Inaugural dissertation

for

obtaining the doctoral degree

of the

Combined Faculty of Mathematics, Engineering and Natural Sciences

of the

Ruprecht - Karls - University

Heidelberg

Presented by

Frauke Kikul, M.Sc.

born in: Kirchheimbolanden, Germany

Oral examination: 21.11.2023

The influence of membrane lipids on glycosylation processes: Activity of Dpm1 in different lipid environments

Referees:

Prof. Dr. Britta Brügger

Prof. Dr. Sabine Strahl

Abstract

An important function of lipids is their ability to form membrane bilayers. Membrane properties such as thickness, fluidity or curvature depend on the lipid composition, which varies among species, tissues, cells and organelles and even between membrane domains. Membranes are cellular barriers but also provide a matrix for proteins and chemical reactions and their properties can affect protein localizations, and enzyme activities of embedded proteins. Most known glycosylation enzymes are integral membrane proteins, that catalyze glycosylation reactions at the ER and Golgi membranes.

With this project, I aimed to study a regulative role of lipids in glycosylation processes. I chose the integral membrane protein Dolichol phosphate mannose synthase (Dpm1) from yeast as a model protein. Dpm1 is an essential protein in eukaryotes, and dysfunction of Dpm1 *in vivo* leads to hypoglycosylation and severe glycosylation defects. The glycosyltransferase catalyzes the synthesis of the mannosyl donor DolP-Man which is required for all protein glycosylation routes.

To investigate a role of membrane lipids in modulating the activity of Dpm1, I used a liposomal reconstitution system. I established a purification and liposomal reconstitution protocol and developed an assay to study the enzymatic activity of yeast Dpm1 *in vitro*. The liposomal lipid composition was then systematically varied, to investigate the effect of the membrane lipids on the enzyme activity of Dpm1. The system was then extended by reconstituting of Dpm1 together with other proteins to study their interaction in different lipid environments. I found that the enzymatic activity of Dpm1 is modulated by the lipid composition of the membrane. An increase in DolP-Man formation was observed at increased membrane fluidity and in the presence of phosphatidylethanolamine. In addition, I showed that yeast Dpm2 (Yil102c-A) can stimulate Dpm1 activity, independently of the membrane environment. By including the O-mannosyl transferase Pmt4 from yeast or *C. thermophilium*, I successfully reconstituted O-mannosylation in liposomes, where the DolP-Man substrate for Pmt4 is provided by the enzymatic activity of Dpm1, demonstrating a successful *in vitro* reconstitution of a coupled enzyme chain reaction. With this work, I have provided the basis for further studies to investigate the role of lipids in regulating the activity of *in vitro* reconstituted enzyme reaction chains of glycosylation.

Zusammenfassung

Eine wichtige Funktion von Lipiden ist ihre Fähigkeit Membrane zu bilden. Membraneigenschaften wie Dicke, Fluidität oder Krümmung sind abhängig von der Lipidzusammensetzung der Membran, welche sich zwischen Spezies, Gewebearten, Zellen, Organellen und sogar Membrandomänen unterscheidet. Membranen fungieren als zelluläre Barrieren, aber sie bieten auch eine Matrix für Proteine und chemische Reaktionen und ihre Eigenschaften können Proteinlokalisation oder Enzymaktivität von eingebetteten Proteinen beeinflussen. Die meisten heute bekannten Glykosylierungsenzyme sind integrale Membranproteine, welche Glykosylierungen an den ER- und Golgi-Membranen katalysieren.

Mit diesem Projekt wollte ich die regulative Rolle von Lipiden in Glykosylierunsprozessen untersuchen. Ich habe das integrale Membranprotein Dolicholphosphat-Mannose-Synthase (Dpm1) aus Hefe als Modelprotein ausgewählt. Dpm1 ist ein essentielles Protein in Eukaryoten, und gestörte Funktion von Dpm1 *in vivo* führt zu Hypoglykosylierung und schweren Glykosylierungsdefekten. Die Glycosyltransferase katalysiert die Synthese von dem Mannosyldonor DolP-Man, welcher für alle Proteinglykosylierungswege benötigt wird.

Um die Rolle von Membranlipiden für die Aktivität von Dpm1 zu untersuchen habe ich ein Aufreinigungsprotokoll und Liposom-basiertes Rekonstitutionsprotokoll etabliert und einen Test entwickelt um die enzymatische Aktivität von Hefe Dpm1 *in vitro* zu untersuchen. Die Lipidzusammensetzung der Liposomen wurde systematisch verändert um den Effekt der Membranlipide auf die Enzymaktivität zu untersuchen. Das System wurde zusätzlich erweitert durch die Rekonstitution von Dpm1 zusammen mit weiteren Proteinen. Ich konnte zeigen, dass die Aktivität von Dpm1 durch die Lipidzusammensetzung beeinflusst wird. Zusätzlich konnte ich zeigen, dass Dpm2 aus Hefe (Yil102c-A) die Aktivität von Dpm1 unabhängig von der Membranzusammensetzung stimuliert. Durch die Hinzunahme der O-Mannosyltransferase Pmt4 aus Hefe oder *C. thermophilium* konnte ich erfolgreich eine gekoppelte enzymatische Reaktionskette in Liposomen *in vitro* rekonstituieren, bei welcher das DolP-Man Substrat für Pmt4 durch die enzymatische Reaktion von Dpm1 gebildet wird. Mit dieser Arbeit habe ich eine Basis gelegt, um die Rolle von Lipiden in der Regulation von Enzymaktivität von *in vitro* rekonstituierten Glykosylierungskettenreaktionen zu untersuchen.

Graphical abstract

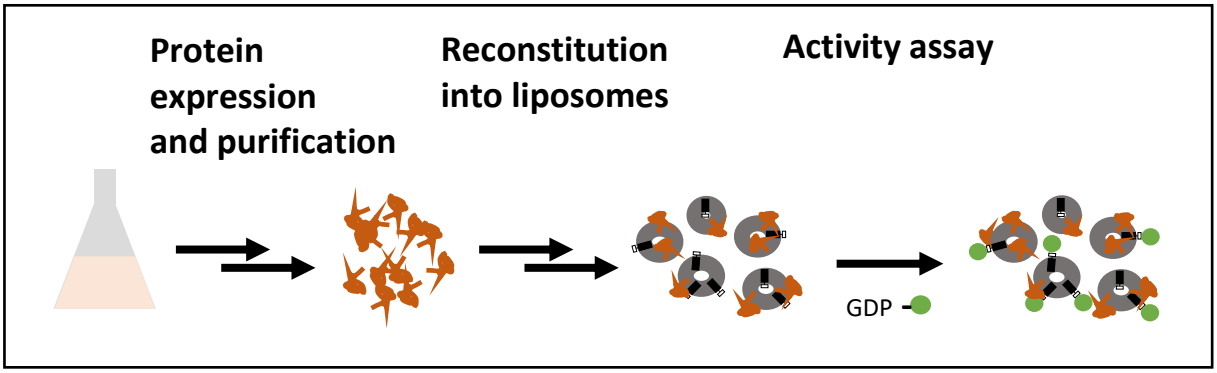


Figure 1 **Graphical abstract**

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Alg	Asparagine-linked glycosylation enzyme
Bio-Beads	Bio-Beads SM2
CDG	Congenital disorder of glycosylation
Chol	Cholesterol
Cit	Citronellol
CitP	Citronellol phosphate
CMC	Critical micelle concentration
DDM	n-Dodecyl-β-Maltoside
DLS	Dynamic light scattering
DMAPP	dimethylallyl pyrophosphate
Dol	Dolichol
DolP	Dolichol phosphate
DolP-Man	Dolichol phosphate mannose
DolPP	Dolichol pyrophosphate
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine, PC (18:1/18:1)
DPMS	Dolichol phosphate mannose synthase
DRM	Detergent resistant membrane
ER	Endoplasmatic reticulum
ER	Endoplasmic reticulum
Erg	Ergosterol
EtNP	Ethanolamine phosphate
FA	Fatty acid
FPP	Farnesyl-pyrophosphate
FU	“Float Up”
GDP	Guanosine diphosphate
GDP-Man	Guanosine diphosphate mannose
GPI	Glycosyl phosphatidyl inositol
GPL	Glycerophospholipid
GSL	Glycosphingolipid
GT	Glycosyl transpferase
GT	Glycosyl transferase
GUV	Giant unilamellar vesicle
IEF	Iso-electric focusing
IMP	Integral membrane protein
IPP	isopentenyl pyrophosphate
LC	Liquid chromatography
LDAO	Lauryldimethylamine oxide
LLO	Lipid-linked oligosaccharide
LUV	Large unilamellar vesicle
MDS	Molecular dynamics simulation
MLV	Multi lamellar vesicle

MS	Mass spectrometry
NP-40	Nonidet P-40 substitute
OG	Octyl-β-D-glucopyranosid
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphoethanolamine
PI	Phosphatidylinositol
PI(4)P	Phosphatidylinositol 4-phosphate
PM	Plasma membrane
PMT	Protein O-mannosyl transferases
Pol	Polyprenol
PolP	Polyprenol phosphate
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, PE (16:0/18:1)
PS	Phosphatidylserine
pαDG	α-Dystroglycan peptide (401-420)
pαDG-Man	Mannosylated α-dystroglycan peptide (401-420)
Rh-PE	Rhodamine-phosphoethanolamine
SDS	Dodecylsulfate-Na-salt
SMA	Styrene maleic acid
SMALP	Styrene maleic acid lipid particle
SL	Sphingolipid
SOPC	1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, PC (18:0/18:1)
SUV	Small unilamellar vesicle
TMD	Trans membrane domain
UDP	Uridine diphosphate

Contributions

Cloning and transformation were done by Thomas Kupke and Alexia Hermann.

Mass spectrometric measurements of DolP and DolP-Man, including derivatization, were performed by Dipali Kale.

Mass spectrometric measurements of PC for the normalization of Data were done by the help of Iris Leibrecht, Timo Sachsenheimer and Jürgen Reichert.

Purified Pmt4 protein was kindly provided by Antonella Chiapparino and Melanie McDowell.

List of Publications

During my PhD, I contributed to the following papers:

Himmelreich, Nastassja et al. “Complex metabolic disharmony in PMM2-CDG paves the way to new therapeutic approaches.” *Molecular genetics and metabolism*, vol. 139,3 107610. 16 May. 2023, doi:10.1016/j.ymgme.2023.107610

Kale, Dipali et al. “Quantification of Dolichyl Phosphates Using Phosphate Methylation and Reverse-Phase Liquid Chromatography-High Resolution Mass Spectrometry.” *Analytical chemistry* vol. 95,6 (2023): 3210-3217. doi:10.1021/acs.analchem.2c03623

1 Introduction

Sugars and lipids are two important biological molecules for life. In this project, I wanted to elucidate the connection between both of them and investigate the influence of membrane lipids on glycosylation reactions. Thus, in the first part of the introduction, I will summarize and shortly present different ways of glycosylation and the relevance of sugar modifications in health and disease. In the second half I will introduce the ER-membrane (membrane of the endoplasmic reticulum), as a major site of cellular glycosylation and lipid biosynthesis, and the role of its lipid composition and introduce ways to study membrane proteins.

1.1 Cellular glycosylation

Glycosylation is the enzymatic attachment of one or more sugar molecules to a substrate. The most common glycan acceptors are proteins, but also lipids are also modified by glycosylation [1]. Recently, even some nucleic acids from non-coding RNA were discovered to be decorated with sugars [2].

Glycosylation is an essential posttranslational modification for cellular functionality. Glycans serve several roles, including stabilizing proteins, supporting proper folding, acting as a signal molecule, and to promoting cell-cell interactions, either intrinsically or extrinsically [3]. The cell surface of eukaryotic and prokaryotic cells is highly decorated with sugars that are attached to proteins and lipids, forming a protective glycocalyx [4]. Around 200 enzymes are involved in cellular glycosylation reactions and a close coordination of the function of these enzymes is required [4]. Misfunction of the glycosylation machinery causes severe glycosylation defects, leading to congenital disorders of glycosylation (CDGs).

Glycan structures are highly divers in shape and size, ranging from single monomers to linear or heavily branched glycan structures. Glycans have a high potential for information storage due to their chemical diversity. In human, 10 different sugar monomers are used for glycosylation, namely glucose (Glc), galactose (Gal), mannose (Man), N-acetyl-glucosamine (GlcNAc), N-acetyl-galactosamine (GalNaAc), glucoronic acid (GlcA), N-acetylneuraminic acid (Neu5Ac), ribose (Rib), xylose (Xyl) and fucose (Fuc) [4]. However, it is not the quantity of the various monosaccharide building blocks, but their variety of chemical linkages that is responsible for the

complexity of the glycome. In contrast to linear peptides or DNA molecules, where monomers can only be connected in a single and linear way, two hexose monomers can be linked in numerous different ways. This diversification of oligosaccharides by the different constitutional- and stereoisomers is leading to a variety of possible chemical linkages and structures. Not all theoretically possible linkages are also found in nature and there is a tight regulation of glycan structure and stereochemistry. Most glycosyl transferases (GTs) were found to be highly regio- and stereoselective for their substrates [5]. Interestingly, glycan structures are not encoded in the genome like the protein sequences. Still, glycans are not getting randomly attached to the protein and there is a strict orchestration of the glycosyl transferases and glucosidases. How exactly the final glycan structure is determined and how the synthesis is regulated is not yet fully understood.

The vast majority of over 85% of the secretory proteins is decorated with glycans [4]. Protein glycosylation occurs while proteins travel through the secretory pathway [3]. It is initiated co- or post-translational in the ER or Golgi and further elongation and modifications of the glycans occur along the secretory pathway.

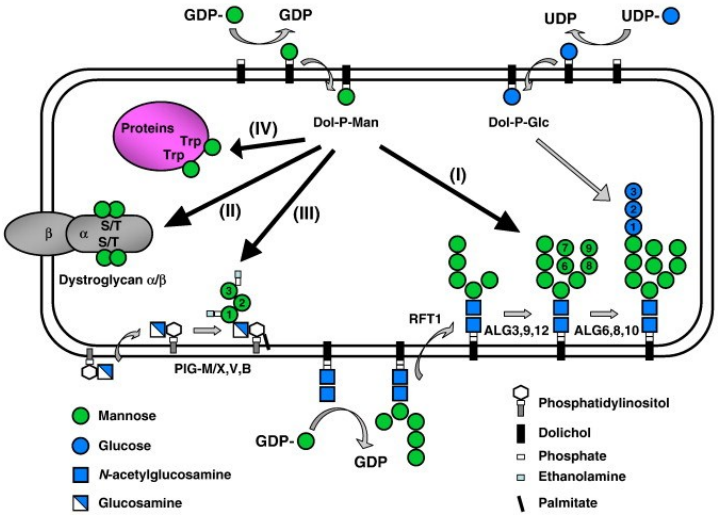


Figure 2 **Glycosylation reactions at the ER**
DolP-Man is used for the synthesis of Lipid-linked oligosaccharides (I), for O-mannosylation (II), GPI-anchor synthesis (III) and C-mannosylation (IV). Image taken from Maeda and Kinoshita [6], license no 5623190090918

Glycans get attached to specific amino acids of the proteins. Depending on the atom the glycan is linked to, the types of glycosylation are categorized into N-, O- and C-glycosylation. In addition, proteins can get C-terminally attached to GPI-anchors (glycosylphosphatidylinositol-anchors), a sugar containing lipid anchor. The glycan

profile of each protein is not limited to a single type of linkage, and often a combination of different glycan types is found on a single protein.

1.1.1 N-glycosylation

The most common and best-studied form of protein glycosylation is the N-glycosylation, where the glycan is attached to the γ-amido group of an asparagine residue. N-linked glycans can be found in all domains of life and their core structure is highly conserved in most eukaryotes [7, 8]. Here, this core structure consists of two GlcNAc residues, nine Man and 3 Glc residues. It gets built up sequentially by various different asparagine-linked glycosylation enzymes (Alg) at the ER. The structure gets first assembled as a lipid-linked oligosaccharide (LLO) on a dolichol pyrophosphate (DolPP) lipid anchor before the glycan gets transferred *en bloc* to the peptide chain.

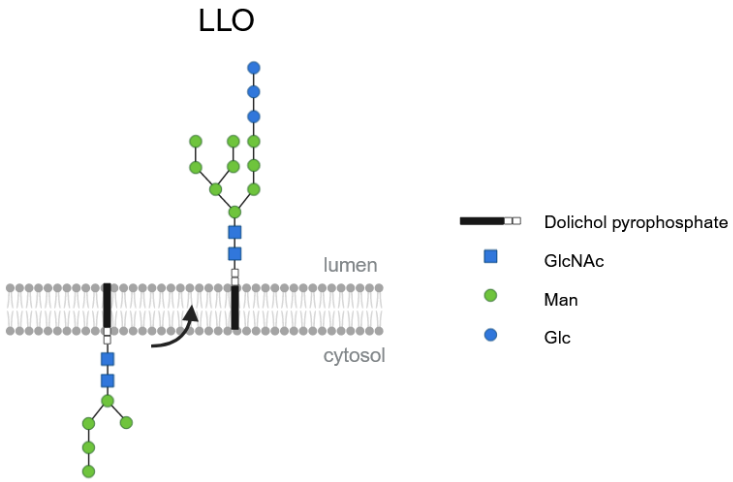


Figure 3 **Core structure of LLO and its assembly at the ER**
The assembly of the LLO starts at the cytosolic side of the ER by the formation of DolPP-$GlcNAc_2Man_5$. The precursor is then flipped into the ER lumen and is further elongated to the full LLO. The image was created using BioRender.com

Assembly of the LLO starts on the cytosolic side of the ER by the addition of GlcNAc-P to DolP by Alg7. The so-formed DolPP-GlcNAc gets elongated by the addition of another GlcNAc by the Alg13/14 complex [9]. Subsequently, five mannose residues are attached by Alg1, Alg2 and Alg11 [9]. All of these cytosolic glycosyl transferases are using the nucleotide sugars UDP-GlcNAc and GDP-Man as sugar donor substrates. The resulting di-antennary DolPP-$GlcNAc_2Man_5$ gets flipped into the ER lumen for further processing. After translocation by the involvement of Rft1 [10], the glycan chain is elongated by the addition of another four mannoses by Alg3, Alg9 and Alg12 [9]. In contrast to the cytosolic mannosyl transferases, membrane-anchored DolP-Man

serves as the mannose donor on the luminal side of the ER. Finally, three Glc residues are added sequentially by Alg6, Alg8 and Alg10 (using lipid-bound DolP-Glc as substrates), before the -$GlcNAc_2Man_9Glc_3$ oligosaccharide gets transferred *en bloc* onto the nascent peptide chain by the OST protein complex. The acceptor motif for the recognition of glycosylation sites by OST is Asn-X-Ser/Thr, where X can be any amino acid except for proline [11, 12]. However, not all of the predicted glycosylation sites are also found to be glycosylated. [13]. Thus, there are additional restrictions due to steric hindrance and accessibility of the Asn residue [7], making it difficult to actually predict site occupancy by glycans.

After the transfer of the oligosaccharide onto the protein, the three glucose residues are subsequently removed by α-glucosidases I and II [14]. DolPP-$GlcNAc_2Man_9Glc_1$ serves as quality control intermediate and is recognized by the ER chaperones Erp57, calnexin and calreticulin [14]. Finally, also the last remaining Glc and a Man residue are removed by the α-glucosidase II and the ER mannosidase and the protein gets transported to the Golgi, where further elongation and trimming of the glycan occurs, leading to a diversified N-glycan pool. Whereas the N-glycan core structure is highly conserved in eukaryotes, modifications occurring in the Golgi are species dependent [7].

The assembly of the LLO is a sequential process, also illustrated by the fact that misfunction of one of the Alg proteins leads to accumulation of the respective precursor LLO and is accompanied by hypoglycosylation of proteins [15]. Even though the preferred oligosaccharide that is transferred by the OST is the complete LLO, truncated glycan structures can still get attached to the protein. However, the reaction is much slower compared to the one of the complete oligosaccharide [16]. Thus, misfunction of the Alg glycosyltransferases does not only lead to reduced glycosylation but also to an altered glycan profile.

1.1.2 O-glycosylation

Another type of glycosylation is O-glycosylation, which is the attachment of sugars to the hydroxy group of an amino acid, usually a Ser or Thr residue. In contrast to N-glycosylation, a sequential attachment of monomers occurs directly to the protein. In addition, O-glycans do not share a common core structure and different sugars were found to be attached to the amino acid residues [4]. Further, there is no O-glycosylation sequence motif, although *in vivo* Ser/Thr rich domains are often favoured [17].

O-glycosylation takes place in the ER and Golgi, depending on the sugar monomer and the glycans can get elongated to form linear or more complex and branched oligosaccharide structures.

The attachment of mannose to proteins (O-Mannosylation) is the only conserved O-glycosylation. While in mammals several sugars were found attached to proteins by O-glycosidic linkage, mannose is the only O-glycan found in yeast so far [18]. O-mannosylation is an important modification, especially in yeast where mannose-rich mannoproteins heavily decorate the cell surface, forming a protective layer around the cell and regulating cell wall permeability [19]. O-Mannosylation is initiated in the ER lumen by protein O-mannosyl transferases (PMTs) [7]. As for the other mannosylation reactions in the ER, DolP-Man serves as the sugar donor for the first mannose residue. This mannose residue can then be further elongated in the Golgi by the use of nucleotide-activated sugars.

In yeast, at least 5 protein mannosyl transferases, which are grouped into Pmt1, Pmt2 and Pmt4 families, catalyze the linkage formation from a mannose to the hydroxyl group of an amino acid side chain [17]. Interestingly, PMTs themselves were found to be mannosylated [18]. Functional O-mannosyl transferases form either homodimers (Pmt4) or heterodimers (Pmt1/2) [20]. These PMT complexes show specific substrate specificity [21, 22] and Pmt4 complexes prefer to mannosylate membrane proteins [23].

In human, only two O-mannosyl transferases, namely POMT1 and POMT2, are known and they form a heteromeric complex [24]. Whereas O-mannosylation is a common modification in yeast, only a few mannosylated proteins were found in human. The best-studied O-mannosylation substrate is α-Dystroglycan (α-DG). α-DG is heavily O-glycosylated and the glycans can make up to 70% of the total protein mass [25]. The protein is not only O-glycosylated in its mucin domain, but also N-linked glycans are present. The major role of dystroglycan, consisting of α-DG and β-DG, is to connect the cytoskeleton with the extracellular matrix by its interaction with Laminin [25]. This function is lost when the protein is fully deglycosylated, thus showing that the sugar modifications are mediating the interaction. However, N-glycans do not seem to be necessary for this interaction [26]. O-mannosylation of α-DG is important for muscle and brain development and hypoglycosylation leads to dystroglycanopathies such as Walker-Warburg syndrome, Fukuyama congenital muscular dystrophy or

muscle-eye-brain disease [25, 27]. Besides α-DG, also cadherins and plexins were found to be O-mannosylated [28].

1.1.3 C-mannosylation

A special type of glycosylation is C-mannosylation, which was first identified in the human RNase II from urine [29, 30]. Protein C-mannosylation is the covalent attachment of a mannose to the indole ring of a tryptophane residue, by the formation of a C-C-bond. These mannoses were not found to be elongated and thus are rather small and embedded in the protein structure [31]. The addition of the polar sugar to the rather unpolar side chain of the tryptophan greatly changes local hydrophobicity and enables additional intra- and inter-protein interactions to aid in protein folding and stability. The mannoses can also stabilize so-called “Thr-Arg-ladders that are often found in thrombospondin repeats, as shown by MDS[32, 33] and NMR [34].

