

# Regulation of dolichol-linked glycosylation

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**Abstract** In the majority of congenital disorders of glycosylation, the assembly of the glycan precursor  $\text{GlcNAc}_2\text{-Man}_9\text{Glc}_3$  on the polyprenol carrier dolichyl-pyrophosphate is compromised. Because N-linked glycosylation is essential to life, most types of congenital disorders of glycosylation represent partial losses of enzymatic activity. Consequently, increased availability of substrates along the glycosylation pathway can be beneficial to increase product formation by the compromised enzymes. Recently, we showed that increased dolichol availability and improved N-linked glycosylation can be achieved by inhibition of squalene biosynthesis. This review summarizes the current knowledge on the biosynthesis of dolichol-linked glycans with respect to deficiencies in N-linked glycosylation. Additionally, perspectives on therapeutic treatments targeting dolichol and dolichol-linked glycan biosynthesis are examined.

**Keywords** CDG · Dolichol · Mevalonate pathway · Statins

## Dolichol biosynthesis and its role in N-linked glycosylation

N-linked glycosylation occurs in all domains of life and the same basic molecular principles underlie this type of post-translational modification [1, 2]. Briefly, an oligosaccharide precursor is assembled on a lipid carrier before the oligosaccharide is transferred to a target protein. In eukaryotes, N-linked protein glycosylation requires the assembly of the oligosaccharide precursor at both the outer and inner leaflet of the ER

membrane [3]. The precursor is first assembled on dolichyl-pyrophosphate (Dol-PP) starting at the cytoplasmic side. The intermediate Dol-PP- $\text{GlcNAc}_2\text{Man}_5$  is then flipped to the luminal side of the ER where the assembly proceeds to Dol-PP- $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ . Note worthily, dolichol (Dol) serves as lipid carrier in eukaryotes and archaea whereas another isoprenoid alcohol, bactoprenol, is used in bacteria. Apart from its role as carrier of the oligosaccharide  $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ , Dol is used to form the activated monosaccharides Dol-P-Man and Dol-P-Glc, which are substrates for glycosyltransferases involved in N-glycosylation, O-mannosylation, C-mannosylation and glycosylphosphatidylinositol (GPI) anchor biosynthesis.

Dol biosynthesis begins at the mevalonate pathway (Fig. 1) [4, 5], which produces essential isoprenoids. Isoprenoids are divided into two classes: sterol isoprenoids, such as cholesterol and steroid hormones, and non-sterol isoprenoids, encompassing polyprenols, ubiquinone, and Dol. Starting from acetyl-CoA via acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is first produced. The reduction from HMG-CoA to mevalonate is catalyzed by the rate-limiting HMG-CoA reductase (HMGCR) enzyme. Mevalonate is then phosphorylated to mevalonate-P by mevalonate kinase (MVK). A second phosphorylation step and subsequent decarboxylation leads to isopentenyl-5-pyrophosphate (IPP), which represents an important building block for isoprenoids. IPP is an activated isoprene unit of five carbon atoms and is used to form farnesyl-pyrophosphate. At this point, the pathway diverges to feed on the one hand the biosynthesis of cholesterol and on the other hand the biosynthesis of non-sterol isoprenoids. To obtain Dol, dehydrololichyl diphosphate synthase (DHDDS) catalyzes the stepwise head-to-tail *cis* addition of IPP to farnesyl-pyrophosphate, thus giving rise to polyprenyl-pyrophosphates of 15 to 19 isoprene units. These polyprenyl-pyrophosphates are then dephosphorylated by pyro- or monophosphatases. The resulting polyprenols are reduced at the  $\alpha 1$  position to become

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Dol of different lengths. Dol is then phosphorylated by Dol kinase (DOLK). Dol-P can then be utilized as the carrier for the oligosaccharide  $\text{GlcNAc}_2\text{Man}_6\text{Glc}_3$  or as a carrier for Man and Glc. Dol-P-Man is produced by the Dol-P-Man synthase (DPM1-3) that transfers Man from GDP-Man to Dol-P [6]. Similarly, Dol-P-Glc is produced by the glucosyltransferase ALG5 that transfers Glc from UDP-Glc to Dol-P [7].

