structure. Similarly, the incomplete oligosaccharide $Man_9GlcNAc_2$ accumulates in CDG-Ic cases where the DolP-Glc-dependent ALG6 glucosyltransferase is deficient[26–28]. These intermediates are suboptimal substrates for OST. As a consequence, glycoproteins lacking complete N-linked oligosaccharide chains result from deficiencies in the assembly pathway of the lipid-linked oligosaccharide. Therefore, the underglycosylation of glycoproteins, such as in the case of the serum protein transferrin, serves as a first diagnostic tool to identify and classify patients suffering from CDG[29].

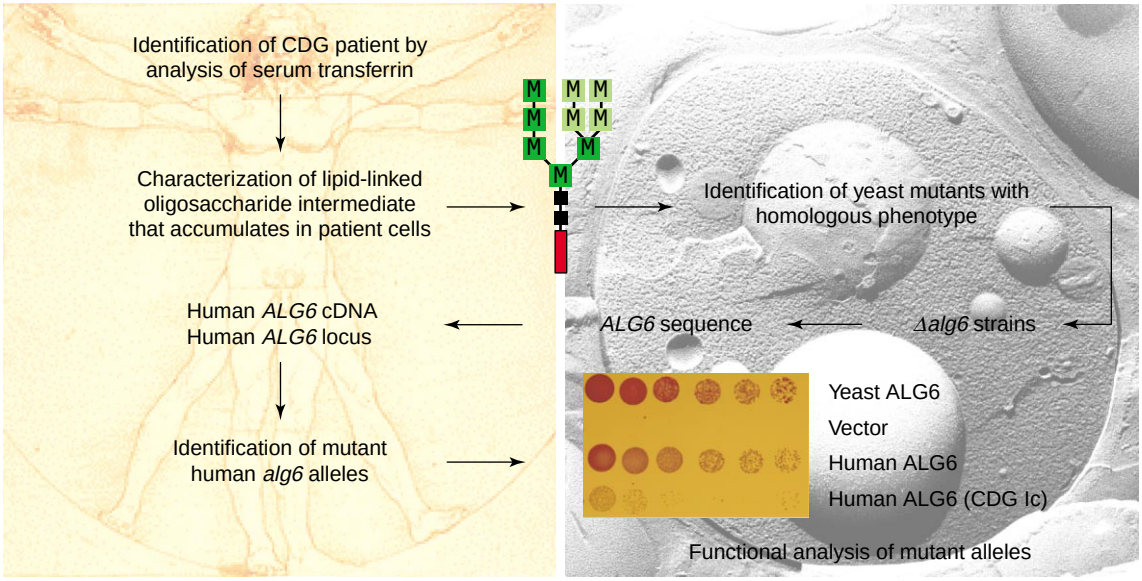

Fig. 3. Yeast as a tool to identify the molecular cause of congenital disorders of glycosylation (CDG). A schematic flow chart that led to the identification of the molecular defect of CDG-Ic is shown. Experiments performed with patient cells are indicated on the left; experiments performed in the yeast *Saccharomyces cerevisiae* are shown on the right. A serial dilution of *ALG6*-dependent yeast cells transformed with plasmid DNA containing the insert DNA indicated is shown as an example of a complementation analysis.

Budding yeast has been a valuable system to identify mutant strains with specific deficiencies in the ER glycosylation pathway[9] (Fig. 2). Owing to the high conservation of the pathway, yeast mutant strains can be directly used to define the molecular cause of a novel type of CDG. In fact, determining the mutant phenotypes and the underlying gene sequences can be important in order to characterize the human defects (Fig. 3). For example, CDG-Ic arises owing to a deficiency of the ALG6 enzyme, and fibroblasts from CDG-Ic patient cells accumulate the lipid-linked $Man_9GlcNAc_2$ oligosaccharide, as do *alg6* mutant yeast cells. The sequence information gained from the yeast *ALG6* locus made it possible to identify the orthologous human gene and to discover alterations in this locus in CDG-Ic patients[27]. In order to show that the mutations observed in the CDG-Ic patients were actually affecting the function of the ALG6 protein, the normal and mutant cDNA was expressed in *alg6*-deficient yeast cells. Indeed, the mutant form of the human ALG6 protein was not able to complement the *alg6* deficiency in yeast, in contrast to the normal human ALG6 protein. This functional assay directly correlated the mutation with a reduced efficiency of the ALG6 glycosyltransferase in CDG-Ic patients[27,30]. The same approach was used to confirm altered mannosyltransferase function conferred by the mutation in *ALG3* identified in a CDG-Id patient[25].