C-mannosylation takes place in the ER lumen and requires DolP-Man as a substrate [35]. The mannosyl transferase that catalyzes attachment of the mannose to the tryptophan is DPY19, a protein substrate for C-mannosylation that was first identified in *C. elegans* [36]. In human, 4 homologs of the protein (DPY19L1-4) were found. So far, mannosyl transfer activity was only shown for DPYL1 and DPYL3 and little is known about the function of the other two homologs. DPYL2 is only expressed in sperm and is important for spermiogenesis [37-39]. The DPY-proteins, specifically mannosylate tryptophan residues in a WxxWxxW motif (x being any amino acid except for proline). All tryptophans within the motif can get mannosylated, however not always all three tryptophans are modified. In human, there is a specificity of the mannosyl transferase for certain tryptophans of the motif. DPYL1 catalyzes the attachment of the mannose to the first two tryptophan residues in the motif, whereas DPYL3 can mannosylate the last tryptophane [39]. Even though a huge number of proteins are predicted to be mannosylated [40], this type of modification was only confirmed in a few proteins, most of them belonging to the thrombospondin type-I repeats and cytokine type-I receptor family [31].

1.1.4 GPI-anchor

The synthesis of GPIs, that serve as membrane anchor for some proteins, also takes place at the ER and requires the attachment of sugars to the PI lipid.

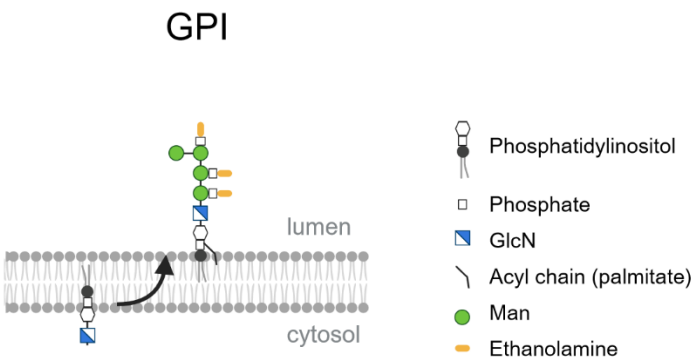


Figure 4 **Structure of the human GPI anchor**
The assembly of the GPI-anchor starts at the cytosolic side of the ER by the formation of Phosphatidylinositol-GlcN. The precursor gets flipped into the ER lumen and is further elongated and modifier to the GPI-anchor. In yeast, the fourth mannose is missing. The image was created using BioRender.com

Like the LLO, the stepwise synthesis of GPI-anchors on the cytosolic side of the ER is initiated by the addition of a GlcNAc residue to a lipid anchor, in this case a PI. In humans, this first step is catalyzed by the heteromeric protein complex GPI-N-acetylglucosaminyl transferase (GPI-GnT) consisting of the catalytic subunit PIGA as well as PIGC, PIGH, PIGQ, PIGP, PIGY and DPM2 [41]. Interestingly, DPM2, which is part of the DPMS complex that catalyzes the synthesis of DolP-Man (see also 1.3), was found to stimulate GPI-GnT activity [42]. After transfer, the GlcNAc sugar is deacetylated by PIGL, before the PI-GlcN is flipped to the luminal side of the ER [41]. In this case, too, the responsible flippase remains to be identified. In the ER lumen, the PI lipid is modified by acylation, usually with palmitic acid, before the diacyl PI get is converted mainly to a 1-alkyl-2-acyl PI and little diacyl PI [43, 44]. Subsequently, further mannose sugars are attached by mannosyl transferases using DolP-Man as substrates. In yeast, four mannoses are required whereas in human three mannoses are sufficient for successful GPI anchoring [45]. These mannoses are further modified by ethanolamine phosphate (EtNP) before the fully synthesized core GPI-anchor gets covalently attached to the C-terminus of the translated protein via the EtNP of the third mannose [41]. After transfer, the EtNP on the second mannose is cleaved off and also the acyl chain is removed before the GPI-anchored protein is transported to the Golgi for further addition of sugars and modification of the PI lipid. GPI-anchored proteins are finally transported to the plasma membrane, where they associate with cholesterol and sphingolipid-rich membrane domains [41].

1.1.5 Sugar donors for glycosylation

Many sugars, that are used for nucleotide sugar synthesis come from *de novo* synthesis after dietary uptake of precursors. The main sugar sources are glucose and fructose, that can be converted by the cell into other sugars to be used for glycosylation [46]. In addition, sugar monomers can be recovered by the glycan salvage pathway after degradation of glycans. [47]. Before sugars can be used for glycosylation, they have to be activated by conversion into energy-rich intermediates. Glycosyl transferases (GTs) are using two different types of activated sugar donors for the assembly of the glycans. GTs that are oriented with their catalytically active domain to the cytosolic side of the ER or to the luminal side of the Golgi use nucleotide activated sugar derivatives as substrates. In contrast, mannosyl- and glucosyl transferases which are facing with their active side the luminal side of the ER require lipid bound sugar substrates.

1.1.5.1 Nucleotide activated sugars

The majority of sugar monomers for glycosylation come directly from nucleotide bound sugars. Nucleotide sugars are soluble substrates for glycosyl transferases and are synthesized by a multistep process in the cytosol [47]. One exception is CMP-Sia, which is instead synthesized in the nucleus [47]. In general, nucleotide sugars consist of a nucleotide diphosphate (GDP or UDP) and the sugar residue. For synthesis, the sugar first needs to be phosphorylated at the anomeric C atom, before the sugar-1-P reacts with a nucleoside triphosphate (GTP or UTP), releasing a PPi [46]. In human, nine different nucleotide sugars are used to build up the diverse glycome [1, 48].

Most glycosylation reactions are localized to the ER and the Golgi. To use the nucleotide-activated sugar donors at the luminal side of organelles, they have to be imported. Nucleotide sugar transporters belong to the SLC35 family of solute carriers and facilitate translocation by an antiport of the respective nucleotide monophosphate [49, 50]. The transporters show high specificity towards the nucleotide sugar substrate and are mostly localized to the Golgi [51]. Nucleotide sugars are required for all glycosylation reactions and defects in formation or import have an impact on multiple glycosylation pathways and lead to an aberrant glycosylation profile.

1.1.5.2 Dolichol-linked sugars

In contrast to the Golgi, there are no ER-localized nucleotide sugar transporters for GDP-Man or UDP-Glc located in humans. These sugars are presented to the luminally oriented active sited of glycosyl transferases as lipid-bound sugars. The use of these lipid bound sugars as donors for N- and O-glycosylation is conserved in all domains of life and offers an additional level of regulation and spatial distribution of glycosylation reactions.

1.1.5.2.1 Structural diversity of polyprenols

The glycan lipid anchor is a long-chain isoprenoid phosphate. Although their function as glycan carrier is highly conserved, their structure is quite diverse. The lipid anchor is composed of several linearly connected isoprene units and can be saturated at the α-isoprene unit next to the phosphate group. Most eukaryotes use α-saturated polyprenols, so-called dolichol phosphates (DolPs), as glycan anchors, whereas fully unsaturated polyprenol phosphate (PolP) derivatives are used by bacteria and plants [52]. Prenol-chains are not present in a single length, but rather are found as pools of species with varying lengths. The average number of isoprene units forming the prenol-chain varies between species, with yeast having 15-16 isoprene units containing chains (75-80 C-atoms) as main species whereas 17 and 18 isoprene units (C85 and C90) per chain are predominant in human [53]. Very long polyprenols with up to C200 chains have also been detected in plants, whereas shorter C55 polyprenols are found in bacteria [52, 54].

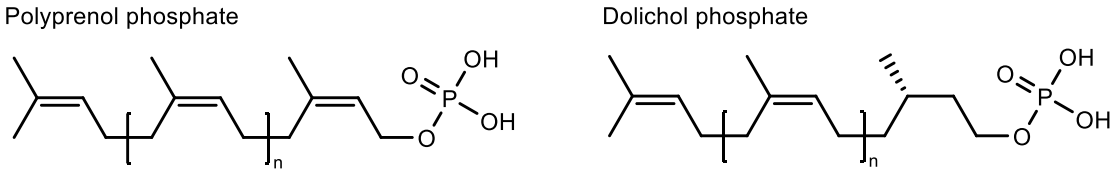


Figure 5 **Structural similarity of Polyprenol phosphate and Dolichol phosphate**
Dolichols only differ from Polyprenols by their α-saturation. Both chains consist of many isoprene units, usually with n=11-21. The structure was drawn using ChemDraw 20.0

The reason and implication of this structural diversity are not yet understood. The α-saturation of dolichols is an important feature, and in organisms that use DolP as glycan substrates, PolP is used only to small extent as substrate when added

exogenously [55]. The α-saturation may stabilize the sugar intermediate or it may change the lipid packing close to the phosphate group [56]. In contrast to the requirement of the α-saturation, mannosyltransferases such as Dpm1 or Alg1 are not dependent on defined lengths of the polyprenol chain, as they can even use very short dolichol derivatives such as citronellyl phosphate (CitP) *in vitro* [57, 58].

DolPs or PolPs are required for all glycosylation reactions. DolPs serve as the platform for LLO assembly in N-glycosylation as presented in 1.1.1. In addition, DolPs are used as lipid anchor for the supply of lipid bound mannose and glucose monosaccharides in the ER. The synthesis of DolP-Man from DolP and GDP-Man by DPMS is essential [9]. Also, reduced or loss of DolP-Glc synthesis, which is synthesized by the transfer of a Glc monomer from UDP-Glc by Alg5, leads to altered glycosylation patterns and can cause cause atypical polycystic kidney disease [59].

1.1.5.2.2 Synthesis of dolichols

Dol (dolichol) and Pol (polyprenol) derivatives are low abundant lipid species in the membranes and account for about 0.1% of total phospholipids in eukaryotes [52]. PolP and DolP *de novo* synthesis starts by the enzymatic condensation of dimethylallyl pyrophosphate (DMAPP) with isopentenyl pyrophosphate (IPP), both produced by the mevalonate pathway. The so-formed geranyl-pyrophosphate is further elongated by the addition of IPP to farnesyl-pyrophosphate (FPP) that is not only a precursor for Pol and Dol, but is also required for cholesterol/ergosterol synthesis, for ubiquinone formation and protein prenylation [60]. This interconnection of ergosterol and polyprenol synthesis was observed in yeast, where the disruption of the ergosterol synthesis pathway leads to an increase in polyprenols, due to the accumulation of the FPP substrate [61]. Further elongation of FPP by the head-to-tail condensation with additional IPP units, is catalyzed by cis-prenyl transferases. In human, only a single cis-prenyl transferase has been identified (hCPT) to date, although other proteins such as NgBR are also involved in the elongation [62]. In yeast, this prenyl elongation is catalyzed by at least two homologous proteins, Rer2 and Srt1 [63]. These prenyl transferases catalyze the elongation of the prenyl backbone until the polyprenol chain reaches its final length. The length is determined by the enzymes, indicated also by *in vivo* experiments, in which replacement of the yeast cis-prenyl transferase by the bacterial UPPS resulted in the corresponding shift in the chain length distribution towards the one of bacteria [60, 64, 65].

After elongation by the addition of IPP units (14-17 in human), the final prenol pyrophosphate (PolPP) gets dephosphrylated to Pol. Subsequently, in eukaryotes, Pols are desaturated at the α-position by prenyl reductase to Dol [66], before being phosphorylated again by the dolichol phosphate kinase to DolP [67]. The dephosphorylation-phosphorylation step is crucial for glycosylation in eukaryotes and represents a key step of regulation of glycosylation. Besides the *de novo* synthesis, released DolPP and DolP are recycled at the ER.

1.1.5.2.3 Flipping of DolP-sugar species

DolP-sugar synthesis is initiated at the cytosolic side of the ER membrane while its use is restricted to luminal-facing glycosyl transferase activity. Thus, the lipid-linked sugars have to be translocated to the other side of the membrane. Due the hydrophilicity of the phosphate and sugar residues, DolP-species cannot spontaneously flip through the hydrophobic ER-membrane but require the assistance of a flippase or scramblase. Extensive research was done to identify the responsible proteins, however the identity of these proteins remains unclear to this date [68].

Four different DolP derivatives have to be flipped through the ER-membrane for functional glycosylation in eukaryotes. For N-glycosylation, the cytosolically pre-assembled DolPP-$GlcNAc_2Man_5$ has to be translocated to the luminal side of the ER for further elongation and transfer onto the protein. It is the most complex DolP-sugar to be flipped, as it harbors seven hexose moieties. Yeast Rft1 was first suggested as a flippase, as depletion of the protein leads to an accumulation of DolPP-$GlcNAc_2Man_5$ [10]. However, subsequent biochemical assays showed that Rft1 is not the flippase itself [69-71], although it is required for functional N-glycosylation.

Likewise, both sugar donor substrates DolP-Man and DolP-Glc have to be translocated to the ER lumen. Translocation activity for both substrates was investigated in biochemical assays by monitoring the translocation of the water-soluble dolichol-sugar analogues CitP-Man and CitP-Glc into sealed rat liver or pig brain microsomes [72-74]. DolP-Man flippase activity was also reconstituted into liposomes from a Triton-X100 extract of rat liver ER membranes [75]. Lec35 (MPDU1) was found to be required for the utilization of DolP-Man and DolP-Glc *in vivo*, but were not required for flipping *in vivo* [76, 77]. Thus, the identity of the responsible protein remains unclear.

Finally, DolP, which is formed on the luminal side of the ER after glycosyl transfer, has must be flipped back to the cytosolic to be recycled. Even though *de-novo* biosynthesis

of DolP takes place on the cytosolic side of the ER, recycling of DolP released from DolP-Man and DolP-Glc as well as by hydrolysis of DolPP is important to prevent accumulation of DolP at the luminal side. However, also this flippase still remains to be identified [68].

For all these flipping events, there is evidence that they are protein dependent, bidirectional and energy independent. However, the nature of the responsible flippases is still unknown. To date, no CDG has been identified that was caused by a flippase, suggesting that flippase activities may be essential. Flipping could also be facilitated by an essential protein as a moonlighting activity. It is also still unclear, whether there is a distinct flippase for each substrate or if there are multiple isoforms or shared substrate specificities with other flippases, which would complicate the identification of the responsible flippase by genetic deletion [68].

1.2 Congenital Disorders of Glycosylation

The cellular glycosylation machinery requires around 200 enzymes for functional glycosylation [4]. Inborn defects in these enzymes, causing reduced enzymatic activities, lead to aberrant glycosylation patterns or hypoglycosylation and are classified as congenital disorders of glycosylation (CDG).

CDGs are a rare type of diseases. A complete knockout of glycosylation enzymes is usually prenatally lethal. Thus, most CDG-patients are heterozygous and show at least some residual enzyme activity of the affected enzyme. This heterogeneity of residual activity leads to a broad spectra of phenotypes, ranging from mild, almost normal phenotypes to severe multiorgan dysfunctions [48]. By now, over 130 different types of CDG have been discovered, with numbers constantly rising [78, 79]. Initially, CDGs were classified into two groups: CDG-I that included all enzymes involved in LLO synthesis up to the transfer of the LLO onto the protein and CDG-II that contained enzymes involved in elongation and trimming of N-glycans. With the increasing discoveries of other CDGs targeting also other types of glycosylation, and the involvement of multiple proteins in multiple pathways, the nomenclature used nowadays includes only the gene name of the protein affected, followed by -CDG [80]. CDGs are not only associated with glycosyl transferases but also with enzymes required for the synthesis of donor substrates, sugar transporters and even enzymes involved in vesicular trafficking or pH homeostasis [48].

PMM2-CDG is the most prevalent type of CDG with over 1000 patients and it was also the first CDG to be identified. [78, 81]. The hypoglycosylation in these patients is caused by defects in the phosphomannomutase II (PMM2), that catalyzes the conversion of Man-6-P to Man-1-P. Man-1-P is a direct precursor for GDP-Man synthesis [81] and as GDP-Man is required for all mannosylation reactions, these PMM2-defects impact all glycosylation pathways. Depending on the severity of the defect, PMM2-CDG patients show a huge variety of phenotypes. Symptoms often include psychomotor retardation, ataxia, strabismus, might involve liver misfunction and mild hepatopathy and many patients show dysmorphic features such as inverted nipples or lipodystrophy [78, 81].

1.2.1 Diagnostics

Initially, diagnostics mostly relied on the altered glycosylation pattern of serum transferrin, which can be detected by isoelectric focusing (IEF) [82]. Transferrin contains two N-glycosylation sites and proteins of healthy controls mainly contain two biantanary glycans that are each capped with negatively charged sialic acids. Altered patterns of CDG patients with defects in the LLO synthesis show a drastic reduction of tetrasialo transferrin and rather present disialo or asialo glycan chains. Trisialo and monosialo patterns indicate issues in glycan trimming and processing in the Golgi [78, 83]. Even though the IEF-method is still used in routine analysis, its use is limited to the identification of N-glycan CDGs. IEF of O-glycosylated serum apolipoprotein C-III can reveal O-glycan CDGs [78]. By using IEF the affected glycosylation pathway can be found, but identification of the responsible enzyme is not possible. To confirm CDGs and identify the responsible genetic defect, the gene candidate is sequenced. Improvement in whole-genome sequencing and untargeted analysis have led to the discovery of several new CDG types caused by enzymes not previously associated with glycosylation [48].

1.2.2 Treatments

To date, there is no universal treatment for CDGs. Research is difficult not only due to the huge diversity and phenotypes, but also because of the lack of suitable cellular models to test drugs and treatments [78]. Thus, most available CDG treatments are only addressing the symptoms.

In some cases, however, dietary intake of sugars or enzymatic co-factors has been found to improve or restore glycosylation. [78, 84, 85] For example, in many patients MPI-CDG can be treated by the oral intake of mannose [85, 86]. This CDG is caused by a defective mannose phosphate isomerase that converts Fru-6-P to Man-6-P. Hence, the enzyme is important for the endogenous formation of mannose formation from glucose. By dietary uptake of mannose this step is bypassed and aberrant mannosylation restored. Mannose treated MPI-CDG patients showed a restored IEF pattern, but the liver disease caused by MPI-CDG was not improved [85].

Treatment of PGMI-CDG and also SLC35A2-CDG patients with Gal was also beneficial in some cases [85]. Oral administration of fucose was shown to restore glycosylation for some SLC35C1-CDG patients [85] and was recently also demonstrated to be beneficial for GFUS-CDG patients [87]. In addition, the supplementation with co-factors can increase glycosylation efficiency. SLC39A8-CDG leads to a deficiency in available Mn^{2+} in the Golgi, which is required for galactosyl transferase (GalT). Additional uptake of this ion could improve galactosylation in some patients [85]. Reduced biotinidase activity in PMM2-CDG patients could also be counteracted by oral supplementation of biotin, leading to improved cognitive abilities [88].

Oral treatment with glycan-precursors and co-factors is usually well tolerated and can restore glycosylation in some cases. However, the success is limited to a few types of CDG and it is mainly restricted to enzymes upstream of LLO synthesis and protein glycosylation reaction. Usually the treatment is based on supplying the defective enzyme with an excess of precursor molecules or shifting the equilibrium to bypassing pathways. Thus, there is still a need for further development of treatments. The increased diagnostics of CDGs and research on the affected enzymes can help in gaining a better understanding on the glycosylation machinery and its interplay with other biological pathways. By studying the underlying mechanisms and effects of CDG-causing mutations, we can learn from naturally occurring genetic variances to better understand glycosylation [3].

1.3 DPMS and its central role in glycosylation

Dolichyl phosphate mannose synthase (DPMS) is an ER-resident mannosyl transferase which plays an essential role in the glycosylation machinery. DPMS catalyzes the transfer of mannose from GDP-Man to the DolP lipid to form DolP-Man,

that serves as a substrate for all downstream mannosylation reactions within the ER [6]. Thus, the enzyme is a central hub in the cellular glycosylation machinery.

1.3.1 Structure and function of DPMS

DPMS activity was first discovered [89] and cloned [90] in *S. cerevisiae*. But soon, other species followed. By now, DPMS genes of 39 different species have been identified and sequenced [91].

By sequence comparison of the catalytically active Dpm1 proteins from different species, the mannosyl transferase can be structurally grouped into three classes. The first class comprises enzymes where the catalytic domain harbors a C-terminal transmembrane domain (TMD), to anchor the protein to the ER membrane [6]. These tail-anchored proteins are fully functional without any additional protein and can substitute each other, as shown for *T. brucei* [92] and *U. maydis* [93] in yeast. Further members of this type are *L. mexicana* [94] and *E. histolytica* [95].

The second class of Dpm1 proteins lack the C-terminal TMD and are thus soluble proteins [6]. Examples of the second class include Dpm1 from human [96], *S. pombe* [96] or *T. reesei* [97]. These Dpm1 proteins depend on the interaction with additional membrane proteins, Dpm2 and Dpm3 in humans [98], for correct localization and function. Dpm2 and Dpm3 are small integral membrane proteins of about 10 kDa, each formed of two transmembrane helices. It has been shown in Dpm2 null mutant Lec15 cells, that Dpm3 directly associates with Dpm1, whereas Dpm2 is not required for Dpm1 anchoring to the membrane and ER localization [98]. Dpm2 was found to stabilize this complex and to increase Dpm1 activity, by interacting with Dpm3 [98]. While class-II proteins can supplement Dpm1 deficiencies of their own class, these enzymes are not able to compensate for the loss of type-I Dpm1, presumably due to the lack of the membrane-anchoring Dpm2 and Dpm3 proteins in these species [6, 96].

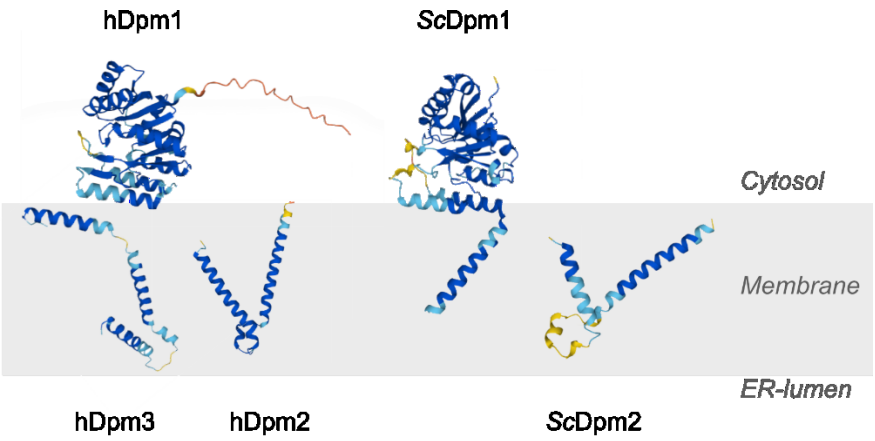


Figure 6 **Structural comparison of DPMS complexes from human and yeast**
The human DPMS complex consists of Dpm1, Dpm2 and Dpm3. In yeast, Dpm1 and Dpm2 (Yil102c-A) form an active complex. Protein orientations in the membrane, localization and relative sizes can differ *in vivo*. Protein structures were obtained from AlphaFoldDB (accession no: O60762 (hDpm1), O94777 (hDpm2), Q9P2X0 (hDpm3), P14020 (ScDpm1), Q2V2P5 (ScDpm2 = Yil102c-A) The image was created using Affinity designer software.