Importantly, Dol levels at the cytoplasmic leaflet of the ER are not only maintained by *de novo* synthesis but also depend on recycling of discharged Dol-PP and Dol-P. Once the oligosaccharide precursor is transferred to a target protein, Dol-PP is released and dephosphorylated to Dol-P by the luminal phosphatase DOLPP1 [8]. Dol-P is then flipped across the membrane to the cytoplasmic leaflet by a yet unknown mechanism [9]. Recycling of Dol-P and Dol-PP contributes significantly to the Dol pool available glycosylation in the ER. Accordingly, a defect of the DOLPP1 orthologue in yeast leads to impaired N-linked glycosylation [10].

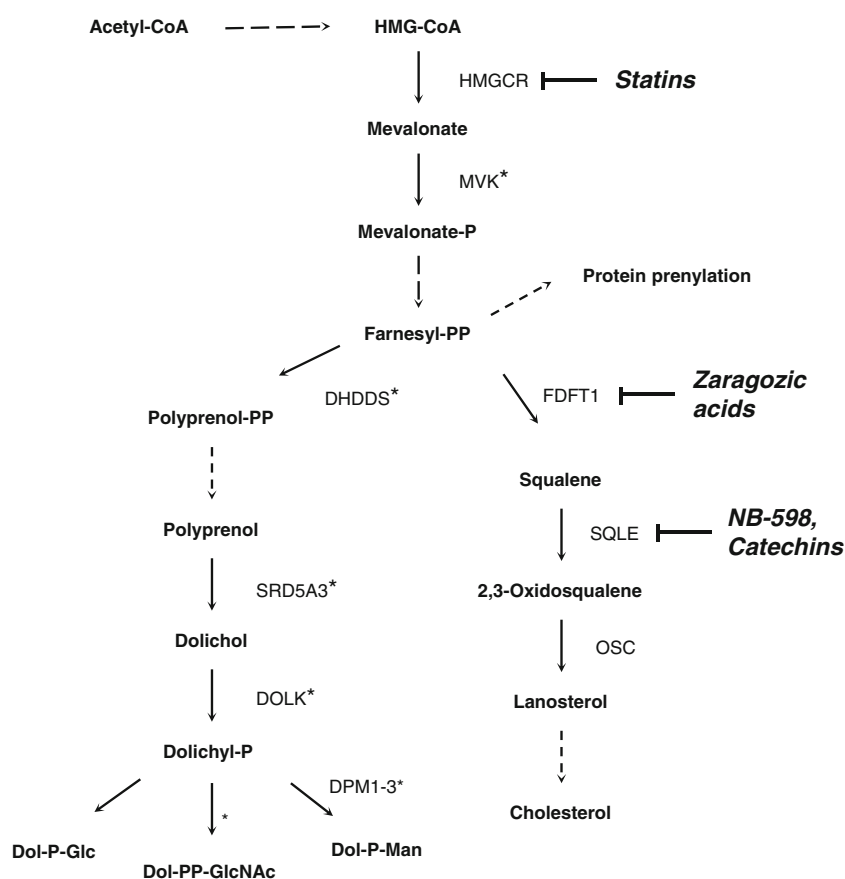
### Deficiency of dolichol biosynthesis—a new family of CDG

Several forms of congenital disorders of glycosylation (CDG) caused by Dol biosynthesis defects have been characterized

recently. The long sought polyprenol reductase has been identified through the description of SRD5A3-CDG [11] and DHDDS deficiency was discovered in a subgroup of retinitis pigmentosa patients [12]. Moreover, the clinical picture of Dol kinase deficiency was extended through the description of new cases presenting with dilated cardiomyopathy [13].

Proximal to Dol, a defect along the mevalonate pathways has also been linked to diseases. Mutations of MVK (Fig. 1) cause of two different forms: mevalonic aciduria (MVA) [14] and hyperimmunoglobulinemia D syndrome (HIDS) [15]. In MVA, MVK activity is reduced to 0–4 % of normal values leading to death in early childhood. Typical symptoms (Table 1) are psychomotor retardation, failure to thrive, progressive cerebellar ataxia, dysmorphic features, progressive visual impairment and frequent febrile attacks [5, 16, 17]. Psychomotor retardation, failure to thrive, dysmorphic features, and visual impairment are also frequently found in CDG, thus suggesting possible glycosylation problems in MVA. HIDS is a milder form of the disease correlating with residual MVK activity of 5–15 % of normal values. In HIDS, febrile attacks and skin rashes start in early childhood and can be triggered by diverse events, such as vaccinations or minor infections. The febrile disposition is believed to be linked to insufficient levels of anti-inflammatory isoprenylated proteins. Low geranylgeranyl-PP in HIDS leads to