In the central step of the glycosylation pathway, the oligosaccharide is transferred from the lipid carrier to selected asparagine residues of nascent polypeptide chains by the OST complex. In yeast, this complex comprises at least eight different subunits, and biochemical and genomic analyses suggest that the composition of the OST is similar in higher eukaryotic cells[10,31]. No specific defects resulting in reduced OST activity have so far been detected as the molecular cause of CDG, but such alterations are expected to result in a CDG phenotype as deduced from the wide range of underglycosylation phenotypes observed in yeast deficient for some OST subunits.

Once attached to the protein, the $Glc_3Man_9GlcNAc_2$ oligosaccharide is trimmed in the ER by glucosidase I, glucosidase II and ER mannosidase to form the $Man_8GlcNAc_2$ oligosaccharide. This trimming process and reglucosylation by the UDP-Glc-dependent glucosyltransferase represent parts of the quality-control mechanism of glycoprotein folding in the ER[32]. Interestingly, a deficiency of glucosidase I was identified recently as the molecular cause of a fatal case of a congential glycosylation disorder, now called CDG-IIb[33]. In the Golgi apparatus, the oligomannose structure is trimmed further[34] and elongated in a species- and tissue-specific manner.

Disease phenotypes

Within the past three years, several new forms of CDG have been identified and characterized at a molecular level (Table 1). This burst of progress has been made possible by a coordinated effort between clinicians and basic researchers. The rapid increase of recognized types of CDG has prompted a need for a consistent nomenclature. The present system groups the disorders affecting the maturation of the lipid-linked oligosaccharide and its transfer onto glycoproteins as CDG-I. The disorders of N-glycan processing as well as those affecting other glycosylation pathways have been tentatively grouped as CDG-II. Notably, some diseases caused by glycosylation disorders have already received a

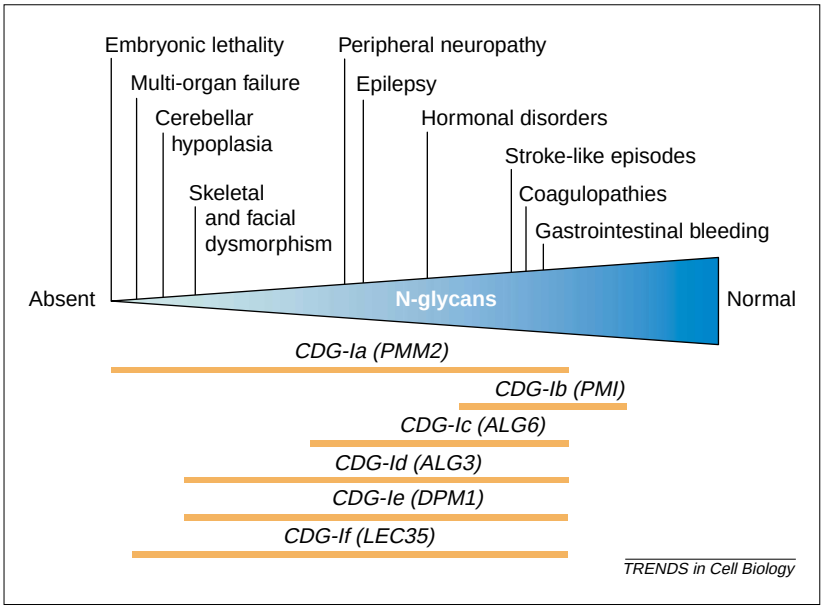

Fig. 4. Relation between clinical manifestations and extent of underglycosylation in congenital disorders of glycosylation (CDG) patients. The blue triangle symbolizes the degree of N-glycosylation on glycoproteins. The symptoms encountered in CDG patients are indicated at the top, and the range of symptoms found in specific types of CDG is shown by the orange bars at the bottom.

distinct name as they were described before the nomenclature rules were established. For such cases, such as the GDP-Fuc transporter deficiency called both LADII and CDG-IIc, it is conceivable that two names might coexist until a consensus emerges.