To date, the only available crystal structure of DPMS was obtained from *P. furiosus* [99], which forms another class of Dpm1. It structurally resembles the full DPMS complex observed in humans, by being comprised of the catalytic domain and four membrane-anchoring TMDs. Structures of *Pf*DPMS were solved with bound GDP and GDP-Man and DolP-Man in the active site [99]. Asp91 and Gln93 were found to coordinate a divalent metal ion (Mg^{2+}/Mn^{2+}) and the nucleotide phosphate in *Pf*DPMS [99]. The phosphate of DolP was coordinated by Ser135, Arg117 and Arg131 in the active center of the *Pf*DPMS [99]. The isoprene chain was found to be positioned between the two amphipathic helices of the catalytic domain, that interact with the first two isoprene units of DolP[99]. Further interactions were seen with one of the TMDs, however, the TMDs were not essential for *Pf*DPMS activity [99].

Besides the crystal structure of *Pf*DPMS, the only other structural information was obtained by a FRET study of yeast Dpm1 with fluorescent DolP analogues [100]. Substrate binding was modelled using the structural similarity to spore-coat protein SpsA, another GT-A protein, and FRET distances.

DPMS belongs structurally to the GT-A fold family, which have a conserved DxD motif that coordinates a divalent metal ion (Mg^{2+}/Mn^{2+}) and the nucleotide sugar [99, 101]. The metal ion preference was shown to be species dependent, with yeast Dpm1 preferring Mg^{2+} and the human Dpm1 preferring the presence of Mn^{2+} [91]. The mannosyl transfer is suggested to occur via an S_N2-like transfer mechanism, leading to inversion of anomeric center of the sugar from α to β [102]. Dpm1 was also found to be

phosphorylated by a cAMP dependent protein kinase at Ser141 in yeast [103] and Ser 165 in humans [104]. Phosphorylation was found to enhance Dpm1 activity *in vitro* by six-fold [103].

1.3.2 DPMS and CDG

Mutations in any of the three subunits of the DPMS complex can disrupt DPMS function and cause CDG. DPM-CDG, formerly classified as CDG-Ie, is a rare type of glycosylation defect. To date, only 24 cases of mutations in either of the subunits were reported.

12 of the reported cases show DPM-CDG mutations in the catalytically active Dpm1 [105-112]. All mutations led to a severely reduced synthesis of DolP-Man. Mutations in the membrane anchoring proteins Dpm2 and Dpm3 also led to hypoglycosylation, even though these proteins are not catalytically active themselves. Six cases of DPM2-CDG mutations [113-115] and further six cases of DPM3-CDG [116-121] have been reported. For Dpm2 it was observed, that mutations in the C-terminal domain caused much less severe symptoms compared to alterations in the N-terminal TMD [114, 115], indicating that the N-terminal region could be more important for Dpm1 activity. Also, a DPM2FY/LS mutation in rat, where Phe21 and Tyr23 were changed to Leu21 and Ser, showed reduced interaction with Dpm3 [98]. Similarly, the C-terminal TMD of Dpm3 was found to be important for interaction with Dpm1 [116].

The majority of DPM-CDG patients had a perinatal or early onset of symptoms. Patients are affected to varying degrees, likely depending on the location of the mutation and residual activity. Common clinical symptoms are developmental delay, intellectual disability, progressive microcephaly, seizures, hypotonia and many patients showed dysmorphic features [78, 114]. Most patients presented a CDG type-I pattern in IEF analysis of serum transferrin, showing the importance of DPMS for N-glycosylation [114]. The presence of muscular dystrophy as a regular symptom, shows the relevance of DolP-Man for O-glycosylation. The reduction in DolP-Man availability leads to severe hypoglycosylation of αDG and causes progressive muscular dysfunction. In addition, reduced N-glycosylation of βDG contributes to the symptoms of muscle dystrophy [119]. Although not routinely analyzed, the lipidome can also be affected by reduced DPMS activity. Lysophospholipids as well as hexosylceramides were found to be reduced in

a Dpm2-CDG patient [114], demonstrating a close connection between lipid homeostasis and glycosylation.

1.3.3 DPMS and other proteins

DPMS could directly or indirectly interact with all mannosyl transferases within the ER. As the DolP-Man product serves as the mannosyl donor for all of them, interaction with DPMS might contribute to the shuttling of DolP-Man in the different glycosylation pathways. However, no interactions of DPMS with other mannosyl transferases have been found by co-immunoprecipitation so far. Thus, it remains unclear whether DPMS plays a role in DolP-Man distribution. DPMS is facing the cytosolic side of the ER, whereas the downstream ER mannosyl transferases are oriented towards the ER lumen. Thus, interaction would most likely take place within the membrane. As DolP-Man has to be flipped to the lumen, the yet-to-be-identified flippase could be involved in the shuttling of DolP-Man rather than the DPMS itself.

To date, only few interaction partners of DPMS have been identified. Dpm2 was found to be involved in the first step of GPI-anchor synthesis in human, by associating with GPI-GnT [42]. This interaction was not required for GPI-synthesis as shown in Lec15 mutants, but the presence of Dpm2 greatly enhanced GPI-GnT activity. In addition, the newly identified yeast Dpm2 (Yil102-A) was found to interact with Spt14, a homolog of the human PIGA that is also part of the GPI-GnT complex [122]. Even though this first step of GPI-anchor synthesis neither involves DolP nor DolP-Man from DPMS, this interaction with Dpm2 could play a regulatory role in glycosylation as it is the first committed step of GPI-glycosylation. Both, DPMS and GPI-GnT activity are oriented towards the cytosolic side of the ER and both, the DolP-Man product as well as the deacylated PI-GlcNAc product are subsequently flipped into the ER lumen [41]. The following sugar that gets attached to the GPI-anchor is a mannose, transferred from DolP-Man after flipping [97]. Thus, the close proximity of DPMS and GPI-GnT could be beneficial for DolP-Man shuttling into the GPI-glycosylation pathway. By specific interaction of GPI-GnT subunits with Erg11, this first step of GPI-anchor synthesis was also found to be co-regulated with ergosterol biosynthesis in *Candida albicans* [123]. This is an interesting fact, considering that also the dolichol synthesis, required for DPMS function, is also linked to ergosterol via the mevalonate pathway (1.1.5.2. Increased

amounts of PolPs, the precursors of DolPs, were observed when the sterol pathway was blocked in yeast [64].

Alg7/GPT activity of the N-glycosylation pathway that catalyzes the formation of DolPP-GlcNAc, was shown to be stimulated by DolP-Man, although no direct interaction with DPMS was found, [124]. As with GPI-GnT, increased activity was found, although DolP-Man is not directly required for the reaction of the first committed step towards LLO synthesis. Vice versa, yeast Dpm1 was found to be activated by DolPP-GlcNAc *in vitro* [125], demonstrating the crosstalk between Dpm1 and N-glycosylation.

Sac1 was found to be a direct interaction partner of Dpm1 in humans [126]. Sac1 is a phosphatidylinositol-phosphate phosphatase that dephosphorylates PI(4)P in the ER and Golgi. The phosphatase cycles between both organelles in a growth dependent manner [127]. Under nutritional growth conditions, Sac1 is transported by COPI-dependent vesicular transport to the ER, where it associates with Dpm1. Upon starvation, it is re-located to the Golgi by COPII vesicles and secretion is slowed down. This Sac1-Dpm1 interaction is also connected to sterol homeostasis, as cholesterol is counter transported by the Osh-dependent PI(4)P translocation to the ER [128]. Dephosphorylation of PI(4)P by Sac1 is contributing to the equilibrium import of PI(4)P to the ER and cholesterol export from the ER. In addition, Sac1 was found to interact with ORM proteins in the ER [129]. ORM proteins are negative regulators of sphingolipid synthesis by inhibiting serine palmitoyl transferase activity [130]. Sac1 deletion in yeast increases most sphingolipids, but reduces inositol phosphosphingolipids [131]. Thus, interaction of Dpm1 with Sac1 shows another potential regulatory link between lipid synthesis and glycosylation.

1.4 The role and structure of membrane lipids

Lipids are essential and ubiquitous biomolecules in life. Besides their key role in forming membrane barriers, lipids are also important for energy storage and take part in signaling [132]. Further, the attachment of fatty acids to proteins is an important post translational modification, that can greatly impact cellular localization, secretion or protein-protein and protein-lipid interactions [133].

In general, lipids are hydrophobic or amphipathic molecules that are immiscible in polar solvents, which includes a huge variety of chemical compounds. Depending on their

chemical structure, lipids are classified into different categories. The most common membrane lipids can be attributed to one of the following groups: glycerophospholipids (GPLs), sphingolipids (SLs) and sterols [134].

Glycerophospholipids consist of two fatty acid chains, linked to a glycerol backbone at *sn1* and *sn2* positions, which harbors a phosphate group at *sn3*. This basic structure results in the simplest GPL class, namely phosphatidic acid (PA). GPLs are further diversified by the addition of functional groups to the phosphate, which gives rise to additional lipid classes. The most common headgroup modification is the addition of choline, resulting in phosphatidylcholine (PC), the major GPL in most eukaryotic membranes by making up for over 50% of total GPLs [132]. Other modifications are the addition of ethanolamine, inositol, or serine to the phosphate head, resulting in the lipid classes phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) respectively [134]. GPLs differ not only in their headgroup. The differences in the two acyl chains also contribute to lipid diversity. The FAs that are attached to the glycerol backbone differ in length and degree of saturation [134]. In yeast, mainly C16 and C18 FAs, either saturated or monounsaturated, are found [135]. In human, also longer and more unsaturated lipid species get incorporated into GPLs and SL [134]. In addition, the *sn1* and *sn2* position of FA attachment gives rise to constitutional isomers. Finally, FA acids can be linked also via an ester or ether bond to the backbone. All these differences ultimately lead to the huge number of lipid species for each class of GPLs.

Sphingolipids are another important class of lipids. In a first step, sphinganine (dihydrosphingosine) is formed by the condensation of serine and palmitoyl-CoA by the serine palmitoyl transferase complex (SPT) [136]. Sphinganine is a conserved precursor for complex sphingolipids in yeast and human [137]. Sphinganine can be further modified by the addition of an N-linked FA, resulting in dihydroceramide. In human, dihydroceramide is further desaturated, resulting in the formation of ceramide (Cer), which is a precursor for complex SLs [63]. Like for GLPs, the length and saturation of the acyl chain attached to the sphingosine backbone can vary, contributing to diverse SL. By the addition of a phosphocholine group from PC lipids to Cer, sphingomyelin (SM) is formed, which is the main class of SLs in mammalian cells [136]. Sphingolipids are often also found to be modified by the attachment of sugars, forming the class of glycosphingolipids (GSL). The first sugar that gets attached to Cer in

humans is either a Glc or Gal, resulting in GlcCer or GalCer respectively. As for protein glycosylation, GalCer synthesis takes place in the ER, whereas Glc gets attached to Cer after transfer to the ER [138]. Glycans can be further elongated in the Golgi, resulting in the formation of sugar-heavy and branched gangliosides [138].

Sterols are less diverse in structure. The main representatives of this non-polar lipid class are cholesterol (Chol) in vertebrates and ergosterol (Erg) in fungi [139]. Other sterols like stigmasterol and sitosterol are important species in plants [139]. Sterols are important structural membrane components and play an essential role in modulation of membrane dynamics [139]. An important feature of sterols in membranes is their ability to form liquid-ordered micro-domains, so called lipid rafts [139-141]. Sterols are not formed by the use of FAs, but from squalene, a polyprene which is synthesized by the condensation of two molecules of FPP from the mevalonate pathway. After completed cholesterol synthesis in the peroxisomes and the ER, most of the sterol gets transported to the plasma membrane. There is a gradual enrichment of cholesterol along the secretory pathway towards the plasma membrane (PM) [142]. Likewise, SLs are found to be enriched in the PM where they are found together with Chol or Erg in lipid raft domains [132, 142].

In eukaryotic cells, most lipids are synthesized in the ER and subsequently distributed to other cellular membranes [134]. The lipid transport occurs either via vesicles or protein mediated at membrane contact sites [143].

Advances in analytical methods such as mass spectrometry based lipidomic analysis revealed the huge diversity and dynamics of the lipidome. Though this helps in recognizing the enormous structural variances of lipids, the reason and mechanisms behind this diversity is less understood.

1.5 Membranes and lipids

The most abundant membrane lipids are GPLs [134]. In aqueous solution, these amphipathic molecules orient with their polar phosphate-containing headgroups towards the solvent, whereas the hydrophobic acyl chains are facing each other. This leads to the spontaneous self-assembly of lipid bilayers which are essential for all forms of life. These membranes form physical and chemical barriers, they hold cells together and shield the cellular machinery from the outside. Membranes of inner organelles are compartmentalizing the cell and separating chemical reactions which

also allows the presence of different chemical environments within the same cell. In addition, membranes offer a platform for chemical reactions. They “solvate” integral membrane proteins and contribute to protein activity and protein complex formation by spatial restriction and organization of chemical reactions. Thus, membranes compartmentalize, structurally organize cellular reactions and sort proteins.

1.5.1 The lipid composition of membranes

In vivo, membranes are composed of many different lipid classes and species. Membrane lipid compositions are heterogeneous and differ between species, cell types and organelles, thus giving a unique lipid profile [134]. In humans, changes in the lipidome were found related to diseases like cancer [144-146], diabetes type II [147] or Alzheimer´s disease [148]. The lipidome is also adapted during growth and the development or due to temperature changes in yeast and bacteria [149].

The lipid composition determines important membrane properties like fluidity or curvature (see 1.5.3) and the compositions of individual subcellular membranes are adapted to their function. For example, the ER membrane shows to be more loosely packed to facilitate protein insertion and transport, whereas the outer plasma membrane is more rigid and forms a stable barrier [134]. This is also reflected in the lipid profile of the respective organelle membranes, as shown in Figure 7.

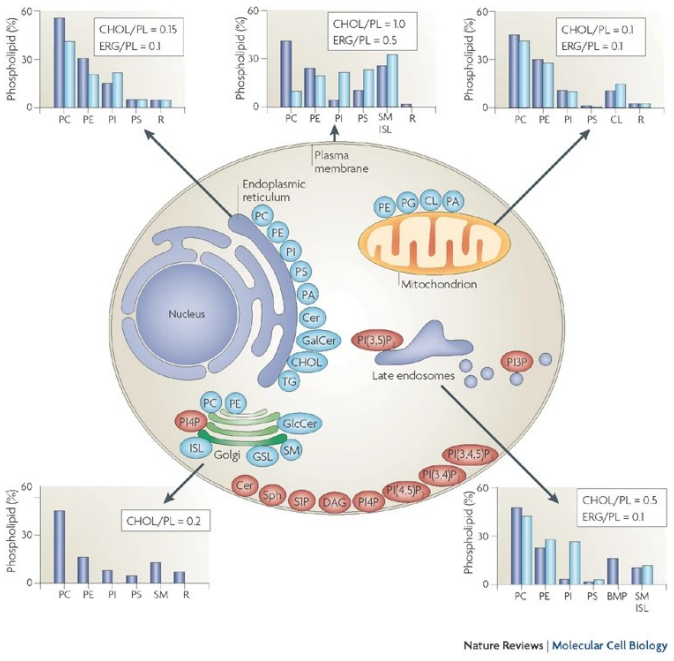


Figure 7 **Lipid composition of different organelles**
The lipid composition varies between different organelles. The illustration shows the lipid profile for mammals (blue) and yeast (light blue) in different organelles. Figure taken from van Meer et al. [132], license 5623740669231

The yeast ER membrane contains mainly PC and PE, making up together for about 58% of the total phospholipids (Figure 8). Another prominent phospholipid class in the ER is PI. PS, PA and CL are only found as minor constitutes of the membrane. The yeast ER also contains other lipids like Ergosterol, which makes up 10-20 % ergosterol of total phospholipids in yeast[132, 150].

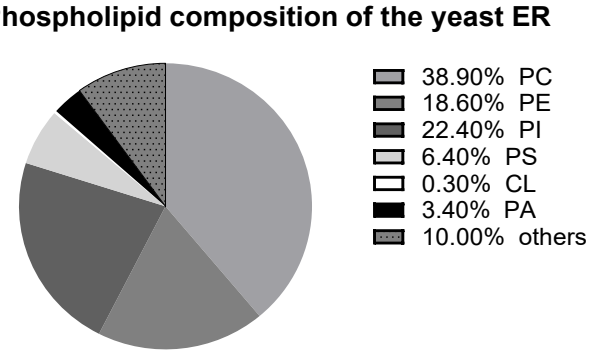


Figure 8 **Phospholipid composition of the yeast ER**
Main GPL species in yeast PC, PE, PI, PS, CL and PA .Data taken as summarized by Klug and Daum [135]

1.5.2 Membrane leaflets and asymmetric bilayer distribution

Phospholipid bilayers are structurally formed by two monolayers, referred to as membrane leaflets. In the lateral dimension, the monolayer structure is not rigid and lipid molecules, as well as membrane proteins, can rapidly diffuse within the leaflet. Due to the polar headgroup, GPLs and GSLs tend to stay within the same leaflet and spontaneous diffusion (translocation) to the other leaflet occurs infrequently [151]. However, lipids are found to be readily translocated between the leaflets by energy independent bidirectional scramblases or by ATP-dependent lipid transporters [152]. This gives rise to asymmetric lipid distribution in bilayers. *In vivo*, it was found that the lipid composition of the leaflets can differ as seen by the increased prevalence of PS and PE in the cytosolic and SM in the extracellular leaflet of the plasma membrane [132, 152].

1.5.3 The lipid composition determines membrane properties

The physicochemical properties of membranes are greatly determined by the lipid composition. Thus, the lipid composition of membranes is constantly sensed and modified by the cell to maintain lipid homeostasis and membrane properties.

Fluidity and lipid packing

The speed of lateral diffusion of lipids and proteins in the membrane depends on the fluidity of the membrane. The fluidity of membranes increases with the elevation of temperature, leading to an energetic increase and faster molecular movement [153]. Temperature induced changes are also sensed by the cell and result in lipid remodeling and homeoviscous adaptation, as for example seen when comparing the lipid profile of yeast that was grown at different temperatures [154]. Another important factor, contributing to the membrane fluidity, are the chemical properties of the lipid constituents. Mono- or polyunsaturated lipid species lead to loosely packed and fluid membranes due to the steric hindrance of the acyl chains. Increased saturation of lipid acyl chains results in tightly packed and liquid-ordered membranes [134]. Also, cholesterol was found to stabilize membranes by inducing tighter packing of SL and interaction with saturated lipid species [155]. Tighter lipid packing reduces lateral diffusion and permeability of the membrane and decreases membrane flexibility for the integration of proteins.

Differences in lipid packing are not only observed between different membranes. Also, accumulation of Chl and SLs in leads to tightly packed membrane domains. These lipid raft domains are more stable to mechanical disruption and detergents, thus also referred to as detergent resistant membranes (DRM) [141]. Lipid rafts are mostly found in the plasma membrane, where they lead to sub-compartmentalization of the membrane and can effect local protein distribution and facilitate protein complex formation [141]. Specific proteins, e.g. GPI-anchored or acylated proteins are preferentially found in raft domains at the PM [141].

Adaptation of the lipid composition and maintaining membrane fluidity plays an important role in cellular stress resistance to e.g. temperature changes or EtOH [142]. An increase in membrane fluidity can be achieved by decreasing lipid saturation, as seen for example upon cold-stress in Arabidopsis [156] or yeast [154], or by inducing negative curvature stress through an increase in PE and by reduction in sterol content, as shown in yeast and humans [154, 157].

Curvature

The shape of GPLs also effects the spontaneous curvature of the membrane [158]. PC and PS are cylindrically shaped lipids, forming flat membranes. Conically shaped lipids, with a relatively small head group compared to the acyl chains such as PE or

PA, induce negative curvature and lipid defects in membranes [134]. In contrast, PIs are lipids with a large headgroup which lead to positive membrane curvature [134]. Heterogeneous lipid distribution of curvature inducing lipids in the membrane leads to differences in local membrane shape. Likewise, local membrane curvature can also be induced by the shape of integral membrane proteins [158]. The membrane curvature shapes the organelles and the ability to locally modulate membrane curvatures is essential for membrane fusion and fission and vesicular transport [158].

1.5.4 How lipids affect integral membrane proteins

Membrane lipids can affect the integral membrane proteins in different ways, depending on the strength of the protein-lipid interaction.

The bulk of lipids are interacting randomly and unspecifically with membrane protein sand are affecting the protein by determining the overall membrane properties like fluidity, curvature or membrane thickness. A change in membrane thickness e.g. by altered acyl chain length can cause a hydrophobic mismatch between the TMD and the membrane, resulting in conformational changes like bending or stretching of the protein [134]. It can also lead to lateral diffusion of the protein into membrane regions with better matching membrane properties [134]. The fluidity of the membrane affects the protein-protein interactions complex formation by determining lateral diffusion rate. Autophosphorylation of human epidermal growth factor receptor (EGFR) was found to be inhibited in the presence of phase separating lipid compositions in the presence of the ganglioside GM3 *in vitro,* that was caused by reduced dimerization [159]. The dimerization of p24, which is part of the COPI machinery, was also suggested to depend on the presence of a specific sphingolipid (SM18) [160], and the presence of tightly packed membrane domains were found to stabilize dimerization of p24 *in vitro* [161]. Thus, changes in lipid packing e.g. in lipid rafts can be sensed by proteins and affects protein localization and protein-protein interactions[134]. Sensing of membrane properties can be used by proteins to discriminate membranes from different organelles and can regulate protein distribution and enzyme activity [134].

Annular lipids show specific lipid-protein interaction with higher protein affinity compared to the bulk lipids [162]. These lipids are found in rather loose contact to the protein and accumulate around the TMDs due to their protein interaction [162]. The presence of annular lipids could change the immediate lipid environment of the protein

and thus effect protein activity and conformation. Interactions of lipids with proteins can occur via the hydrophobic chain or the headgroup of the lipid. Protein-annular lipid interactions are dependent on the residues and charges exposed on the TMD of the protein [163]. A layer of ordered cholesterol and phospholipid molecules was found to be bound to the TMDs of the human ABCB1 transporter, specifically in the substrate bound state when the TMDs were kinked, thus presumably mediating protein conformation [164].

In addition, proteins can be regulated by non-annular lipids that act like co-factors [162, 163]. These lipids show specific and tight interaction with the membrane protein and are characterized by high residence time at the protein-lipid interphase [162]. They are often found between TMDs or at the interface of two membrane proteins [163] and are most times co-purified with the integral membrane proteins because of their strong interaction with the protein. For example, PI and PE were co-purified with the Eukaryotic Purine Symporter UapA and were found to promote dimerization [165]. Other examples for non-annular lipids are cardiolipins and PI species in the Cytochrome bc_1 complex that are required for protein activity [162, 166, 167].

In conclusion, lipids can influence protein localization, conformation and activity either directly by specific protein-lipid interactions or by the membrane properties.

1.6 Reconstitution systems to study membrane proteins

Cellular membranes do not only consist of lipids, but also accommodate a large amount of different membrane proteins. *In vivo* studies of protein-membrane interactions are challenging, due to the diversity of lipids and membrane components. In addition, modulations of membrane lipid compositions do not only affect the target protein, but probably also interfere with other cellular pathways. Thus, more detailed information on protein regulation and activity by the surrounding lipids can be gained by studying isolated membrane proteins *in vitro* in a less complex lipid environment.