**Fig. 1** Dolichol and cholesterol biosynthesis pathway. Two acetyl building blocks are required for HMG-CoA formation. HMG-CoA reductase (HMGCR) catalyzes the rate limiting step, the formation of mevalonate. Mevalonate kinase (MVK) phosphorylates mevalonate in a CTP-dependent manner. Sequential action of dehydrodolichyl diphosphate synthase (DHDDS), pyro- or monophosphatases,  $5\alpha$  steroid reductase type 3 (SRD5A3), and dolichol kinase (DOLK) produce Dol-P. Dol-P-Man synthase (DPM1-3) transfers a mannose to Dol-P. Squalene synthase (FDFT1) produces squalene from farnesyl-PP and subsequent action of squalene epoxidase (SQLE), and 2,3-oxidosqualene cyclase (OSC) leads to lanosterol. Dotted arrows indicate simplifications of the biosynthetic pathway and \* point out known congenital defects in dolichol biosynthesis of the respective enzymes



**Table 1** Disorders of dolichol biosynthesis and their respective symptoms

	MVA	HIDS	DHDDS- CDG	SRD5A3- CDG	DOLK- CDG <sup>a</sup>	DOLK- CDG <sup>b</sup>	DPM1- CDG	DPM3- CDG
Cardiomyopathy				*	**	**		*
Cerebellar ataxia / malformation	**	**		**			**	
Coagulopathy				**			**	
Dysmorphic features	**			**	**			
Failure to thrive	**							
Febrile attacks	**	**						
Hypoglycemia					**			
Hypotonia					**		**	
Microcephaly	**	*			**		**	
Muscular dystrophy							**	*
Myopathy							**	*
Ocular malfunctions	**		**	**	**		**	
Psychomotor retardation	**	**		**	**	*	**	
Seizures	**	*			**		**	
Skin disorders	**	**		*	**	*		

Symptoms are distinguished between \*\* for typical/dominant symptoms and \* for sporadic symptoms or symptoms of moderate severity

<sup>a</sup>Classical DOLK-CDG, <sup>b</sup>DOLK defect in dilated cardiomyopathy

caspase-1 activation and IL-1 $\beta$  secretion [18, 19]. Other symptoms found in HIDS (Table 1) are mental retardation, ataxia, ocular symptoms and epilepsy [17]. Diagnosing HIDS involves the detection of increased mevalonic acid in urine and increased serum immunoglobulin D and A. Apart from febrile attacks, the clinical features of MVA and HIDS are reminiscent of CDG. Indeed, metabolic labelling experiments with [<sup>14</sup>C]galactose in MVK deficient fibroblasts revealed lower secretion of the radio-labelled macromolecules indicating impaired glycosylation, which is possibly due to decreased formation of Dol and Dol-P [20]. However, systematic analysis of glycosylation in HIDS has not been performed yet.

Recently, DHDDS deficiency (Fig. 1) has been linked to inherited retinitis pigmentosa [12], a disorder causing retinal degeneration with an estimated incidence of 1 in 3,000–4,500. The single mutation identified was present in a very small subgroup of retinitis pigmentosa patients [21]. Interestingly, DHDDS patients did not show typical CDG symptoms despite the central role of DHDDS in Dol production (Table 1). This fact suggests that *DHDDS* mutations associated with retinitis pigmentosa only partially impair polyprenol-PP formation and that only few photoreceptor-specific proteins may be sensitive to a reduced polyprenol-PP pool. Note worthily, inhibition of N-linked glycosylation by tunicamycin also leads to retinal degeneration in *Xenopus* [22, 23]. Retinitis pigmentosa is also associated with MVK deficiency and with cases of phosphomannomutase 2 deficiency in PMM2-CDG [24].