Defects in the biosynthesis pathway of the oligosaccharide core limit the substrate availability for the transfer onto nascent glycoproteins, hence causing a general decrease of N-glycosylation. N-linked protein glycosylation is an essential process in eukaryotic cells, and the total absence of N-linked oligosaccharides is lethal[3]. The types of CDG that are caused by deficiencies in the biosynthetic pathway of N-linked oligosaccharides in the ER mostly represent partial losses of function in specific biosynthetic steps. Therefore, the extent of underglycosylation largely depends on the degree of enzymatic impairment. For example, in CDG-Ia patients, the severity of the disease correlates with the reduction of the enzymatic activity measured in the *PMM2* mutant alleles[35]. Globally, the reduction of N-glycan chains on glycoproteins leads to variable features, ranging from dysmorphism to coagulopathies[4,5]. In the most severe cases, patients die within the first years of life of cardiac insufficiency or liver failure (Fig. 4). Cerebellar hypoplasia is a recurrent feature found in PMM2 deficiency, indicating the importance of N-glycosylation in the development of this brain structure. On the other hand, patients with PMI deficiency do not show any neurological alterations. These patients generally have symptoms of gastrointestinal bleeding accompanied by progressive liver fibrosis. Notably, it is possible to correct the underglycosylation phenotype in PMI deficiency by oral mannose supplementation[15].

CDG patients are often plagued by frequent stroke-like episodes. This life-threatening condition reflects an increased risk of thrombosis caused by decreased serum levels of the coagulation-controlling proteins antithrombin-III, protein C and protein S. Levels of factor XI are also diminished, and low levels of factor VIII and IX can be detected[36,37]. However, it remains unclear whether these findings relate to an impaired secretion of these proteins or to an increased clearance from the circulation. In any case, it appears that proper glycosylation is important for the maintenance of blood homeostasis. Another intriguing feature of CDG patients is that female patients often do not undergo puberty, although males do. Notably, the glycoprotein hormones follicle-stimulating hormone and luteinizing hormone bear a specific sulfated N-glycan structure, which has been shown to regulate the half-life of the hormones after release into the blood[38]. Correspondingly, the levels of these two hormones are often found to fluctuate in CDG patients[39,40].

Impairments in the processing of N-glycans in the Golgi apparatus and in other glycosylation pathways such as the formation of proteoglycans often yield different phenotypes to those encountered in CDG-I patients. For example, the deficiency of the xylose β1-4 galactosyltransferase leads to proteoglycans lacking glycan chains[41,42]. This feature renders the skin of the patients fragile, which causes a progeroid syndrome.

Outlook

Because of the diverse functions of N-linked glycans, the symptoms observed in CDG patients are highly variable. The complexity of these symptoms is probably the reason why this family of diseases has only been recognized recently[43]. The variability of symptoms means that an accurate diagnosis of the diseases is difficult, explaining why CDG remains largely underdiagnosed in most countries. The precise diagnosis of some specific types of CDG, such as CDG-Ic, -Id and -Ie, still requires an elaborate analysis of lipid-linked oligosaccharides, and, for many patients with clearly defined CDG symptoms, the molecular cause of the disease is still not known.

The elucidation of the molecular cause of different types of CDG represents a good example of how experimentally accessible model systems can be essential tools for a rapid molecular diagnosis of a novel type of a congenital disease, and it is evident that the yeast genetic system will be important to characterize novel types of CDG. However, one single-model system is not sufficient to address the many different types of disease: a novel type of CDG (If) was recently attributed to a deficiency of the human LEC35 protein (B. Schenk, T. Imbach, T. Hennet and M. Aebi, unpublished). This protein is not present in yeast, but a lack of this protein in Chinese hamster ovary (CHO) cells results in a reduced efficiency of N-glycosylation in the ER[44,45]. This example shows that other model

Acknowledgements
We thank all the members of our research groups for their continuous efforts and the participants in Euroglycan for their collaboration. The authors are supported by the Swiss National Science Foundation Grants 31-57082.99 (M.A.) and 31-58577.99 (T.H.).

systems, such as CHO cell mutants[46], are needed to characterize deficiencies in novel types of CDG.

Until now, most of the known CDG types affect the early pathway of N-linked glycosylation, and only a few patients with a defined deficiency in the Golgi-localized pathways have been identified. Considering the marked tissue-specificity of Golgi glycosylation, it is very likely that such deficiencies would result in more restricted symptoms than encountered in CDG-I. Because Golgi glycosylation pathways differ significantly between human and yeast, the latter model will be of little help to characterize these types of diseases. However, CHO cell lines[46] and mouse models with targeted glycosyltransferase gene disruptions[47] will provide essential clues to define the biological processes affected by altered Golgi glycosylation and hence allow identification of the symptoms expected in novel types of CDG.

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