Membrane proteins can be directly embedded into the membrane or anchored to the membrane e.g. by the attachment to lipid anchors or by interaction with other membrane resident proteins. Integral membrane proteins (IMPs) are not soluble in aqueous solutions, due to the hydrophobic nature of their transmembrane regions. Thus, their isolation requires the use of detergents or other molecules such as SMAPLs (styrene maleic-acid lipid particles). Like phospholipids, detergents are amphiphilic

molecules that have a polar group and a hydrophobic part. In aqueous solutions, detergents form micelles with the polar group facing outwards, shielding the hydrophobic residues. Detergents can also shield the hydrophobic part of IMPs, thus rendering the proteins soluble. Detergents are commonly classified into ionic-, non-ionic- and zwitterionic detergents, depending on their chemical structure or by their strength in extracting membrane proteins [168]. During the solubilization process, integral membrane proteins get stripped off their native lipid environment and detergent micelles often do not offer a sufficient membrane environment to maintain protein function. Thus, solubilized proteins need to be re-inserted into a lipid bilayer to study their activity.

Artificial membranes can be prepared from purified natural or chemically modified lipids. Reconstitution systems also allow the study in a chemically well-defined lipid environment and can be useful to stabilize the protein for structural studies, but also to study the effect of specific lipids on the membrane protein. However, they do not resemble the native lipid environment in its complexity of lipids and proteins and represent a simpler membrane system. The most common reconstitution systems are liposomes and nanodiscs, two membrane mimicking systems that will be discussed in detail the following.

1.6.1 Liposomes

Liposomes are spherical lipid bilayers. They can serve as lipid platforms and supply a lipid environment for membrane proteins, enabling the study of integral membrane proteins. The potential to encapsulate chemical compounds and therapeutics into the vesicles, led to the development of liposome-based drug delivery systems. A prominent, recent application of liposomes is their use for vaccine delivery, as done by Pfizer/BioNTech and Moderna with the mRNA-based vaccinations against SARS-CoV-2.

Liposomes are often distinguished by size. Small unilamellar vesicles (SUVs) are smaller than 100 nm, large unilamellar vesicles (LUVs) range from 100-1000 nm and liposomes above 1000 nm diameter are called giant unilamellar vesicle (GUVs) [169].

1.6.1.1 Liposome preparation

Liposome preparations usually start off with a dried lipid film of the chosen membrane lipids. Upon hydration of the lipid film with aqueous buffer and vortexing, lipid vesicles are formed spontaneously. However, simple hydration usually leads to large multi-layered vesicles (MLVs) with broad distribution in size [170]. To produce uniform SUV and LUV preparations, these MLVs are further processed and reduced in size.

SUVs are commonly prepared by extensive sonication of MLVs. Sonication leads to rupture of the large MLVs finally resulting in SUVs. Due to the small size and high membrane curvature, however, they are not ideal for accommodation of membrane proteins. Due to the surface tension, SUVs are also prone to aggregation below the phase transition temperature of the membrane lipid [171].

LUVs are most commonly used for protein reconstitution. They can be formed by different methods such as sonication, freeze-thawing, extrusion, or ethanol injection. Sonication can also be used to produce LUVs, when less extensively applied compared to SUV preparations. However, the resulting liposomes often remain inhomogeneous due to the poor control of energy and starting size. In addition, high energy of sonication leads to local heating and can cause lipid oxidation [172]. Repeated freeze-thawing also leads to rupture of large MLVs, however efficiency greatly depends on the lipid composition [173]. Another commonly used method is extrusion. Here, the MLV suspension gets continuously passed through a filter membrane with 100-400 nm pore size. MLVs rupture due to sheering forces and the resulting liposomes are usually unilamellar and uniform in size. Efficiency and liposomal size depend of the pore size, number of passages as well as lipid and buffer composition [170, 174]. Extrusion is often combined with either freeze-thawing or sonication to reduce initial particle size. Other methods, like ethanol injection or other reverse phase evaporation techniques do not start off with a dried lipid film, but instead with a lipid solution in organic solvents such as ethanol. Liposomes are formed upon injection of the organic lipid solution into aqueous solution [175]. To obtain aqueous liposome solutions, the organic solvent has to be removed by heating or using a rotary evaporator, however traces might remain in the liposome solution. Alternatively, detergent solutions can be used instead of organic solvents. Nonetheless, they also have to be removed and the liposome size depends a lot on speed or detergent removal.

GUVs with a size over 1 µm are large enough to be studied by microscopy. These giant liposomes are most commonly prepared by the electroformation technique [170]. Here, a thin lipid film is dried on electrodes and subsequently hydrated in the presence of an alternating electric current. The procedure consists of a growing, swelling and rebounding step, that are modulated by the amplitude and voltage [170]. Other methods of GUV preparation are by gentle hydration in the absence of current, by the use of microfluidics or by droplet emulsification [170].

1.6.1.2 Proteoliposome formation

To study membrane proteins in liposomes, they have to be inserted into the membrane. Proteoliposomes can be obtained either by preparation of liposomes in the presence of the detergent-solubilized protein or by reconstitution of proteins into pre-formed liposomes. The latter strategy is often superior, as sonication, extensive freeze-thawing or the presence of organic solvents during liposome preparation are not beneficial to maintain proteins in their native state [172]. Thus, most proteoliposome protocols use a step-wise preparation-reconstitution method. Liposomes are first formed by one of the methods presented above and thereafter, the purified membrane protein is reconstituted. As discussed, the purification of membrane proteins requires the use of detergents to extract the protein from the native lipid environment and to stabilize the protein in solution. Hence, also reconstitution usually takes place in the presence of detergent.

A simple way to insert proteins into liposomal membranes is by mixing the liposomes with the detergent solubilized IMP. Ideally, the resulting decrease in detergent concentration leads to less stabilized micelles and the membrane proteins spontaneously insert into the liposomal membrane to prevent exposure of the hydrophobic parts to the aqueous buffer. Even though in some cases this method led to sufficient reconstitution, it was found to be restricted to SUVs and certain lipid compositions. In addition, this type of reconstitution led to inhomogeneous preparations with a wide range of size [176].

Better results can be achieved when using a detergent mediated approach by pre-incubating the liposomes with detergent to also maintain a detergent concentration that stabilizes the protein. The addition of the detergent to liposomes has to be carefully controlled and optimized, as detergent monomers get integrated into liposomes and thereby destabilize the lipid membrane which ultimately leads to complete

dissolvement of the pre-formed liposomes at high detergent concentrations [176]. Thus, this method requires optimization to maintain liposomal integrity as well as protein stabilization by the detergent. Subsequently, the detergent has to be removed from the liposome-protein-detergent mixture. Reduction of the detergent concentration under its critical micelle concentration (CMC) results in a breakdown of detergent micelles, that drives the transition of the IMP from the micelles into liposomes. When using detergents with high CMC, simple dilution can be used to insert proteins into the lipid vesicles [172]. However, detergent remains present and the dilution results in an increased sample volume. Usually, complete detergent removal is required to study reconstituted membrane proteins, as residual detergent might inhibit protein activity or destabilize the liposomal membrane. Another method to reduce detergent concentration is by gel-filtration. This technique relies on the different elution of detergent monomers and mixed micelles from large proteoliposomes. However, this technique is less frequently used as it also leads to sample dilution and inhomogeneous proteoliposome preparations [172].

A common way for detergent removal to obtain detergent free liposome preparations is dialysis. This gentle method uses the diffusion of detergent monomers through the membrane of the dialysis bag into an excess of detergent free buffer, while proteoliposomes are retained. This method is mostly suitable for detergents with high CMC, which are get much faster removed compared to detergents with low CMC and the resulting liposomes are usually uniform in size [172]. A major drawback is the time requirement of several days, that is often deleterious to protein activity.

A faster alternative, that is also suitable for detergents with low CMC, is the use of detergent adsorption by polystyrene beads. These hydrophobic polystyrene particles preferentially bind to detergent molecules, pulling them slowly out of solution and thus leading to reduction of detergent concentration. After full removal, beads can be removed by gravity sedimentation, short centrifugation, or filtration. The speed of removal can be controlled by the number of beads used.

Proteoliposomes offer the possibility to study membranes in simplified lipid environments. They are large enough to accommodate several proteins and also allow the co-reconstitution of different proteins to study protein-protein interactions. The sealed lipid membrane offers the possibility of compartmentalization and membrane potential and can be used to study translocation reactions. Proteoliposomes can also be used to study protein mediated membrane fusion [171]. The stability of liposomes

depends on their size and the lipid composition as well as the buffer composition. Due to the membrane curvature and the small luminal volume, proteins are usually oriented biased, with a preference of orienting the lager part towards the outside [177] The protein orientation after reconstitution is difficult to predict and depends on factors like the size of the protein, membrane curvature and method of reconstitution.

1.6.2 Bicelles

Bicelles are disc shaped membranes. They form upon mixing of a bilayer forming, long chain phospholipid (e.g. dimyristoylphosphatidylcholine, DMPC) and a short chain phospholipid (e.g. dihexanoylphophatidylcholine, DHPC) or detergent (e.g. CHAPS), that is found at the rim of the membrane [178]. Bicelles are often used for structural studies of membrane proteins by NMR, as they are very small and align spontaneously in the electromagnetic field [179].

1.6.3 Nanodiscs

Structurally, nanodiscs consist of a discoidal phospholipid bilayer, that is surrounded by membrane scaffolding proteins (MSP) which are wrapped belt-like around the lipid disc. MSPs are helical proteins, typically derived from human serum apolipoprotein A1, that self assembles into disc-like structures in the presences of phospholipids [170]. Recently, also polymer based and peptide-based scaffolds were developed [180].

Nanodiscs are typically 8-16 nm in diameter, and thus are much smaller than most liposomes. [170]. The lipid membrane consists only of about 150 phospholipid molecules compared to approx. 230.000 lipids in a 140 nm LUV [181, 182]. An even smaller lipid environment is provided by so called peptidiscs, that only contain of co-purified, annular lipids of the protein [183]. The size of nanodiscs can be modulated by modifications of the MPS [184].

The membrane of nanodiscs is planar and has nor curvature compared to the spherical liposomes. Hence, also reconstitution and protein function are not affected by curvature. However, the MSP also constrains the lipid bilayer and thus limits lateral diffusion [185]. In contrast to proteoliposomes, both sides of the protein are accessible in nanodiscs and thus protein orientation after reconstitution is irrelevant. Hence, reconstitution leads to more uniform particles compared to liposomes which is beneficial for structural and spectroscopic studies [180].

The reconstitution of membrane proteins into nanodiscs requires the optimization of the protein-phospholipid-MSP ratio. The ratio of phospholipid-MSP can determine the yield of nanodisc formation, whereas the nature of the MSP and its ratio to the protein affects the size and number of reconstituted protein per nanosdisc [184]. The most common way to reconstitute membrane proteins into nanodiscs is by mixing purified, detergent solubilized proteins with phospholipids and MSP. Alternatively, phospholipids and MSPs can be added already during detergent solubilization to stabilize the protein in detergent solution [184]. Upon detergent removal by the use of Bio-Beads or dialysis, nanodiscs harboring the membrane protein are formed.

1.6.4 SMALPs

SMALPs (styrene maleic acid lipid particles) are a further development of nanodiscs, using a styrene-based polymer as scaffold [186]. Similar to MSPs, SMAs (polystyrene maleic acid) polymers wrap around the hydrophobic part of the protein, rendering it soluble. The benefit of SMAs and other SMA-based polymers is their capability to directly extract the membrane proteins from native membranes without the need of detergents [187]. Extraction occurs together with the immediate lipid environment, which helps in stabilization of the protein and protein complexes [187] . SMALPs are useful to study structure and function of membrane proteins. However, they often form heterogeneous particle populations and can be sensitive to low pH and divalent cations [180, 188, 189].

1.7 Objectives

Most known glycosylation enzymes are integral membrane proteins. Membranes can serve as platforms for reactions, but can also modulate localizations and activities of membrane-embedded proteins by direct or indirect interaction (1.5). To date, little is known about the importance of lipids for glycosylation reactions.

The aim of this project was to study the role of lipids in regulating enzymatic glycosylation processed. As a model protein, I chose yeast Dpm1, which catalyzes the synthesis of the mannosyl donor DolP-Man. Dpm1 is an essential protein in eukaryotes and malfunction of Dpm1 leads to severe glycosylation defects in all glycosylation routes (1.3). As an integral membrane protein and by providing the essential the sugar donor DolP-Man for all mannosylation reactions within the ER, Dpm1 was a promising

target to investigate a link between glycosylation and membrane lipids. To study Dpm1 activity in different membrane environments, I chose a liposomal *in vitro* reconstitution system, as it provides a simple and chemically well-defined lipid environment for membrane proteins. With this, the effect of membrane alterations on the enzyme activity of Dpm1 can be directly studied by modifications of the liposomal lipid composition.

In vivo, cellular membranes are composed of many different membrane proteins, that can form molecular complexes or influence each other by direct interactions or changes in the membrane environment. Thus, the idea was to enlarge the proteoliposomal model of Dpm1 by co-reconstituting Dpm1 with other proteins. The stepwise increase of complexity of liposomal protein constituents could be used not only to study the effect of lipids on additional proteins but also to investigate their role in mediating interactions between different proteins. Two proteins were chosen for co-reconstitution experiments. The yeast Dpm2 homolog (Yil102c-A), recently identified and not previously studied *in vitro*, was an interesting candidate to study a role of Dpm2 in modulating Dpm1 activity in different lipid environments. Another target protein was the O-mannosyl transferases Pmt4, as reconstitution of the O-mannosylation pathway by including Pmt4 is a first step to study channeling of DolP-Man into different glycosylation pathways *in vitro*.

As previous studies only used Dpm1-enriched detergent extracts, a major aim was to establish a method to purify Dpm1 in high purity and sufficient yields. This could then be used for protein reconstitution into liposomes. After optimization of the protein reconstitution, a fast and robust *in vitro* activity assay should be established to study the effect of lipids on Dpm1 activity. Extending the liposomal assay by the addition of other glycosylation proteins could then be used to study the effect of lipids on protein-protein interactions as well as to investigate their role on Dpm1 activity.

Further, we aimed to develop a mass spectrometry-based analysis method for dolichol phosphate and dolichol phosphate mannose.

2 Results

Within the work of this thesis, I studied the effect of the membrane environment on the membrane resident yeast protein Dpm1. Therefore, I developed and optimized a purification method, to obtain enzymatically active protein in high quality and yield. The protein was reconstituted into liposomes to study Dpm1 activity in a defined lipid environment. The reconstitution method was optimized, and a fast and simple activity assay readout, based on previous studies, was established. Method development and optimization are described in Part I. Using the optimized assay, I investigated the activity of Dpm1 in different lipid environments and its interaction with other proteins involved in the glycosylation process, namely Dpm2, and Pmt4. These results will be presented in Part II of the results section of this thesis.

Part I Method development

2.1 Purification of enzymatically active Dpm1

The first aim was to purify yeast Dpm1 in an active form with high quantity and purity. Previous activity studies of Dpm1 were mostly done by using detergent-solubilized membrane protein fractions of various organisms. Early studies on rat DPMS were performed by Jensen et. al by the use of rat DPMS, and the protein was extracted and enriched in 1% NP-40 from rat liver microsomes [190]. For studies of native yeast Dpm1, the enzyme was solubilized and enriched in a multistep enrichment procedure in the presence of 0.5% Triton and 0.18% SDS [191, 192]. Recombinantly expressed yeast Dpm1 was analyzed using NP-40 as detergent [193]. However, no suitable method had been published for the preparation of highly purified Dpm1 protein.

Therefore, we decided to clone yeast Dpm1 as a His tagged construct to highly purify the protein via Ni-affinity purification and to use the purified protein to test Dpm1 activity in the absence of other proteins. A schematic overview of the purification procedure can be seen in Figure 9.

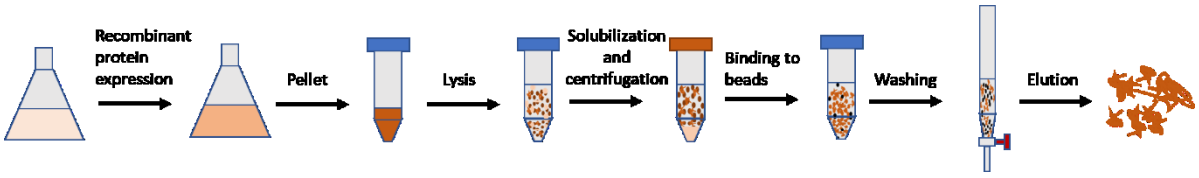


Figure 9 **Schematic overview of Dpm1 purification**
His-tagged Dpm1 was recombinantly expressed in *E. coli.* After harvesting, cells were lysed and solubilized in 0.75% Sarkosyl. Solubilized Dpm1 was purified using Ni-affinity chromatography. After elution with imidazole, the protein was aliquoted and stored at -80°C until further use.

2.1.1 Detergent screen for Dpm1 solubilization

Since Dpm1 is an integral membrane protein, I was able to detect the recombinantly expressed protein only in the membrane fraction of the lysate of the over-expressing bacteria. Therefore, detergent had to be added to solubilize the protein. It was important to choose a detergent that would sufficiently solubilizes the protein while not completely denaturing the enzyme in order to obtain enzymatically active protein. My first purifications of recombinantly expressed Dpm1 with 0.5% Triton, as previously used to solubilize yeast Dpm1 from native membranes [192] , resulted in a low yield of purified protein (data not shown). Also, NP-40 showed low solubilization efficiency (compare Figure 10).

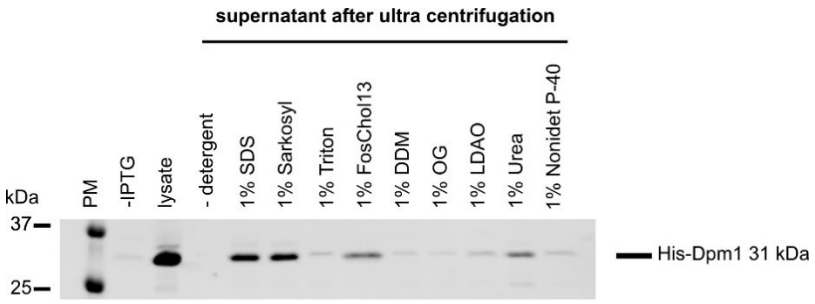


Figure 10 **Detergent screen for Dpm1 solubilization**
E.coli lysate, containing overexpressed Dpm1, was incubated with 1% of the respective detergent for 15 min at RT. Solubilized protein was separated from insoluble material by high speed centrifugation at 153.700 xg for 45 min and the amount of Dpm1 in the supernatant was compared by western blot. The blot was developed using an anti-His antibody.

Since a sufficient amount of purified protein is needed for reconstitution, I wanted to further optimize the solubilisation efficiency. Hence, I performed a detergent screen in order to find the best detergent to solubilize the recombinantly expressed protein. After incubation of the lysate with 1% of the respective detergent, samples were centrifuged

with high speed and the amount of solubilized Dpm1 in the respective supernatants was compared by western blot.

As shown in Figure 10, most mild detergents, such as Triton, OG, LDAO, DDM, and NP-40, only poorly solubilized Dpm1 from the lysate. Urea partially dissolved Dpm1, but was not a suitable detergent for enzyme activity measurements because of its denaturing nature. Similarly, SDS was found suitable for solubilization but rendered the protein inactive. I achieved the best recovery when using Sarkosyl. Even though it is an ionic detergent, it is rather mild: The protein was not active in the presence of Sarkosyl, but activity was restored when reconstituting the protein into liposomes and removing the detergent by the use of Bio-Beads.

Sarkosyl (N-Lauroylsarcosine) is an ionic detergent, derived from Sarcosine (Figure 11).

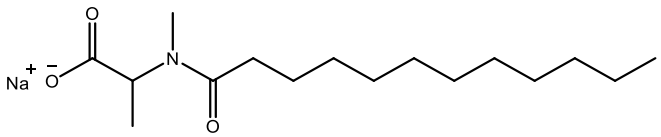


Figure 11 **Chemical structure of Sarkosyl**
The structure was created using ChemDraw 20.0

The solubility of Sarkosyl in water is reported to be high with 293 g/l and its CMC was determined to be around 14 mM in water at RT (Sigma Aldrich, product information of product no. 61745). Sarkosyl is commonly used for the purification of proteins. [194-196]. Sarkosyl was found to selectively solubilize inner membranes of Gram-negative bacteria [197, 198] and can also be used to enrich plasma membrane fractions in the Sarkosyl insoluble fraction [199]. In addition, it can be used for solubilization of inclusion bodies [200], also by the help of other detergents like CHAPS and Triton X-100 [195, 201].

In the presence of Mg^{2+}, Sarkosyl forms highly insoluble Mg-Sarkosyl crystals. I found crystal formation to be enhanced in diluted detergent solutions with higher Mg-Sarkosyl ratio. Historically, these crystals were used to isolate membrane bound DNA and RNA, by the so-called M-band technique [202, 203]. The crystals strongly bind membrane components and form a specific band after density centrifugation. Even though these Sarkosyl-Mg crystals can be of great use for the isolation of membrane bound molecules like DNA, these crystals are not compatible with protein purification. The membrane protein gets bound to the crystals and precipitates with them. I could not find a condition to dissolve the crystals again and release the bound components.

Thus, Mg^{2+} in buffers and solutions should be avoided during protein purification with Sarkosyl.

In the initial screen, I found that Dpm1 can be solubilized using 1% Sarkosyl. To test whether this is also the optimal concentration, a second screen was performed. I tested different Sarkosyl concentrations, ranging from 0-2%, in order to find the optimal detergent concentration for the purification. Solubilization was again checked by comparing the band intensities of Dpm1 in the supernatant after solubilization and high-speed centrifugation.

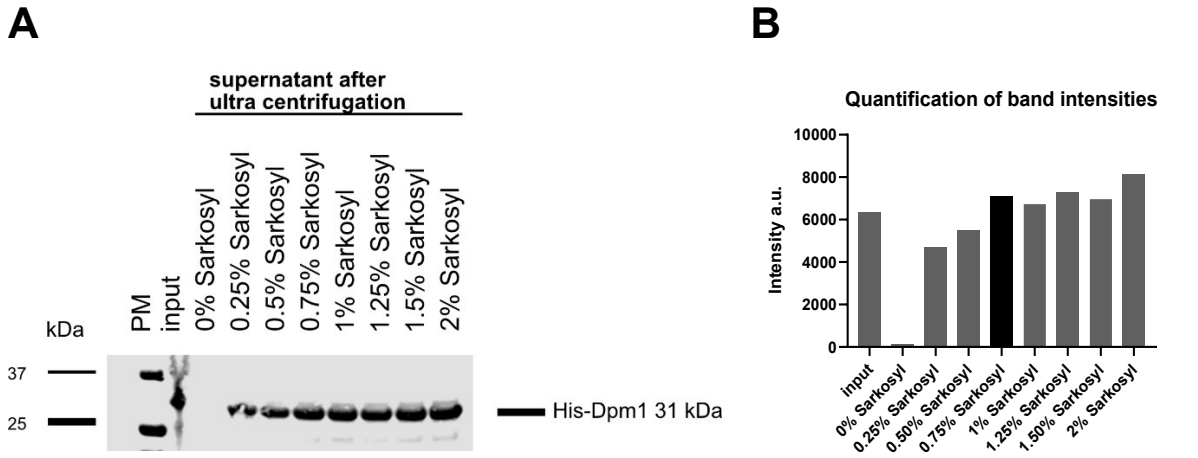


Figure 12 **Optimization of the Sarkosyl concentration for yeast Dpm1 solubilization**
A Western blot of solubilized yeast Dpm1 *E. coli* Lysate, containing recombinantly expressed Dpm1, was incubated with different amounts of Sarkosyl for 15 min at RT. Solubilized protein was separated from insoluble material by high speed centrifugation at 153.700 xg for 45 min and the amount of Dpm1 in the supernatant was compared by western blot. The blot was developed using anti-Dpm1 antibody. **B** Quantification of band intensities. Band intensities were quantified and compared, showing 0.75% Sarkosyl (black) as the optimal detergent concentration. Quantification was done using Image studio lite Ver 5.2

As shown in Figure 12, large amounts of protein were recovered in the soluble fraction with as little as 0.25% (9.3 mM) Sarkosyl. With increasing concentrations of detergent, more protein could be solubilized, reaching a plateau at 0.75%. Above this concentration no further increase in solubilized Dpm1 was observed. Therefore, I chose 0.75% (28 mM) Sarkosyl, the lowest concentration with best solubilization efficiency as optimal concentration for my purifications. This concentration is also 2-fold above the reported CMC of 14 mM.