The third disorder of Dol biosynthesis is a recently described type of CDG, in which the *SRD5A3* gene (Fig. 1) is affected [25, 26]. *SRD5A3* encodes the steroid 5 $\alpha$  reductase

type 3 protein, which reduces the  $\alpha$ -isoprene in polyprenol to form Dol. Symptoms of SRD5A3-CDG are typical for glycosylation disorders and encompass ocular malformations, cerebellar vermis hypoplasia, skin lesions, psychomotor retardation, and facial dysmorphism (Table 1). Strikingly, these multisystemic manifestations strongly differ from those found in DHDDS-CDG despite the proximity of the two enzymes in the Dol pathway. Loss of whole N-glycan chains on proteins has been described in SRD5A3-CDG. Different mutations of *SRD5A3* lead to truncated forms of the protein resulting in loss of function. Residual levels of Dol in SRD5A3 deficient cells suggest an alternative pathway for Dol biosynthesis.

Dol kinase deficiency, characterizing DOLK-CDG, impairs the phosphorylation of Dol to Dol-P (Fig. 1). DOLK-CDG presents with severe phenotypes including hypotonia, skin disorders, and the loss of hair [27]. Moreover, cardiomyopathy, seizures, hypoglycemia, microcephaly, and visual impairment can occur as well (Table 1). Due to the severity of the symptoms, most *DOLK* mutations generally cause a severe disease associated with infant lethality. Reduced DOLK activity decreases the availability of Dol-P and thus impairs the assembly Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> and N-linked glycosylation. Recently, 11 children with dilated cardiomyopathy (DCM), a disease possibly linked to sudden cardiac death and heart failure, were found to have a novel mutation in DOLK [13]. Besides DCM, only few patients exhibit additional symptoms like ichthyosis, failure to thrive, and mild neurological involvement. Biochemical analysis of Dol-P dependent glycosylation pathways of the DCM heart tissue indicated reduced O-mannosylation of  $\alpha$ -dystroglycan [28]. Remarkably, abnormal N-glycosylation of serum transferrin was equally pronounced

in all forms of DOLK-CDG. The phenotypes of DCM strongly contrast with the originally described features of DOLK-CDG [27], thus suggesting a possible tissue-specific regulation of Dol-P dependent glycosylation.

Human Dol-P-Man synthase (DPM1-3, Fig. 1) consists of 3 subunits. DPM1 is the catalytic subunit and DPM2/3 are regulatory and membrane anchoring proteins residing in the ER membrane [6]. So far, two forms of Dol-P-Man synthase deficiency have been associated with mutations in the *DPM1* and *DPM3* genes. DPM1-CDG is characterized by recurrent seizures, hypotonia, developmental delay, dysmorphic features, microcephaly, visual impairment, and in some cases ataxia and coagulopathy (Table 1) [29–32]. Lower Dol-P-Man levels lead to impaired Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> assembly, abnormal N-linked protein glycosylation, and decreased formation of GPI anchored proteins. DPM3-CDG appears to be a milder form of Dol-P-Man synthase deficiency, for which only one patient has been found so far [33]. The symptoms included mild myopathy, a dilated cardiomyopathy, moderate muscular dystrophy, and a single stroke-like episode. N-glycosylation of serum transferrin was found to be only slightly abnormal in DPM3-CDG. In DPM1-CDG and DPM3-CDG, the severity seems to reflect the molecular functions of the affected subunits. A deficiency of the catalytic subunit DPM1 leads to much more severe phenotypes than a deficiency in the tethering subunit DPM3.

In general, the clinical severity of Dol-related diseases does not correlate with the relative position of the deficient enzymes along the biosynthesis pathway. Defects of Dol biosynthesis are severe since not only the assembly of N-glycosylation is impaired, but also GPI anchor formation and O-mannosylation are affected. This notion is confirmed in SRD5A3-CDG that present with severe multisystemic phenotypes. However, the discovery of DHDDS-CDG with a very specific phenotype and lack of the typical CDG symptoms suggests a complex regulation of Dol-dependent glycosylation. Future efforts addressing Dol biosynthesis will improve our comprehension of the regulation of N-glycosylation and provide therapeutic perspectives.