2.1.2 Successful purification of enzymatically active Dpm1

By using 0.75% Sarkosyl, I could successfully solubilize most of the recombinantly expressed protein and purify the protein in high purity and quantity (Figure 13A). In general, purification of 1 l cell culture yielded in 500-800 µg of purified protein.

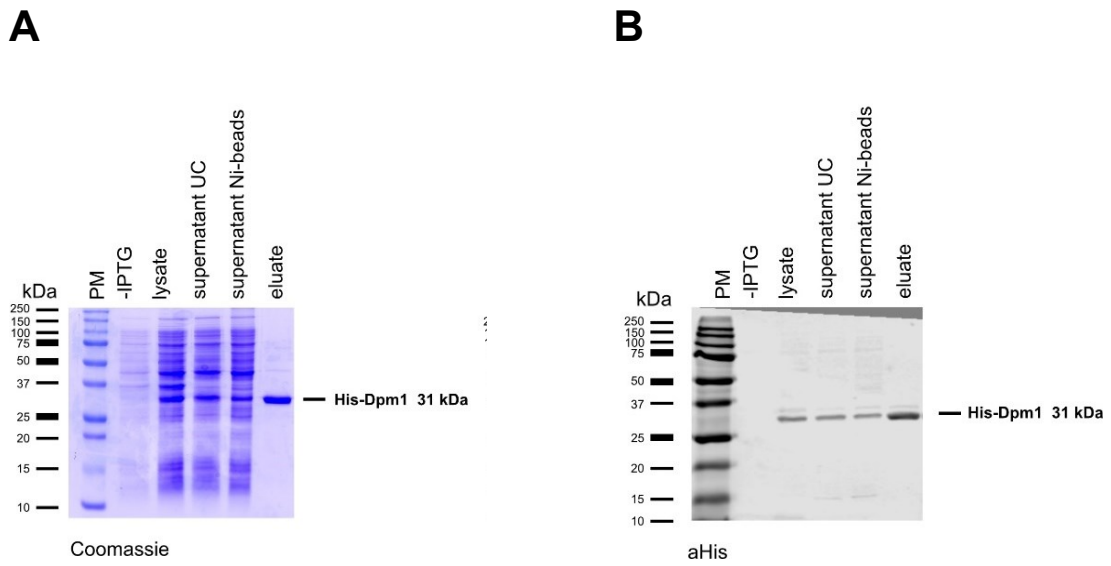


Figure 13 **Yeast His-Dpm1 purification from *E.coli***
A: Coomassie stained SDS-gel shows high purity of the Dpm1-eluate **B:** Western blot developed using anti-His antibody verifies presence of tagged Dpm1 after IPTG induction; loading for both gels: -IPTG…cell lysate before induction, lysate…containing overexpressed Dpm1, supernatant UC…Sarkosyl solubilized fraction, supernatant Ni-Beads…fraction that did not bind to Ni-beads, eluate…pooled eluted protein fractions

I detected the presence of Dpm1 in the eluate fraction by western blot using an anti-His-antibody (Figure 13B) or alternatively with a specific yeast Dpm1-antibody (not shown). Both antibodies detected the same band at the expected 31 kDa height. In addition, samples were sent for proteomics analysis, confirming that the purified protein is indeed Dpm1 (sequence alignment see Supplement).

2.2 Proteoliposome formation

As the aim was to study protein activity in a membrane environment and analyze the effect of different lipids and proteins on enzyme activity, the purified protein had to be re-inserted into a membrane. I chose a liposome reconstitution system, which allowed me to study enzyme activity in a detergent free system. In addition, both the enzyme and the substrate DolP can be incorporated together into the liposomal membrane. Since the lipid composition of liposomes can be easily controlled during preparation, this method was suitable to study the effect of different lipids on enzyme activity.

For the reconstitution of Dpm1 into liposomes I used a detergent-mediated approach, in which proteins are reconstituted into pre-formed liposomes that were destabilized by the addition of the detergent. After equilibration and addition of the protein, the

detergent was removed by adsorption to Bio-Beads SM2. A schematic overview of the final reconstitution process is shown in Figure 14 and details will be discussed in the following chapters.

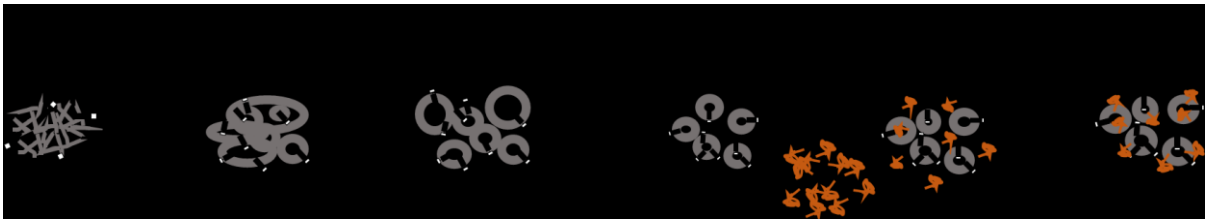


Figure 14 **Schematic overview of optimized reconstitution workflow**
Liposomes were pre-formed by hydrating dried lipids in buffer, performing 10 freeze-thaw cycles to properly suspend all lipids and to reduce the size and lamellarity of liposomes. To obtain uniform, unilamellar liposomes the lipid mixture was further extruded 21x through a 100 nm filter using an Avanti mini extruder. For protein reconstitution, liposomes were mixed with 0.75% Sarkosyl for destabilization and then the protein was added. Liposomes were diluted under the CMC of Sarkosyl and the detergent was removed by incubation with Bio-Beads SM2 (1x1h, 1x1.5h)

2.2.1 Liposome formation

For liposome preparation, lipids from $CHCl_3$ stocks were mixed in defined ratios and the solvent was evaporated under a stream of N_2, resulting in a thin lipid film. I found that it is important for the stability of liposomes that the film is as thin and dry as possible before solvation in buffer. Traces of the organic solvent led to instable liposomes and aggregation of the lipids during reconstitution. Thus, lipids were further dried under reduced pressure over night to get rid of last traces of organic solvent. In addition, glass tubes where used to avoid extraction of softeners from plastic containers by the organic solvents of the lipid stock solutions.

Dried lipids were then hydrated in buffer, resulting in an inhomogeneous lipid suspension. The solution was heated to 40 °C and briefly vortexed to increase solubilization. I tested different methods for the formation of uniform and reproducible liposomes. First, sonication was used to reduce the size of lipid particles and to obtain unilamellar liposomes. However, this method led to very inhomogeneous and not well reproducible liposome preparations. This was most likely due to an inhomogeneous sonication efficiency of the water bath sonicator used for sonication, as the efficiency depended a lot on positioning of the sample in the bath. Different average sizes of liposomes resulted in either clear or opalescent lipid solutions. Due to the inconsistent efficiency of sonication, I did not use this technique in further experiments. I decided to use an Avanti mini extruder with a 100 nm membrane instead, which resulted in

comparable liposome sizes. Reproducibility and lipid recovery were further improved by additional 10 freeze-thaw cycles before extrusion, by alternately placing of the tube in liquid N_2 and a 40°C water bath to reduce initial liposome size.

2.2.2 Protein reconstitution

Next, I wanted to reconstitute the purified protein into the pre-formed liposomes. As simple mixing of liposomes and purified protein did not lead to good reconstitution efficiency (compare in 2.2.2.3), a detergent mediated reconstitution approach was used.

2.2.2.1 Detergent stability of liposomes

In detergent-mediated protein reconstitution, liposomes are destabilized by the addition of detergent to allow protein incorporation into the membrane. Since the addition of detergent might lead to complete rupture and dissolving of liposomes, I tested the detergent stability of liposomes. Pre-formed liposomes were incubated with different detergent concentrations for 1h at RT and thereafter the size of liposomes was measured by dynamic light scattering (DLS).

Sarkosyl [%]	Sarkosyl [mM]	lipids [mM]	Sarkosyl/lipid ratio
0	0	11.25	0.00
0.25	8.5	11.25	0.76
0.5	17	11.25	1.51
0.6	20.5	11.25	1.82
0.7	23.9	11.25	2.12
0.75	25.6	11.25	2.28
0.8	27.3	11.25	2.43
0.9	30.7	11.25	2.73
1	34.1	11.25	3.03
2	68.2	11.25	6.06

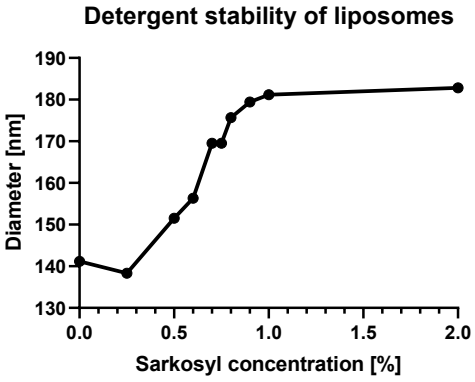


Figure 15 **Detergent stability of liposomes**
Pre-formed DOPC-liposomes were incubated with different Sarkosyl concentrations (0-2%) for 1h at RT and the size was measured using DLS. **Left:** Table of concentrations and the lipid-detergent ratio of mixtures tested **Right:** Liposome size in different detergent concentrations, measured using DLS (Wyatt Nanostar instrument).

As shown in Figure 15, liposomes were stable in Sarkosyl over a range of 0-2% Sarkosyl. An increase in liposome size from about 140 nm diameter to 180 nm was observed which was caused by the incorporation of the detergent into the liposome

membrane. Liposome size increased with increasing detergent concentrations, reaching a plateau at around 1% detergent. Concentrations above 5% detergent lead to a polydisperse lipid solution, indicating disintegration of liposomes. A concentration of 0.75% Sarkosyl was chosen for reconstitution, as it was the half-max of the destabilization curve and provided the best conditions for protein solubilization (see Figure 15).

2.2.2.2 Detergent removal by the use of Bio-Beads SM2

After destabilization of liposomes and addition of protein, the added detergent has to be removed. Since dialysis is rather time consuming, I decided to test detergent removal by the use of Bio-Beads SM2 (recently used for the removal of Sarkosyl [204]). These polystyrene beads are designed to bind to small hydrophobic compounds such as detergents and therefore can be used for detergent removal.

To verify that the detergent was effectively removed, I used the absorbance of Sarkosyl solutions. Sarkosyl shows a concentration dependent absorption peak between 180 nm and 240 nm and the absorbance around 215 nm can be used to determine Sarkosyl concentration of detergent solutions [204, 205].

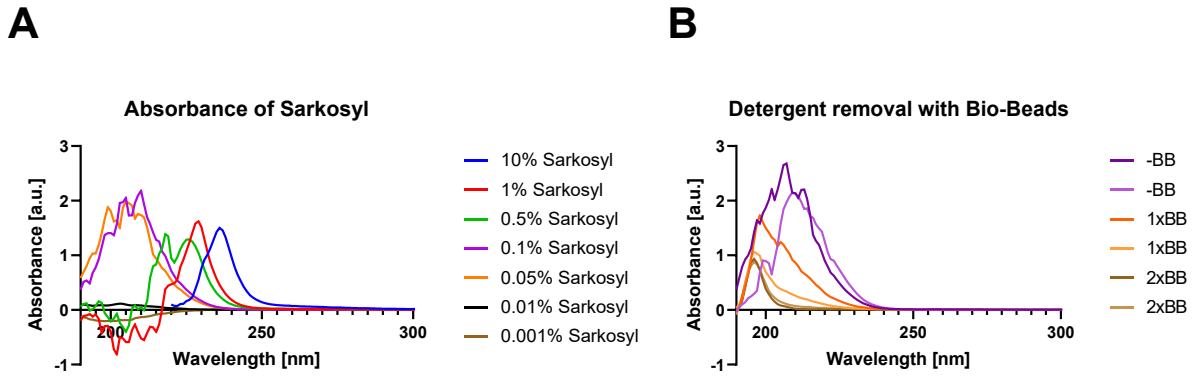


Figure 16 **Absorbance of Sarkosyl and detergent removal**
A: Different detergent solutions were prepared by sequential dilution of a 10% stock solution of Sarkosyl in liposome buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl) and the absorbances of Sarkosyl solutions were measured by the use of a NanoDrop 2000 instrument. **B:** Detergent removal with Bio-Beads was followed by measuring the absorbance of solutions after incubation. Incubations were performed in the absence of lipids and protein. The experiment was done in duplicates. **sample labelling:** -BB…before Bio-Bead incubation; 1xBB after first aliquot of BB and 1h incubation; 2xBB…after second incubation with Bio-Beads for another 1.5h

However, quantification of Sarkosyl by the use of the absorption is difficult. As shown in Figure 16, the absorbance maximum of Sarkosyl depends on the detergent concentration and shifts to higher wavelengths with increasing concentrations.

Therefore, no exact concentration was determined. However, I could see a decrease of the absorption maximum of a Sarkosyl solution upon incubation with Bio-Beads. The detergent concentration could be further decreased by a second incubation with a new batch of Bio-Beads.

2.2.2.3 Verification of reconstitution by flotation assay

To check for successful protein reconstitution into liposomes upon Bio-Bead incubation, I performed flotation assays, using a 3-step sucrose gradient. On this gradient, liposomes float to the top of the gradient during high speed centrifugation, while non-reconstituted protein aggregates at the bottom of the tube as illustrated in Figure 17A and shown for DOPC liposomes in Figure 17B.

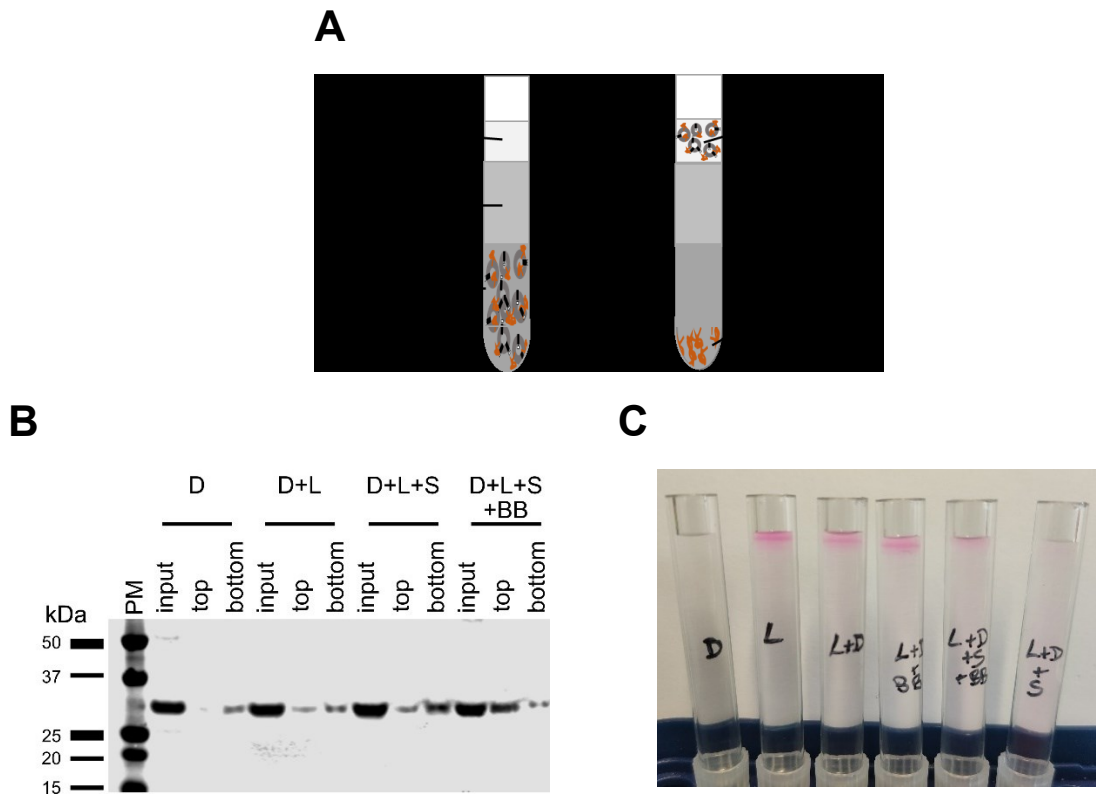


Figure 17 **Floatation of liposomes on a sucrose gradient**
A: Schematic picture of the flotation assay. The liposome sample, adjusted to 30% sucrose was overlaid with 25% sucrose and buffer without sucrose. After centrifugation (1h, 164800 xg) liposomes and reconstituted protein is found in the top fraction and non-reconstituted material at the bottom **B:** Western blot of floated and pelleted material, showing efficient reconstitution after detergent removal with Bio-Beads. The western blot was developed with anti-Dpm1 antibody. **C:** Picture of centrifugation tubes after centrifugation. Liposomes can be seen as a pink fraction at the top of the tube. **Sample labelling:** D…Dpm1, L…liposomes, S…0.75% Sarkosyl, BB… 2x incubation with Bio-Beads

Samples were loaded on the bottom of the gradient and after centrifugation the top fraction was checked for the presence of lipids and protein. As seen in Figure 17B, in

the absence of liposomes protein remains at the bottom of the gradient, while liposomes float to the top in the absence of protein (Figure 17C). The protein was partially reconstituted by simply mixing liposomes and Dpm1 protein, however, reconstitution efficiency was poor. Therefore, liposomes were destabilized by adding detergent prior to the addition of protein. The same detergent concentration as for protein purification was chosen to reduce the risk of protein precipitation. Without addition of Bio-Beads and in the presence of detergent, liposomes did not properly float to the top and only a small fraction of protein was found to be reconstituted. Upon detergent removal, however, most of the protein was reconstituted and found in the liposomal fraction of the gradient. Thus, this method was found to be suitable for the reconstitution of Dpm1 into liposomes.

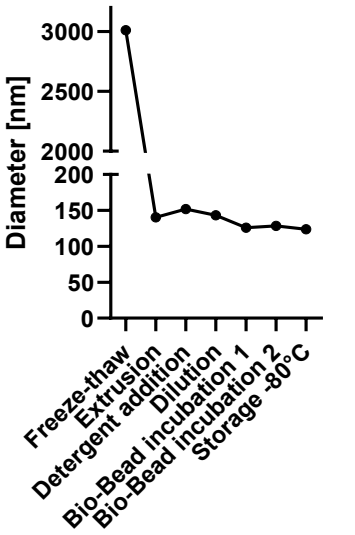
2.2.3 Optimization and important parameters of proteoliposomes

Since I wanted to compare liposomes with different membrane compositions, reconstitution had to be as reproducible as possible. Thus, special care was taken to control liposomal size, lipid amount and protein reconstitution efficiency.

2.2.3.1 Size of liposomes

An important parameter to ensure comparability of different proteoliposome preparations, is the size of the liposomes. This is particularly important since DolP, a substrate of the enzymatic reaction, is part of the membrane and its availability should be constant across different batches and membrane conditions. Therefore, I analyzed the size distribution and average diameter of liposomes during reconstitution. Samples were taken at different steps of reconstitution and the particle size was measured using DLS.

A



B

Step of preparation	Average diameter [nm]
Solubilization	n.d.
Freeze-thaw	3010 (multimodal)
Extrusion	140
Detergent addition	152
dilution	143
Bio-Bead incubation 1	126
Bio-Bead incubation 2	128
Storage -80°C	124

C

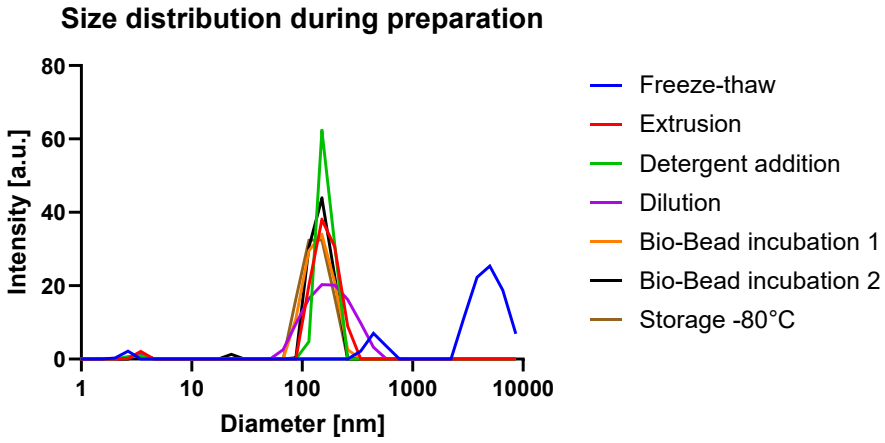


Figure 18 **Analysis of liposome size by DLS**
Liposomal size is relatively stable during preparation after extrusion. Proteoliposomes were prepared and the liposomal size was measured after each step of preparation by DLS (Wyatt Nanostar instrument) **A:** Graph showing the change the of average liposome diameter during proteoliposome preparation **B:** Table of average liposomal size of liposome solutions **C:** Average size distribution of liposomes at different steps of preparation

As shown in Figure 18, liposome size is relatively constant during liposome reconstitution and liposomes have a diameter of around 140 nm. It also shows, that freeze-thaw cycles reduced liposome size and made the sample more uniform, but only after extrusion the sample was found to be homogenous with respect to liposome size. Initial liposome sizes after suspending lipids could not be measured by DLS due to inhomogeneity and polymodal distribution.

2.2.3.2 DolP quantification

Since DolP is one of the enzymatic substrates of Dpm1 it is important to keep its concentration constant between different preparations. To check for comparability and to compensate for different lipid loss during the preparation of proteoliposomes, the amount of DolP was analyzed. A good way to measure DolP concentration is by mass spectrometry. This method allows an identification and exact quantification of the lipid substrate of Dpm1. In addition, DolP species can be separated and thus chain length distributions can be analyzed. However, at the beginning of my thesis, there was no fast and efficient method for DolP quantification available. We therefore started to develop a method for DolP quantification mass spectrometry. I was involved in the work of Dipali Kale, who developed a LC-MS based method for DolP quantification and the final method was published in 2023 [53]. In the meantime, I used indirect methods to monitor DolP loss during liposome preparation, by the quantification of other membrane lipids to estimate the average lipid loss.

2.2.3.2.1 Indirect DolP quantification by Rh-PE fluorescence

One way to indirectly determine DolP concentration is to measure the fluorescence of Rhodamine-phosphoethanolamine (RhPE) of the sample. 0.2% of the pink PE analog was added to each lipid composition, mainly to visualize liposomes during preparation. In addition, the fluorescence intensity can be used to compare the RhPE content and therefore serves as a measure for lipid concentrations in different liposome preparations. Thus, I developed an assay to analyze the RhPE concentration by using the fluorescence of RhPE. The optimal excitation wavelength was found to be at 550 nm. RhPE concentration in liposomes was calculated by comparing the emission at 590 nm to a standard concentration curve of RhPE.

This method was mainly used in initial experiments to optimize the reconstitution workflow, as it offered a fast and quantitative readout of the lipid loss. High sample amount and the need of detergent to solubilize the lipid standard and liposomes were the major drawbacks of this method.

2.2.3.2.2 Indirect DolP quantification by lipidomics

Another way to indirectly quantify DolP is to analyze the concentration of other membrane lipids by mass spectrometry. Analysis methods for the main lipid species in the liposome preparations were already established and routinely used in the lab. I

therefore subjected all liposomes used to PC analysis by mass spectrometry. Lipids were extracted using a modified Bligh-Dyer extraction protocol and analyzed via direct infusion mass spectrometry. The PC concentration was used to calculate the total lipid and DolP concentration.

Table 1 **Lipid loss of DOPC liposomes measured by PC analysis**
n=9 for after reconstitution, n=10 for after FU, total comparison not batch wise, outlier test was performed ROUT (Q = 1%) with no outlier detected

	Total lipid concentration	**Recovery (% of theoretical)**
Theoretical input	2.5 mM	
After reconstitution (DOPC)	1.4 mM +/- 0.2 mM	55 % +/- 10%
After FU (DOPC), normalized to input	0.6 mM +/- 0.4 mM	26 % +/- 15%

With the optimized reconstitution protocol, lipid loss during preparation was relatively comparable. However, huge differences in lipid recovery after flotation were seen, especially when comparing different lipid compositions or different protein content.

2.2.3.2.3 DolP analysis by mass spec

The analysis of DolP by mass spectrometry is challenging. First of all, DolP is a low abundant lipid (~0.1% in eukaryotes) [52]. Paired with its high chemical diversity, due to different chain lengths, the abundance of individual DolP species is even much lower. Differences in isoprene chain length also significantly change the lipophilicity of DolP species, making it difficult to extract all species with similar efficiency using the same method. Due to the unmasked phosphate group, DolPs show a bad running behavior with broad peak shapes on LC-columns. In addition, DolP only poorly ionizes and signals are often suppressed by highly abundant lipids.

Thus, I was involved in the developmental work of Dipali Kale, who established a LC-MS based method for qualitative and quantitative analysis of DolP species from biological membranes [53]. She implemented an efficient derivatization method, using TMSD as methylation reagent. Methylation of the phosphate group by the use of TMSD greatly helped in ionization and strongly improved the running behavior on reversed phase columns. This derivatization method, paired with optimized LC-MS conditions proved to be a useful tool to analyze the DolP content of tissue samples. Since we were interested in the total DolP content of cells, in biological samples all attached sugar moieties were cleaved by alkaline hydrolysis prior to derivatization. This allowed

us to analyze the total cellular DolP pool and also increased the concentration of DolP species in the sample. The developed method was highly sensitive (LOD ~1 pg) and covered a broad molecular range, allowing the quantification of DolP species ranging from C65 to C105.

We used this LC-MS method also to analyze the species distribution of commercially available DolP-Mix, which I used as DolP substrate throughout the work of this thesis (Figure 19).

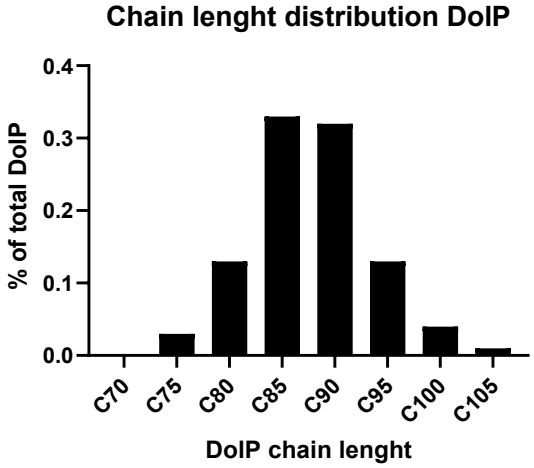


Figure 19 **Chain length distribution of DolP substrate**
Chin length distribution of DolP-Mix (Avanti 900201) that was used as substrate. DolP species were methylated using TMSD and analyzed via LC-MS. Chain lengths are depicted as % of total DolP species detected. n=1; DolP extraction, derivatization and analysis was performed by Dipali Kale

We verified the presences of C65-C105 DolP, as also stated in the product information. C85 and C90 were found to be the main species, accounting for about 65% of the total DolP content. Thus, the DolP used in this work had a species composition that was intermediate between the natural composition of yeast (major species C75 and C80) and human (major species C90, C95 and C100).

2.2.3.3 Protein quantification by western blot

Since the amount of protein reconstituted into liposomes was quite low (around 0.5 pmol Dpm1/µl liposomes solution), reconstituted protein could only be analyzed by western blot. Higher protein concentrations could not be used due to limitations by the protein/DolP-ratio in liposomes. Too high protein concentration would lead to rapid consumption of the DolP substrate. Therefore, western blots were used for protein quantification. Band intensities of reconstituted protein were compared to a loading control of purified protein to calculate protein concentration.

Table 2 **Protein reconstitution and recovery**
n=9 for after reconstitution, n=10 for after FU, total comparison not batch wise, outlier test was performed ROUT (Q = 1%) with 2 outliers detected and removed for “after FU”

	Total protein concentration	**Recovery (of theoretical)**
Theoretical input	0.5 µM	
After reconstitution (DOPC)	0.38 µM +/- 0.26 µM	76 % +/- 53 %
After FU (DOPC), normalized to input	0.08 µM +/- 0.03 µM	16 % +/- 6 %

I found the average recovery of protein after reconstitution to be around 76 %. Recovery after flotation was 16 % of the theoretical input and around 22 % of the input from flotation (=after reconstitution).

The protein amount was found to be comparable between different lipid compositions and liposomes prepared on the same day, but differences were observed when comparing different batches of protein and liposomes. Thus, the enzyme activity data were not only normalized to enzyme amount, but I also decided to use a batch-by-batch comparison for data analysis.

2.2.3.4 Dpm1-DolP ratio

2.2.3.4.1 Theoretical calculations

As DolP is a substrate for the enzymatic reaction, its concentration within the liposomes as well as per enzyme hast to be high enough to follow enzymatic reaction. Therefore, it is important to keep the Dpm1/DolP ratio comparable to have similar substrate concentrations and to be able to compare enzyme activities between samples.

The average number of DolP per liposomes can be estimated by using the liposomal size to calculate the number of lipids per liposome. Liposomal diameter was approximately 140 nm, according to DLS measurements. When assuming a membrane thickness of 5 nm and an average surface area of 0.5 nm^2 per lipid [182], each liposomes consist of about 230000 lipid molecules, with 2300 DolP molecules per liposome. In the conditions chosen, liposome concentration was therefore 19 nM. The number of Dpm1 molecules per liposome, calculated by the theoretical input, was 48. Thus, leading to a Dpm1/DolP ratio of about 1:50.

2.2.3.4.2 Average Dpm1-DolP ratio in liposomes

There was a slight loss in protein and lipid after flotation resulting in an increased DolP/Dpm1 ratio, as seen in Table 3. However, the difference between DolP/Dpm1 ratio before and after flotation was not significant when the data was analyzed using an unpaired t-test (95% confidents level). Thus, the flotation did not significantly change the DolP/Dpm1 ratio and liposomes were comparable before and after flotation.

Table 3 **Calculations of DolP/Dpm1 ratio**
n=9 for after reconstitution, n=10 for after FU, total comparison not batch wise, outlier test was performed ROUT (Q = 1%) with 2 outliers detected and removed for "after FU" for Dpm1 and DolP/Dpm1

	Dpm1	**DolP**	**DolP/Dpm1**
Theoretical	0.5 µM	25 µM	50
After reconstitution (DOPC)	0.38 µM +/- 0.26 µM	14 µM +/- 2 µM	37 +/- 54
After FU (DOPC), normalized to input	0.08 µM +/- 0.03 µM	6 µM +/- 4 µM	75 +/- 103

2.3 Activity assay

Dpm1 is a mannosyl transferase, that catalyzes the formation of DolP-Man from GDP-Man and DolP (Figure 20).

Figure 20 **Reaction catalyzed by Dpm1**
Mannosyl transfer form GDP-Man onto DolP that is catalyzed by Dpm1

Liposomes, containing reconstituted Dpm1 protein and DolP acceptor substrate were used and the assay was started by the addition of GDP-Man to the reaction mixture. The reaction was terminated after a defined time and the amount of DolP-Man product was analyzed. To analyze changes in enzyme activity caused by the membrane environment, the DolP-Man product formation was compared under different conditions.

To follow Dpm1 activity, theoretically either the reduction of the two substrates or the formation of the two products can be used. However, as both substrates are added in excess, their reduction during product formation represents only a small change in the total amount and is hardly measurable. Thus, it is more reliable to monitor the formation GDP or DolP-Man. The release of GDP was also used to monitor Dpm1 activity [206],

however, this method lacks the proof that the mannose is indeed attached to the DolP lipid. Thus, I chose to directly detect and monitor DolP-Man formation.

2.3.1 DolP-Man analysis

To measure Dpm1 activity, the amount of DolP-Man present in the reaction mixture after a certain time was analyzed. A sensitive method to measure the mannosylation of DolP is by the use of radio labelled mannose, as also used in the majority of previous studies on DPMS activity. The transfer of mannose from the aqueous GDP-Man fraction to the liposomal or lipid fraction indicates the attachment of mannose to the lipid substrates. I used the radioactivity detected in these lipid fractions as a readout for enzyme activity.

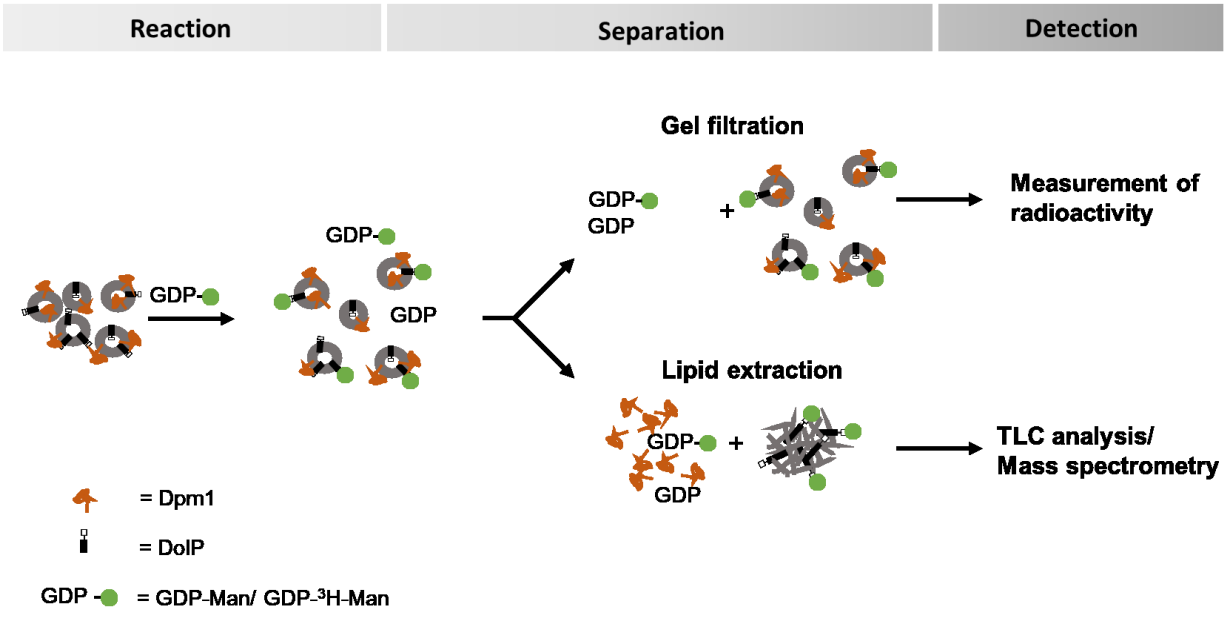


Figure 21 **Schematic overview of the enzyme activity assay**
Proteoliposomes, containing Dpm1, are incubated with GDP-Man to start the mannosyl transfer reaction. After the reaction the DolP-Man product is separated from an excess of GDP-Man before the mannosyl-transfer can be quantified. Two methods were used in this thesis: 1) separation by gel filtration and detection by scintillation counting 2) lipid extraction wad detection of transferred mannose either radioactivity after TLC separation or by mass spectrometry.

There are two options to separate the excess of GDP-Man from the DolP-Man product (Figure 21). Due to the different polarities substrate and product can be separated by lipid extraction methods, where the hydrophobic DolP-Man is found in the organic fraction, whereas GDP-Man stays in the aqueous phase. This method was commonly used in earlier experiments, where additional DolP substrate was added exogenously to the reaction mixture [90, 191]. Verification of DolP-Man formation was be done after

extraction, using either TLC analysis [75, 90] or liquid scintillation counting of the organic extract [105, 207] or using a biphasic scintillation cocktail [190, 208]. I used the TLC based detection method, adapted from Orlean et al.1988 [90], in initial experiments to check for enzyme activities of protein preparations and proteoliposomes (see 2.3.1.1). However, quantification of the mannose transfer is difficult using this technique, as no radiolabeled internal standard was available to test for lipid loss during extractions. When using unlabeled GDP-Man, this extraction method can also be used for a mass spectrometry-based activity assay, where DolP and DolP-Man possibly could be detected simultaneously and extraction efficiency can be normalized to an internal standard.

In the liposomal assay I established, the transferred mannose is bound to the DolP on the liposomes. As the product is embedded into the liposomal membrane, DolP-Man can also be separated from excess GDP-Man by isolating liposomes. Within the work of this thesis, I therefore established a workflow to separate product and substrate using size exclusion columns (2.3.1.2).

2.3.1.1 DolP-Man extraction, TLC and ß-imager

To test if Dpm1 is enzymatically active after reconstitution, I performed an initial experiment, (modification of Orlean et. al 1988 [90]). Liposomes containing reconstituted Dpm1 and DolP as substrate were tested for product formation. I used detergent solubilized yeast membrane fractions (containing endogenous DolP and Dpm1) from Dpm1 over expressing cells as a positive control for Dpm1 activity, while membrane fractions that were heat inactivated at 70 °C for 10 min served as a negative control. Dpm1 activity of protein purifications, either in 0.5 % Triton or 0.75% Sarkosyl, were tested without reconstitution by using heat inactivated membrane fractions as DolP source. In order supply additional DolP for the reconstituted protein, heat inactivated membrane was also added to one liposome sample.

Previous methods required the presence of detergents for Dpm1 activity [90, 209]. Therefore, samples without liposomes were supplemented with low detergent concentrations. Samples 1-3 were analyzed in the presence of 0.5% Triton. Sample 4 contained 0.75% Sarkosyl, to test if Dpm1 is also active in the presence of the detergent it was purified in. In addition, samples containing liposomes in the absence of detergent were analyzed.

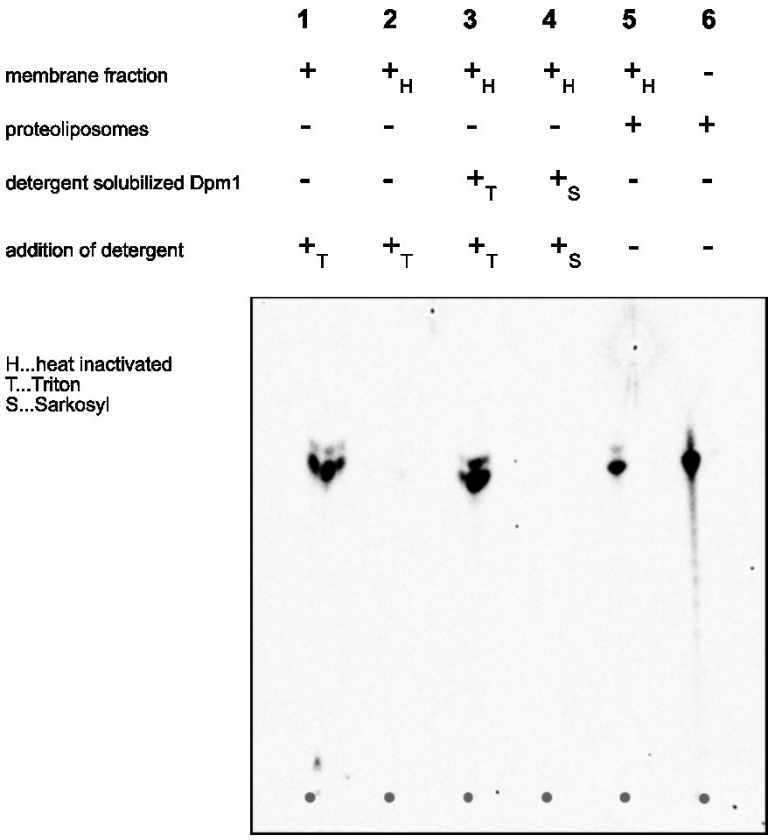


Figure 22 **TLC of DolP-Man**
Solubilized yeast membrane fractions, proteoliposomes or eluates of enzyme purifications were used as enzyme source. The DolP acceptor for the reaction was either supplied with the liposomes and/or as yeast membrane fractions. Samples were mixed as indicated and incubated with 20 mM radio labelled GDP-^{3}H-Man for 20 min at RT. The DolP-^{3}H-Man product was extracted two times into $CHCl_3$/MeOH 2:1. The extracts were dried under a stream of N_2, dissolved in a minimal amount of extraction solvent and spotted onto a TLC. The TLC was developed in $CHCl_3$/MeOH/H_2O/AcOH 65:35:4:1 and imaged using a ß-imager (Biospace Lab). DolP-Man: Rf 0.72

As shown in Figure 22, yeast membrane fractions containing endogenous Dpm1 and DolP showed mannosyl transfer activity, as radiolabelled mannose was found in the lipid fraction after incubation. This activity was lost when proteins were denaturated by heating the sample to 70 °C for 10 min prior to the assay. The activity could be restored by the addition of purified Dpm1 in 0.5 % Triton. However, using a preparation of Dpm1 in 0.75 % Sarkosyl did not allow to restore mannose transfer activity in the presence of Sarkosyl. This was most likely due to the presence of Sarkosyl that was not able to support Dpm1 activity. As the assay buffer also contained Mg^{2+}, it is also possible that Sarkosyl-Mg crystals formed that rendered the enzyme inactive. Liposomes containing reconstituted Dpm1 were active in the absence of detergents. This Dpm1 activity could be seen in the presence and absence of inactivated membrane fractions, demonstrating that DolP in the liposomal membrane can serve as subsrate for the enzyme even in the absence of detergents.

This first assay showed that Dpm1 activity can be measured in the absence of detergent when using liposomal reconstituted enzyme.

2.3.1.2 Size exclusion column and scintillation counter

Compared to liposomes with a 100-150 nm diameter, GDP-Man is small molecule. Thus, I wanted to test whether substrate and product could be separated by size exclusion. I chose commercially available “NICK-columns”, with a cut-off volume of 10 kD that are commonly used for the purification of labelled DNA. As a first test, the liposomal assay mixture, containing liposomes and radiolabeled GDP-Man, was applied to the column and stepwise eluted in 100 µl fractions. The presence of radioactivity in each fraction was monitored using a scintillation counter. Figure 23 shows the elution profile of the liposomal activity assay mixture a NICK-column.

Free GDP-Man was retained by the column and eluted as a single peak over fractions 10-24. When the assay mixture contained active Dpm1 in liposomes with DolP substrate, a second peak appeared upon incubation with GDP-Man. This second peak was eluting earlier in fractions 5-9, indicating the bigger size of the liposomes (Figure 23A). This peak was due to the formation of DolP-Man, as it was not seen with liposomes lacking DolP acceptor. The lack of radioactivity in the front peak in the absence of DolP also showed, that free GDP-Man was not associated with Dpm1 itself, thus confirming that radioactivity in the first represents successful transfer of the mannose onto the DolP substrate. The presence of proteoliposomes in the front fraction was confirmed by western blot detection of Dpm1 in the corresponding fractions (Figure 23B).

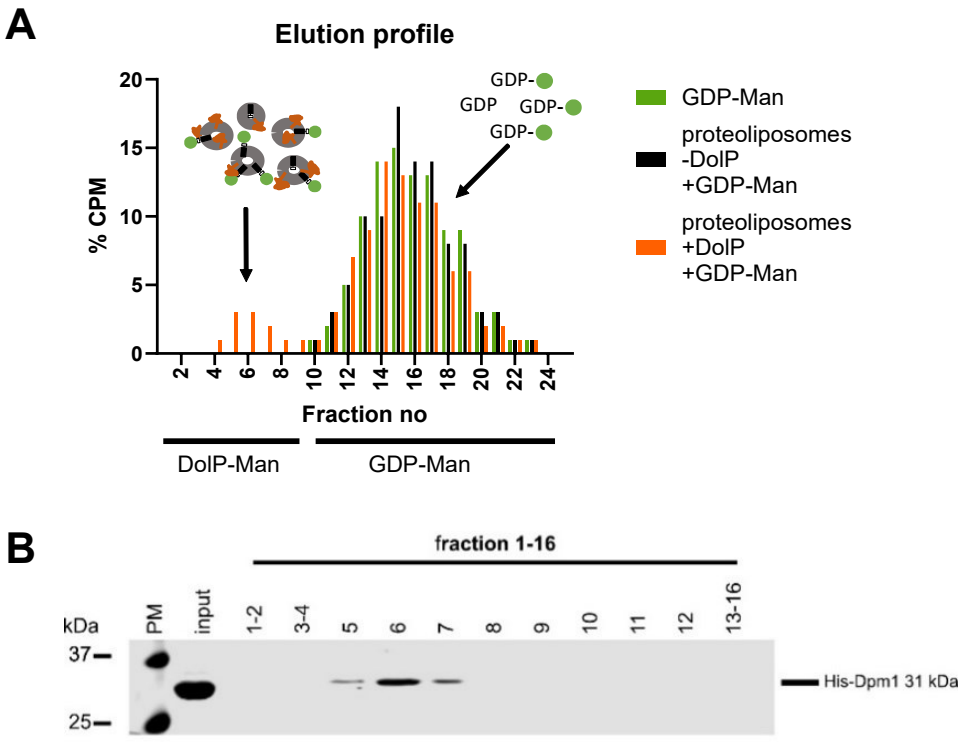


Figure 23 **Elution profile of liposomes on size exclusion column**
Liposomes were incubated with 20 mM GDP-Man in a total volume of 100 µl at 25°C. After 1 min, the sample was applied onto a pre-equilibrated NICK column. The sample was eluted by the stepwise addition of 25x 100 µl aliquots. **A:** Radioactivity eluted in different fractions was measured using scintillation counting. Values are represented as %CPM of total eluted radioactivity per sample **B:** Western blot of eluted fractions. Protein was detected using anti-His antibody

To measure and compare activity of Dpm1, I used the distribution of tritiated mannose in the two peaks. Therefore, I collected two fractions: the liposomal fraction containing DolP-Man product and the free GDP-Man fraction. Activity was determined by the amount of radioactivity found in the liposomal fraction compared to input of radioactivity. Input was either measured by taking an input sample directly from the assay or calculated by the sum of radioactivity found in the DolP-Man and GDP-Man fraction. Both input references gave similar results.