### Therapeutics targeting dolichol

To date, only two forms of CDG are treatable. MPI-CDG can be successfully treated by oral mannose supplementation [34, 35]. In GFTP-CDG, deficient GDP-fucose transport can be compensated by nutritional fucose supplementation [36]. The hypomorphic nature of most CDG mutations allows for a compensatory approach, for instance by providing more Dol to overcome the compromised assembly of Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub>. Along this line, inhibition of squalene synthase (FDFT1, Fig. 1) was shown to improve N-linked protein glycosylation and GPI-anchored protein expression in

DPM1-CDG fibroblasts [37]. Additional drugs such as clofibrate were also tested as a way to increase GPI anchor availability in DPM1-CDG fibroblasts. Clofibrate decreases cholesterol levels by targeting lipid metabolism via activation of peroxisome proliferator-activated receptors, especially PPAR $\alpha$  [38, 39]. Indeed, surface expression of the GPI anchored protein CD59 was increased upon clofibrate treatment (unpublished data). Similarly, the adenylate cyclase activator forskolin [40] increased surface expression of CD59 as well. This was in accordance with the notion that high levels of cyclic AMP lead to higher activity of DHDDS and thus increased Dol levels [41, 42].

New therapeutic possibilities arise for CDG with the advance of non-statin cholesterol lowering drugs, which target the late pathway of cholesterol biosynthesis. Presently, statins are the prevalent medication to lower cholesterol and decrease cardiovascular disease [43]. Statins are inhibitors of HMGCR (Fig. 1), the rate limiting enzyme at the beginning of the mevalonate pathway. The effect of statins on Dol levels has not been investigated systematically. Hela cells treated with pravastatin showed a decrease in Dol-P by 65 % [44]. However, rats treated with lovastatin did not show a change of total Dol-P levels but increase of Dol-P in the liver [45]. The potential of different statins to upregulate Dol could be subject of further investigations.

Inhibition of the first committed enzyme of cholesterol biosynthesis, squalene synthase (FDFT1, Fig. 1), has proven effective in lowering cholesterol production [46]. Prominent among these inhibitors are zaragozic acids, which were identified in a screen of fungal compounds for cholesterol lowering activity [47]. Changes in Dol-P patterns were observed in human fibroblasts treated with zaragozic acid A. Also, DPM1-CDG fibroblasts showed improved N-linked glycosylation upon treatment with zaragozic acid A [37]. Moreover, the expression of the GPI-anchored CD59 protein could be normalized in DPM1-CDG fibroblasts after treatment. Since zaragozic acid is well tolerated in animals [48, 49], this class of FDFT1 inhibitors might be considered for clinical testing in CDG.

Squalene epoxidase (SQLE) catalyzes the next committed step of cholesterol biosynthesis after FDFT1 (Fig. 1). Inhibition of SQLE by the compound NB-598 ((E)*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3'-bithiophen-5-yl)methoxy]benzene-methanamine) successfully reduced cholesterol levels [50, 51], although no changes in Dol levels were detected in treated HepG2 cells [52]. In spite of this early finding, newly developed SQLE inhibitors should be considered as promising candidates for an upregulation of Dol in CDG cells. Moreover, green tea has been attributed a cholesterol lowering effect, which was linked to SQLE inhibition [53]. In a recent study, oral supplementation with the main green tea catechin epigallocatechin gallate was shown to lower low density lipoprotein associated cholesterol [54]. The effects of epigallocatechin gallate on Dol

have not been tested yet. Catechins might represent another family of compounds with the potential to upregulate Dol in CDG cells by inhibition of SQLE.

In conclusion, inhibitors of late cholesterol biosynthesis have a potential increasing effect on Dol levels and should be accordingly tested in CDG cells. Much effort is invested in the development of cholesterol lowering drugs in the context of cardiovascular diseases. Therefore, potential CDG therapies could profit from the discovery of novel cholesterol lowering drugs in the future.

## Outlook

The recent descriptions of tissue-restricted symptoms in DHDDS-CDG and of a novel pathology associated to DOLK deficiency suggest a pronounced tissue specificity of Dol-related biology. Accordingly, additional diseases are likely to be associated with local alterations of Dol biosynthesis in the near future. The study of these tissue-restricted diseases represents valuable models to better understand the regulation of Dol biosynthesis and its impact on various types of glycosylation. Novel therapeutic approaches may be deduced from such insights. The treatment of CDG is still very limited and merits further attention. Interference of cholesterol production and thus upregulation of Dol biosynthesis certainly represents a valuable approach to improve glycosylation in CDG.

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