Thus, the use of size exclusion columns was a fast and simple method, to separate the substrate GDP-Man from the DolP-Man product. Elution was reproducible and comparable between different days, with minimal background activity. The method allowed the measurement of Dpm1 activity without the involvement of organic solvents and time-consuming TLC and was therefore used for all further experiments.

2.3.2 Kinetics

As I wanted to analyze Dpm1 activity under different conditions, I also checked whether reconstituted Dpm1 shows characteristic enzymatic properties. One important point was to make sure that product formation was not measured at the endpoint of the enzymatic reaction. Therefore, I monitored Dpm1 activity after different time points. Within the first 2 minutes, a linear increase in DolP-Man concentration was seen and velocities were reproducible under the same assay conditions (Figure 24).

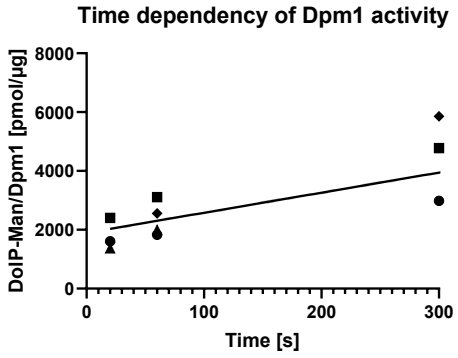


Figure 24 **Time dependency of Dpm1 activity**
DolP-Man concentration is increasing linearly within the first 300s of the reaction. Graph showing the amount of DolP-Man formed over time at 5 µM GDP-Man concentration. DolP-Man was normalized to the protein amount. Data of four replicates, with at least three time points within the first 600 s. Velocity was calculated using a linear fit and was found to be 10.8 +/- 5.2 pmol/µg/s.

Another important factor is the enzyme concentration as product formation should increase linearly with enzyme concentration. Thus, Dpm1 dependency was tested during method development (data not shown). Indeed, a doubling in product amount was observed when reconstituting the double amount of enzyme. Also, doubling the amount of proteoliposomes that were used in the activity assay lead to a doubling of Product amount. To make liposomes more comparable the enzyme amount used for reconstitution was kept constant in all future conditions. To compensate for differences in reconstitution, product amount was further normalized to enzyme concentrations of the respective proteoliposomes.

Enzymatic turnover is also influenced by substrate concentration. Dpm1 is an enzyme that requires 2 substrates, namely DolP and GDP-Man. On one hand, the amount of GDP-Man can be easily controlled by the addition of different amounts at the start of the reaction. GDP-Man is water soluble and by using GDP-Man spiked with only a low amount of tritiated GDP-Man, radioactivity could be kept moderate even at high GDP-Man concentrations.

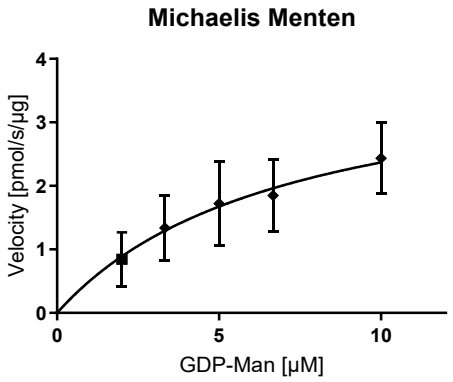


Figure 25 **Kinetics of Dpm1**
Michaelis Menten plot of Dpm1 activity in depenece of GDP_Man concentration, with K_m = 7.1 µM and v_{max} = 4 pmol/s/µg. DOPC-proteoliposomes were prepared and the enzymatic activity of Dpm1 was measured at different GDP-Man concentrations and 4 time points (20s, 40s, 60s, 80s). The velocity was calculated by the slope of the increase over reaction time. Calculations were preformed using GraphPad Prism 9, n=3

Dpm1 activity in DOPC liposomes with 1% DolP was measured in the presence of different GDP-Man concentrations. Figure 25 shows the Michaelis Menten plot. The K_m for GDP-Man was found to be 7.1 µM and the v_{max} was determined as 4 pmol/s/µg. These findings are in accordance with earlier studies of yeast Dpm1 in the presence of detergent, where also a K_m of 7 µM was calculated [191].

On the other hand, DolP is part of the liposomal membrane itself. It is very hydrophobic and only soluble in organic solvents or detergent solutions. This makes it difficult to modify the DolP concentration at other points than at the very beginning of liposome preparation. Earlier studies added additional DolP to the reaction mixture to increase DolP-concentration. DolP was either dried with Mg-EDTA or added solubilized in detergent or organic solvents [90, 191, 207]. Since my aim was to measure enzyme activity in the absence of detergents and with DolP embedded in a lipid membrane, this method was not feasible. Thus, to analyze liposomes with varying DolP concentrations in the liposomal membrane, different liposome preparations had to be used. The activities of Dpm1 in liposomes with different DolP concentrations was measured (see also 2.4.2.) As expected, Dpm1 activity is higher at higher substrate concentrations. However, no kinetic parameters were measured.

In order to compare different membrane compositions and to determine kinetic parameters the substrate concentration has to be comparable between samples and remain relatively constant within a sample over the reaction time. Considering the difficulties in controlling DolP availability, I decided to keep its concentration constant at 1 % of total lipids in all conditions tested. In addition, GDP-Man was used at a

concentration of 20 µM and only the product amount after 1 min was used for comparison. These pre-set parameters allowed me to study the effect of the membrane environment on Dpm1 activity.

Part II Activity of Dpm1 in different membrane environments

2.4 Activity of Dpm1 in different lipid environments

Together, the reconstitution system and the newly established assay allowed me to analyze Dpm1 activity in a very defined lipid membrane environment. Such a simplified and well-controlled system is needed to study effect of single lipid species and to better understand the role of lipid diversity. Proteoliposomes with a defined lipid composition were prepared and their Dpm1 activity was compared to analyze the effect of lipids on enzyme activity.

2.4.1 DolP-Man species distribution and chain length preference

DolP, the substrate of Dpm1, consists of a long α-saturated isoprene chain and a terminal phosphate group. Whereas the phosphate gets attached to the sugar and is needed for subsequent transfer of the sugar, the isoprene chain serves as a membrane anchor. Dolichol derivatives are used as anchors for mannose, glucose and the LLO in the ER (1.1.5.2The number of isoprene units in the Dol chains varies between different species from 12-21 (C60-C105) [52]. In human tissues predominant chain lengths were found to be C85 and C90, whereas in yeast C75 and C80 species are most prominent, as shown in our paper by Kale et.al. [53]. The reason for these differences and the diversity is not understood yet.

As I was using a commercially available DolP mixture, that consisted of a mix of DolPs with different chain lengths, I was interested to see if a specific DolP species is preferentially getting mannosylated. Therefore, we used our newly developed DolP analysis method to analyze the species distribution of the DolP substrate in liposomes. In addition, DolP-Man was extracted from liposomes after the addition of GDP-Man, and the chain length distribution was analyzed. Due to the lack of internal standard for DolP-Man, no quantification or correction for different ionization efficiencies were made for the product of the Dpm1 reaction. The species distribution for DolP and DolP-Man was gained by relative comparison of peak intensities of all detected species.

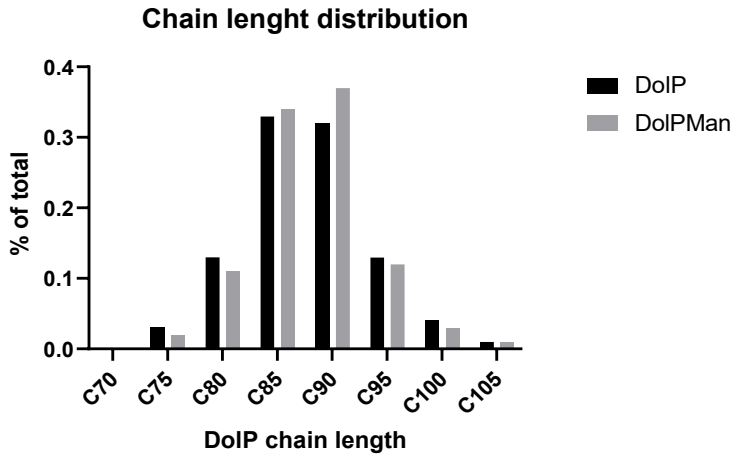


Figure 26 **Chain length distribution of DolP species**
DolP species from proteoliposomes were extracted either directly after reconstitution (DolP) or after 1h incubation at RT with 20 µM GDP-Man (DolP-Man). Lipids were extracted by Bligh-Dyer extraction and samples were methylated by the use of TMSD. DolP and DolP-Man chain length distribution was measured by LC-MS. n=1 Extraction, derivatization and measurements were performed by Dipali Kale.

Comparison of the chain length profile of DolP and DolP-Man, showed that all DolP species were mannosylated with comparable efficiencies (see Figure 26), and no preference for a specific DolP species was seen. Note, that these findings have to be handled with care as they were only performed once. In addition, the reaction time was 1 h, meaning that the extraction and analysis was probably performed at the end of the reaction, even though only a fraction of available DolP was mannosylated and only a slight reduction in DolP was seen upon incubation of liposomes with GDP-Man.

Repetition of the experiments with shorter incubation time could reveal whether the DolP-Man distribution remains constant over the whole reaction time or if at the beginning of the reaction specific DolP species gets preferentially mannosylated, before other, maybe less favorable substrate is used.

Nonetheless, the experiment indicated that yeast Dpm1 is not restricted to certain chain lengths and also accepts other DolP species than the endogenous ones. Even longer DolP species, not found naturally in yeast, were used as acceptor substrate by yeast Dpm1.

2.4.2 Effect of the polyprenol chain on membrane environment

The Dolichol chain consist of many isoprene units, that are embedded into the hydrophobic part of the membrane. To test, whether the tail alone interacts with Dpm1 and if it affects enzyme activity by changing membrane properties or binding to the

protein, I prepared liposomes containing DolP and Dol. Dol is structurally similar to DolP consistent of the same α-saturated isoprene chain as DolP, but contains an alcohol group instead of a phosphate. By lacking the phosphate group, it does not serve as substrate for the enzyme. The Dol species had the same chain length distribution as DolP. Thus, they can be directly compared and can be used to study the effect of the isoprene chain on enzyme activity.

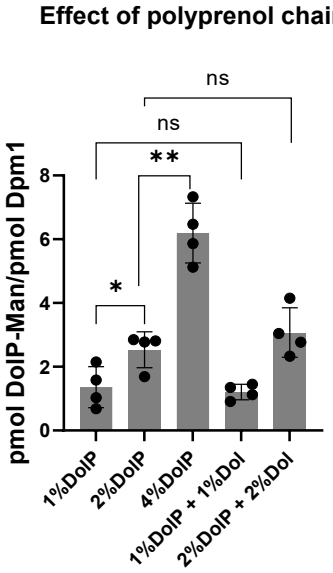


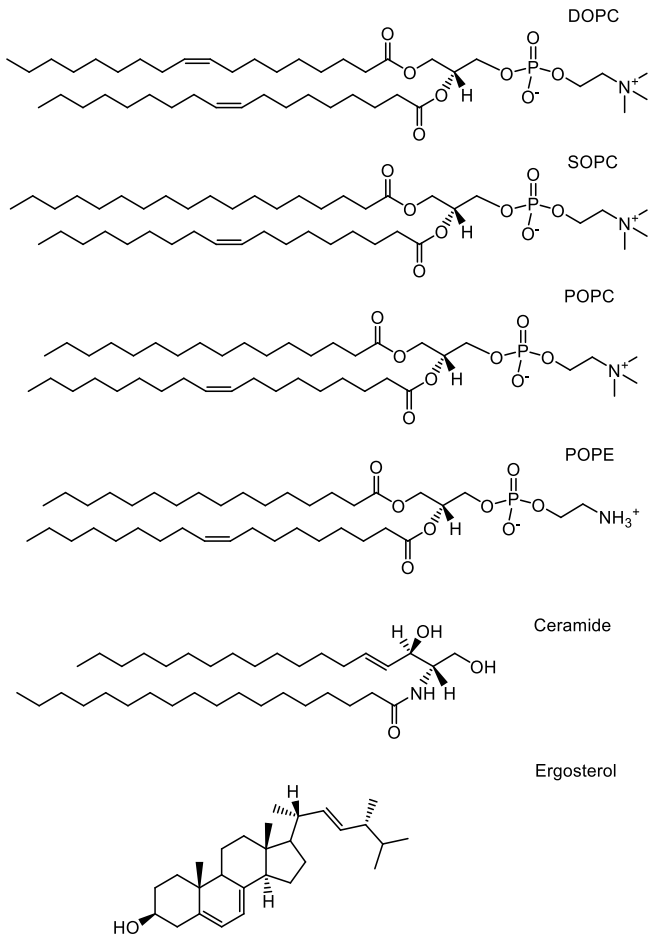
Figure 27 **Effect of polyprenol chain**
The activity of Dpm1 depends on the DolP substrate concentration and is not altered in the presence of Dol. Dpm1 activity was measured in liposomes with different substrate concentrations and with or without the addition of Dol. The assay was performed with radiolabeled 20 µM GDP-Man at 25°C for 1 min. The amount of radioactivity in the liposomal fraction, normalized to protein amount was used for comparison. n=4, statistical analysis was performed using a paired ratio t-test (GraphPad Prism, 9.5.1)

As shown in Figure 26, activity of Dpm1 is not affected by the isoprene chain under the conditions used. Increasing the amount of DolP also increases the formation of DolP-Man, as expected in enzymatic reactions when substrate concentration is increased. Addition of Dol, however, did neither increase nor decrease Dpm1 activity, as the amount of DolP-Man solely depended on the DolP concentration. Thus, Dol does not compete with DolP and does not regulate Dpm1 activity directly. In addition, the change in membrane properties by the addition of the polyprenol chain did not affect enzymatic activity in the range tested.

2.4.3 Influence of lipid composition on Dpm1 activity

A major aim of this project was to study the effect of membrane compositions on enzyme activity. Therefore, I reconstituted Dpm1 into liposomes with varying lipid

compositions and measured Dpm1 activity, using the newly established activity assay. A list of lipid compositions tested can be found in Figure 28.



LIPID COMPOSITIONS	
DOPC (18:1/18:1 PC)	As standard lipid composition
SOPC (18:0/18:1 PC)	To see effect of increased saturation of fatty acids
POPC (16:0/18:1 PC)	To see the effect of shortening chain length
DOPC/POPC 1:1	To see if saturation has an optimum
DOPC/POPC/POPE 5:1:4	To further increase fluidity, PE is known from the literature to increase the activity of DPMS [193]
DOPC/Ceramide 95:5	To promote domain formation, alters fluidity
DOPC/Ergosterol 95:5	To increase rigidity and membrane thickness, alters membrane permeability and promotes domain formation
DOPC/Ergosterol 80:20	To further increase rigidity

Figure 28 **Chemical structure of lipids and lipid compositions used for liposome preparation**
Left: Chemical structure of lipids used for liposome preparation **Right:** All lipid compositions tested to study the effect of the lipid environment of Dpm1 activity. Lipid compositions were partly taken from: [210]

To ensure comparability, I kept the DolP substrate amount constant at 1 % of total lipids for all liposome compositions. The amount of DolP-Man formed after the addition of GDP-Man over the time of 1 min was normalized to the enzyme concentration and used for comparison and to asses membrane dependent differences in enzyme kinetics. DOPC liposomes were used as a reference. In addition, I wanted to see, if the lipid composition had an effect on reconstitution efficiency and lipid recovery.

2.4.3.1 Reconstitution efficiency of Dpm1 into different liposomes

To be able to compare enzyme activity under different conditions, the same amounts of enzyme should be reconstituted. Reconstitution efficiency is presented as pmol Dpm1/pmol DolP in Figure 29. The total protein concentration was measured by

quantification of protein bands on western blots. The DolP concentration was measured indirectly by analyzing the PC content of liposomes via mass spec and calculating DolP as 1 % of the total lipid content. The calculations were based on the assumption that the amount of DolP is proportional to the total lipids and therefore also to the number of liposomes and can be used to normalize and compare reconstitution efficiencies.

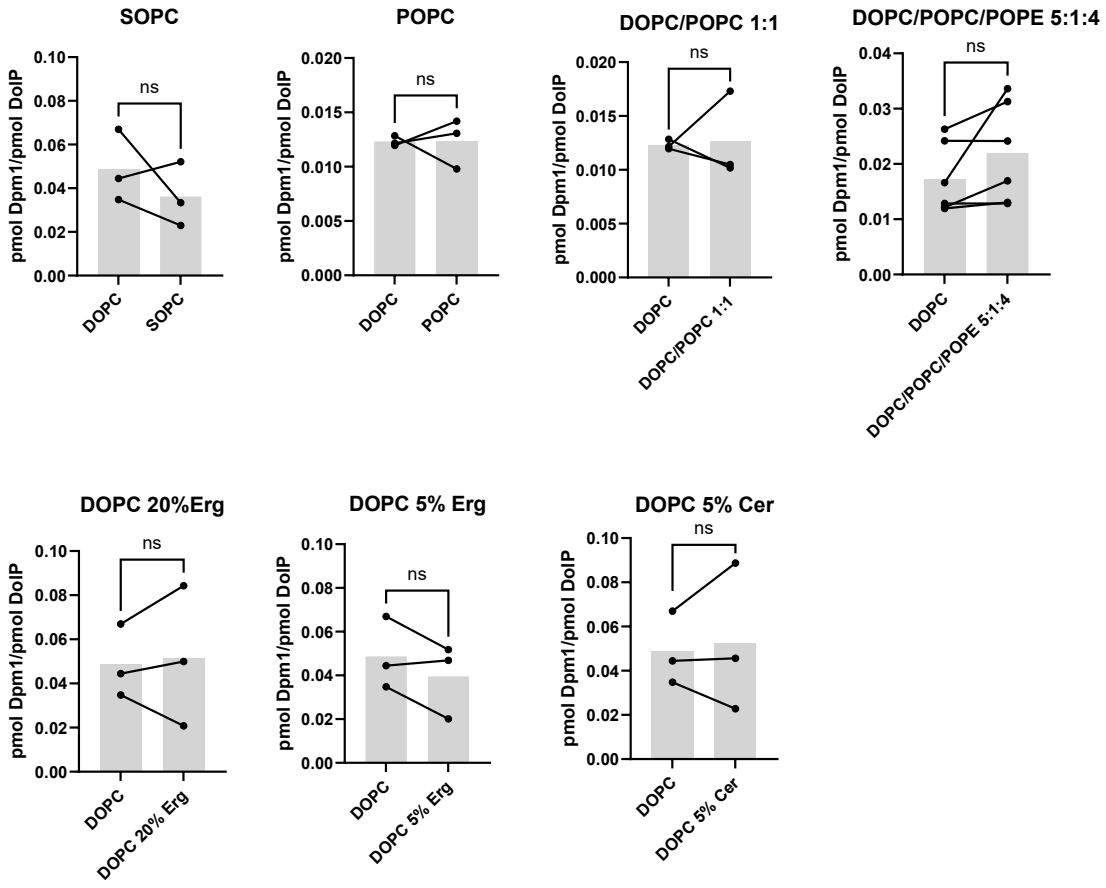


Figure 29 **Reconstitution efficiency of Dpm1 into different liposomes**
Protein concentration in liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. DolP concentration was indirectly quantified by MS-measurement of PC content. Results are presented as pmol Dpm1/pmol DolP. Statistical analysis was performed batch wise for each lipid composition in comparison to DOPC liposomes. A paired ratio t-test was used (GraphPad Prism, 9.5.1)

As seen in Figure 29, the amount of reconstituted Dpm1 enzyme into liposomes of different lipid compositions did not significantly differ from corresponding DOPC liposomes, prepared on the same day. However, I observed differences in protein amounts between different batches prepared on different days, which led to high standard deviations across batches and days. Thus, all data was analyzed batch wise

and paired. DOPC was always used as a reference and was therefore included in all liposome preparations as a control.

2.4.3.2 Activity of Dpm1 in different liposomes

I found Dpm1 activity to be dependent on the membrane environment. The product amount normalized to reconstituted protein can be seen in Figure 30.

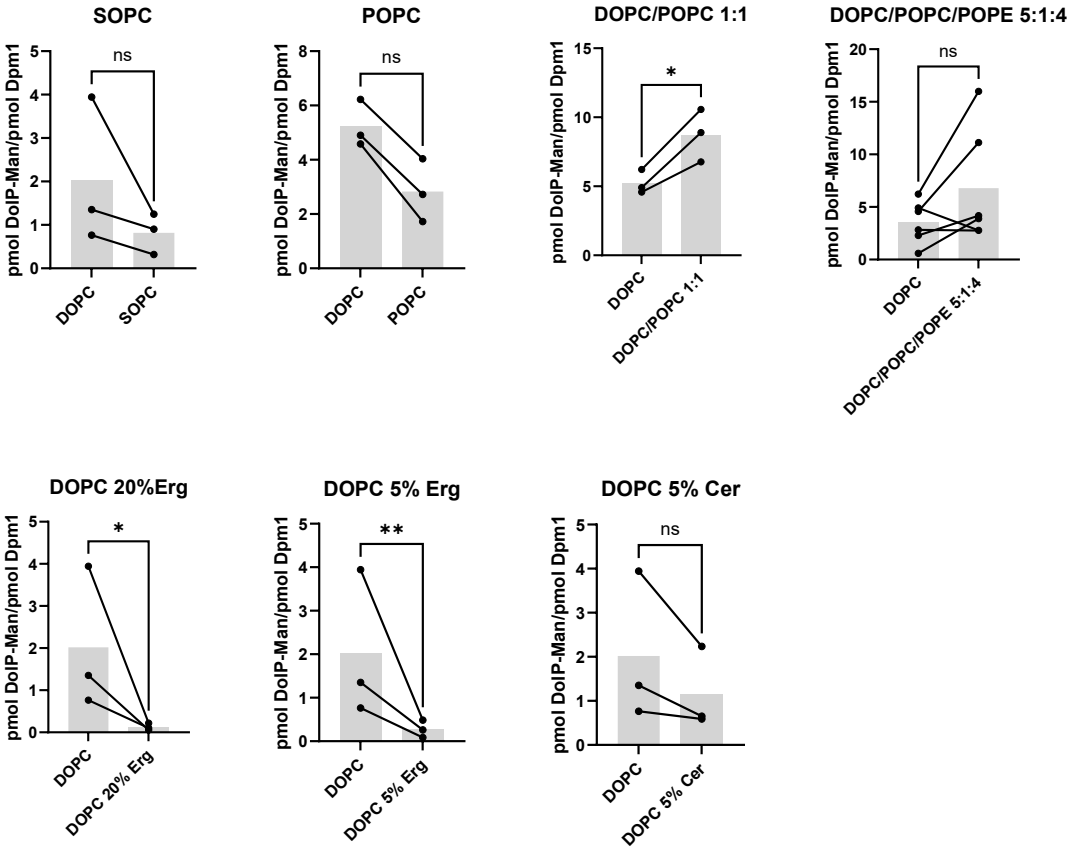


Figure 30 **Activity of Dpm1 in different liposomes**
Protein activity is presented as pmol DolP-Man formed within 1 min normalized to protein amount (pmol DolP-Man/pmol Dpm1). Protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes. A paired ratio t-test was used (GraphPad Prism, 9.5.1)

I first tested liposomes composed of either DOPC, SOPC or POPC to study the effect of saturation on Dpm1 activity. Saturation of the fatty acids leads to a tighter lipid packing [134]. When the amount of unsaturated fatty acid side chains was reduced to 50 % by the use of SOPC (18:0/18:1) instead of DOPC (18:1/18:1), the DolP-Man product amount was reduced by about 50 %. Also, when using POPC (16:0/18:1) a reduction in activity was seen, similar as observed for SOPC. However, the reduction was not significant, most likely to the low sample number. Still, these results indicate

that unsaturation, which leads to a more fluid membrane phase, is beneficial for Dpm1 activity. However, when using a mixture of DOPC/POPC 1:1 (resulting in an overall unsaturation/saturation ratio of 3:1 in the FAs) enzyme activity was greatly enhanced compared to DOPC controls. Thus, suggesting that there is an optimal concentration of fatty acid saturation to promote Dpm1 activity.

Small changes in the fatty acid chain length did not have an impact on Dpm1 activity, as the activity in POPC (16:0/18:1) liposomes did not significantly differ from the one in SOPC (18:0/18:1) liposomes. Whether this is due to the rather insensitivity of Dpm1 to fatty acid chain length or whether reduction from 18 to 16 carbon atoms in the fatty acid chains was too subtle, has to be studied in further experiments, e.g. by using significantly shorter or longer fatty acid-containing PC species.

Addition of PE, as known from literature [190, 193, 208], significantly increased Dpm1 activity. Liposomes composed of DOPC/POPC/POPE (5:1:4) showed highest activity from all liposome compositions tested.

Strikingly, no activity was seen in the presence of ergosterol. Liposomes with either 20 % or 5 % of ergosterol were tested, but none of the preparations of showed activity above background. This is interesting, as the ER is the place of ergosterol biosynthesis and contains 10-20 % ergosterol of total phospholipids in yeast [132, 150]. However, sterols are known for domain formation in the plasma membrane and raft like microdomains were also suggested to exist in the ER [211]. Thus, this membrane segregation may not support Dpm1 activity.

I also analyzed the effect of 5 % ceramide on Dpm1 activity. Like ergosterol, ceramide can promote domain formation and is synthesized at the ER [212]. As seen in Figure 30, Dpm1 was also hardly active in the presence of ceramide. Like for Erg, also here the raft formation of Cer may cause to the inhibition of Dpm1 activity.

2.4.3.3 Comparison +/- float up

In this first set of experiments (2.4.3.2), liposomes were used without further purification after detergent removal. In the absence of lipids, Dpm1 precipitated upon detergent removal by the use of Bio-Beads. Samples were shortly centrifuged to remove Bio-Beads and no protein was found in solution anymore after the second incubation of Bio-Beads (data not shown). Therefore, I concluded this was also true for

non-reconstituted protein and that all detected protein was reconstituted. To verify this assumption, in a second set I used liposome preparations after flotation on a sucrose gradient. As only liposome associated protein floats to the top fraction, this step allowed me to test whether Dpm1 reconstitution was dependent on the lipid environment. I chose three lipid compositions of the liposome panel tested to further investigate whether reconstitution efficiency is affected by the lipid composition.

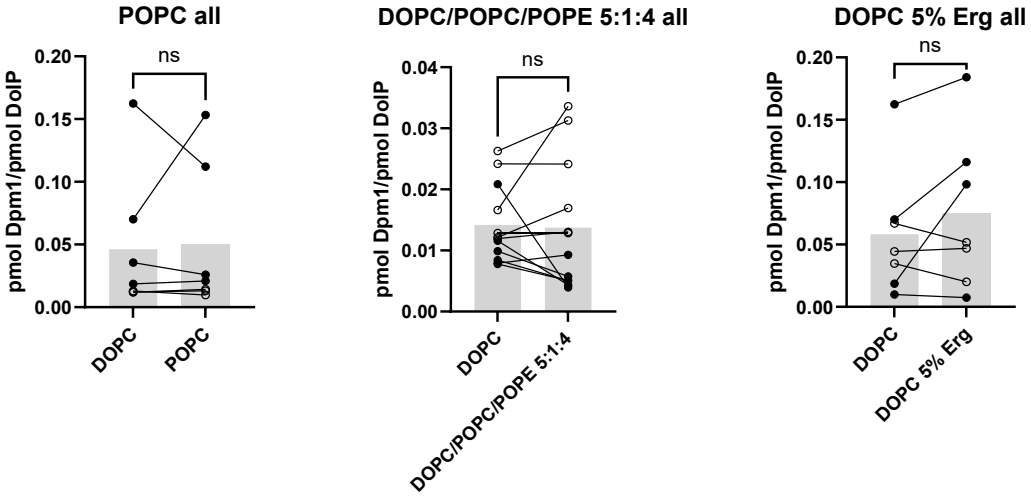


Figure 31 **Comparison of protein to lipid ratio before and after flotation**
Results are presented as pmol Dpm1/pmol DolP. Protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. DolP concentration was indirectly quantified by MS-measurement of PC content. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes. A paired ratio t-test was used (GraphPad Prism, 9.5.1) **Sample labelling:** Open circles…without flotation, filled circles…with flotation

As shown in Figure 31, Dpm1/DolP ratio with and without flotation was comparable for all lipid compositions tested. The enzyme/lipid ratios remained constant, even though absolute values of recovered proteoliposomes varied with the lipid compositions. This confirmed that after detergent removal only reconstituted protein remained in solution. Therefore, also liposomes before flotation can be used to assess the efficiency of Dpm1 reconstitution into liposomes.

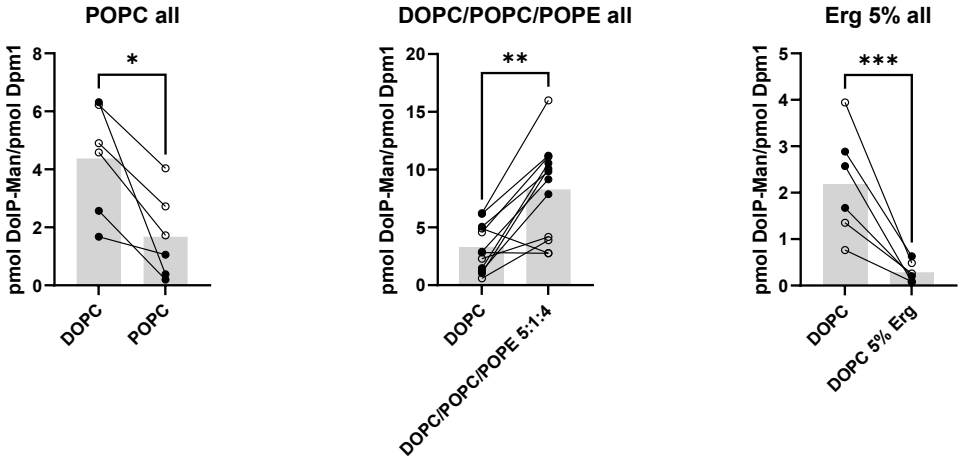
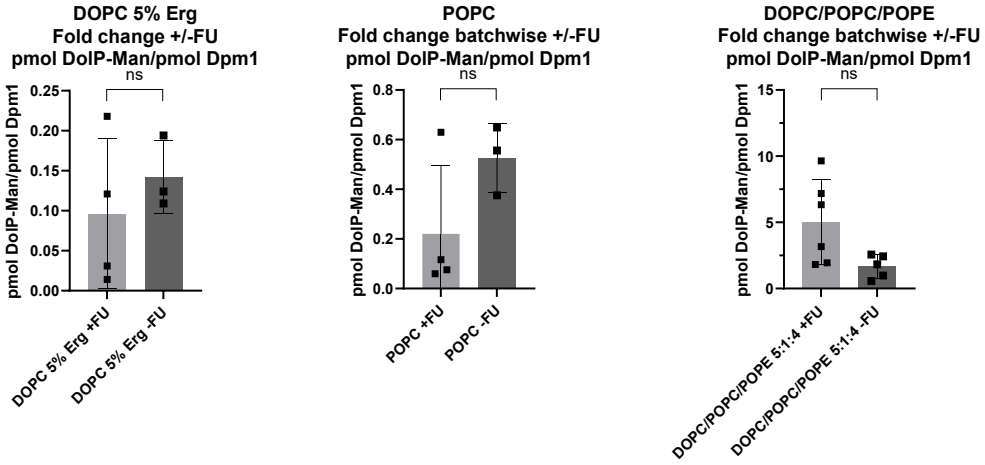


Figure 32 **Comparison of Dpm1 activity +/- floatation**
Enzymatic activity does only depend on lipid composition and does not differ significantly with (open circles) or without flotation (full circles). Protein activity is presented as pmol DolP-Man formed within 1 min and was normalized to protein amount. Protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes. A paired ratio t-test was used (GraphPad Prism, 9.5.1)

Also, the normalized Dpm1 activity (pmol DolP-Man/pmol Dpm1) did not change by the additional flotation step. Thus, the additional centrifugation step after reconstitution did not lead to significant loss of Dpm1 activity.

2.4.3.4 Fold change

Absolute values varied from batch to batch and between liposomes compositions. For better comparison of the liposomes with and without flotation, I also calculated the fold change of enzyme activities in different liposomes, batch wise normalized to DOPC. As shown in Figure 33, the fold change in activity (pmol DolP-Man/ pmol Dpm1), when normalized to DOPC liposomes, did not significantly differ before and after flotation. Thus, showing again that the flotation had no effect on the normalized activity.



.

Figure 33 **Fold change of Dpm1 activity +/- flotation**
Activities of liposomes (pmol DolP-Man/pmol Dpm1) with different lipid compositions were batch wise normalized to DOPC liposomes. The fold change with and without flotation was compared using a t-test and no significant difference was observed (GraphPad Prism, 9.5.1)

In conclusion, the results show, that Dpm1 is indeed affected by the lipid composition of the membrane environment (Figure 34). Activity was found to be increased in the presence of PE and with an unsaturation/saturation ratio of 3:1, whereas a 1:1 ratio decreased (SOPC, POPC) decreased Dpm1 activity compared to DOPC liposomes. Ergosterol inhibited Dpm1 activity under the conditions tested. Also, ceramide reduced enzyme activity.

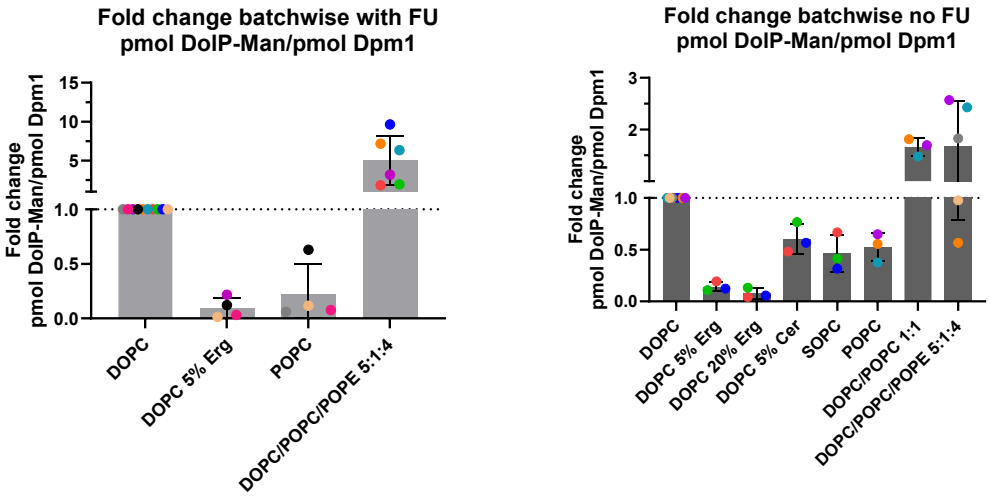


Figure 34 **Fold change of all tested liposome compositions**
Fold change of Dpm1 activity normalized to protein amount in different liposomes. Liposomes were measured after flotation (left) or before (right). Colors correspond to same batches. n ≥ 3

But not only the lipid composition of the membrane can regulate Dpm1 activity. Also, other proteins can interact with the protein and modulate its activity. Thus, the

liposomal assay was extended to include other proteins by co-reconstitution to test their effect on Dpm1 activity.

2.5 Interaction of Dpm1 with other proteins

Dpm1 is part of the cellular glycosylation machinery, which consists of various glycosyl transferases. As the attachment of sugars is a sequential process, all enzymes have to be tightly regulated and orchestrated for efficient glycosylation and defects in any of the involved enzymes lead to severe hypoglycosylation. For my studies, I chose two proteins of interest to further study their interaction with Dpm1. Firstly, I included the yeast protein Yil102c-A in my assay, that was found to have an essential Dpm2 functionality in yeast [122]. Secondly, I studied the interplay of Dpm1 with Pmt4, as a first step toward understanding the distribution of DolP-Man and the interplay of the different glycosylation routes.

2.5.1 Interaction of Dpm1 with Dpm2

In human, the catalytically active Dpm1 protein requires interaction with the smaller membrane proteins Dpm2 and Dpm3 for proper function. Disruption of this so-called DPMS complex leads to reduced enzyme activity. For a long time, it was believed, that in yeast the C-terminally anchored Dpm1 was the only protein required for mannosylation of DolP. However, in 2020, Piłsyk et al.[122] identified the yeast protein Yil102c-A as a functional homologue of human Dpm2. In their study, deletion of Yil102c-A in yeast was lethal but could be rescued by the Dpm2 gene from *Trichoderma reesei*, thus suggesting some essential function of Yil102c-A (Dpm2) in the yeast glycosylation pathway.

Therefore, I decided to include Yil102c-A (Dpm2) into my studies to further investigate the role of this protein. Using the *in vitro* reconstitution system, I could measure Dpm1 activity already in the absence of Dpm2. No additional proteins were needed for enzyme activity, in contrast to the *in vivo* findings of the study by Piłsyk et al. where the enzyme was found to be essential. Nonetheless, the presence of Dpm2 might increase Dpm1 activity by stabilization of the enzyme or by helping to position the DolP substrate. In addition, *in vivo* Dpm2 might assist in the mannosylation process by interacting with other glycosyl transferases and handing over DolP-Man substrate.

Thus, I was interested to see, whether the addition of Dpm2 enhances Dpm1 activity in the reconstitution assay.

2.5.1.1 Co-reconstitution of Dpm1 and Dpm2

In a first step, I wanted to see whether additional Dpm2 in the liposomes would lead to increased DolP-Man formation. Therefore, I recombinantly expressed and purified Dpm2 to include it in the *in vitro* reconstitution system. Dpm2 was successfully co-reconstituted by simple co-addition of the protein during protein reconstitution step. Reconstitution was verified by the presence of Dpm1 and Dpm2 in the liposomal top fraction after sucrose gradient flotation (Figure 35).

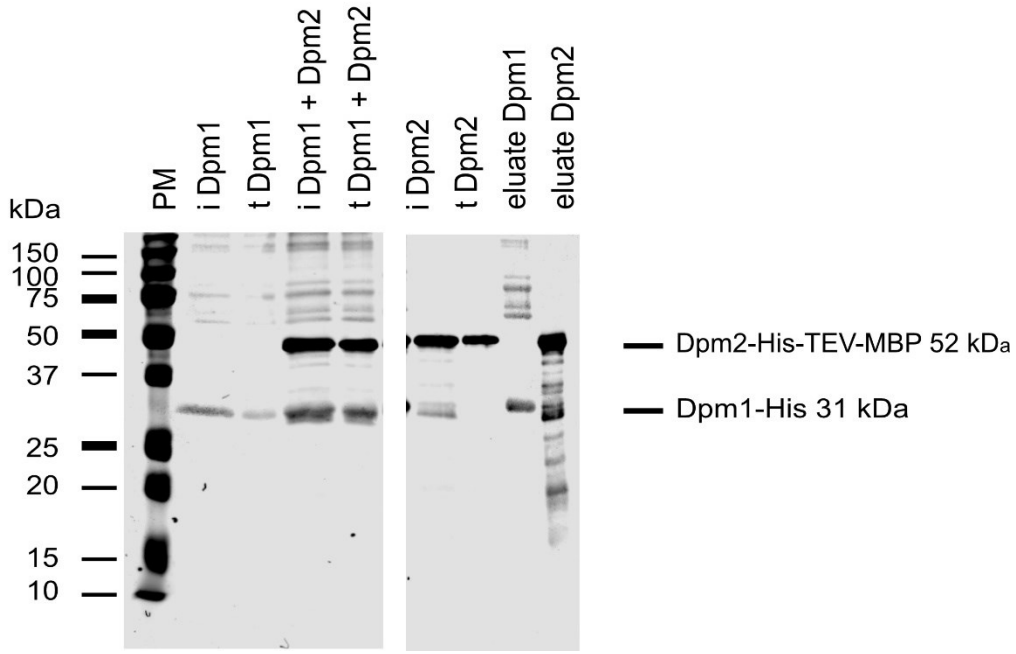


Figure 35 **Co-reconstitution of Dpm1 and Dpm2**
Dpm1 and Dpm2 was reconstituted individually and together in DOPC liposomes. Western blot of input (i) and floated liposomes (top). Eluate concentrations on the blot equal theoretical concentrations in liposomes of the input. The blot was developed using anti-His antibody.

Reconstitution efficiency of Dpm1 was not increased in the presence of Dpm2, as seen when using anti-Dpm1 antibody (blots not shown). The increase of Dpm1 band intensities of Dpm1+2 liposomes in Figure 35 was due to His-tagged impurities in the Dpm2 preparation that ran at the same height as Dpm1 (see lane eluate Dpm2 in Figure 35).

2.5.1.2 Tag positioning

In co-IP studies Piłsyk et al. [122] found, that C-terminally myc tagged Dpm2 protein interacted with Dpm1, whereas this interaction was not observed with N-terminally tagged protein. Thus, also the potential effect of Dpm2 on Dpm1 activity might be affected by C- or N-terminal tags on Dpm1. I therefore tested different constructs, that resulted in either tagged N- or C-terminally tagged Dpm1. Expression and purification of His-tagged Dpm2 protein, especially when tagged C-terminally, showed to be difficult due to low expression rate. Therefore, we designed His-Tev-MBP constructs that were expressed in *E. coli* in good quantities. The Tev cleavage site was included to enable reduction of the size of the tagged fusion protein, in case the bulky MBP hindered protein-protein interaction. Protease-mediated release of the MBP in the purified protein, however, was not successful. This was most likely due to inactivity of the Tev-protease in Sarkosyl. Because Dpm2 showed an effect on Dpm1 activity even with the MBP tag attached, no further efforts were made to remove the MBP.

When analyzing Dpm1 activity in the presence of C-terminally tagged Dpm2-His-Tev-MBP, a strong increase in activity was seen (Figure 36). In contrast, N-terminally tagged protein failed to stimulate Dpm1 activity.

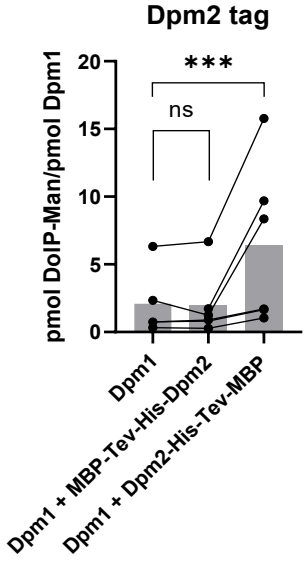


Figure 36 **Effect of tag positioning in Dpm2**
Either C-terminally or N-terminally tagged Dpm2 was co-reconstituted with Dpm1 in DOPC liposomes and the activity was compared to Dpm1 only liposomes. Dpm1 activity is presented as pmol DolP-Man formed within 1 min and was normalized to Dpm1 amount. The protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes. A paired ratio t-test was used (GraphPad Prism, 9.5.1)

These findings are in line with Piłsyk et al. [122], who showed that a N-terminal tag interfered with Dpm1-Dpm2 interaction. However, the reconstitution efficiency and purity of the N-terminal Dpm2 was lower than that of the C-terminally tagged protein. Moreover, N-terminally tagged Dpm2 showed a degradation band in the western blot analysis. Nonetheless, the activation of Dpm1 by C-terminally tagged Dpm2 confirmed its relevance in yeast by activating DolP-Man synthesis.

2.5.1.3 Dpm2 and different lipids

Not only enzymes can be influenced by the lipid environment, but also protein-protein interaction can be mediated by lipids. Thus, I wanted to see if Dpm1-Dpm2 interaction was affected by the membrane compositions. Therefore, I co-reconstituted Dpm1 and Dpm2 into liposomes with different lipid compositions, and again measured the Dpm1 activity using the *in vitro* assay.

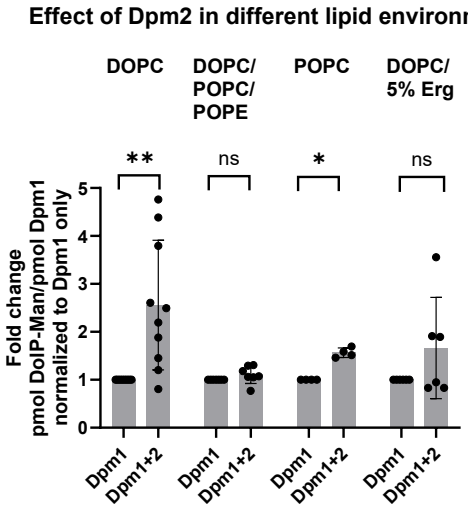


Figure 37 **Effect of Dpm2 on Dpm1 activity in different lipid environments**
Data for Dpm1+2 liposomes were normalized to the corresponding Dpm1 only liposomes to compare the increase of activity caused by Dpm2 in different lipid environments. Results d are presented as fold change of pmol DolP-Man/pmol Dpm1. Protein activity is calculated as pmol DolP-Man formed within 1 min. amount and was normalized to Dpm1 amount. Protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes. Two outliers were removed (in DOPC/POPC/POPE and POPC) after performing an outlier test and a paired t-test was performed to find significant differences (GraphPad Prism, 9.5.1). n=5-9

In all tested lipid environments except DOPC/POPC/POPE, Dpm1 was more active in the presence of Dpm2, as seen in Figure 37, although statistically significant increase was only seen in DOPC and POPC liposomes. The strongest effect was observed for DOPC liposomes, where Dpm1 activity was increased by up to 250 %. A smaller increase to approx. 150 % was observed in POPC and DOPC/Erg liposomes. In the

latter lipid composition, however, overall activity remained only slightly above background and therefore exhibited a large variance and the change in activity was not statistically significant. No increase in activity by the addition of Dpm2 was observed in DOPC/POPC/POPE liposomes. With this lipid composition, Dpm1 activity was already high in the absence of Dpm2, as discussed in section 2.4.3.2. Addition of Dpm2 failed to further significantly increase the activity of Dpm1. Thus, these data suggest that the maximal rate of enzyme reaction (v_{max}) can be either reached by changing the lipid environment or by the addition of Dpm2. In conclusion, the data shows that the lipid environment affects not only enzymatic speed of Dpm1 but also Dpm1-Dpm2 interaction and might play a role in regulation of Dpm1 activity.

In a next step, I tested whether the enhancing effects of lipids and Dpm2 were synergistic or independent. Therefore, I checked if the increase in activity due to the lipids was comparable in the presence or absence of Dpm2. Figure 38 shows the Dpm1 activity in the presence and absence of Dpm2. Data was normalized to the corresponding DOPC liposomes with the same protein composition.

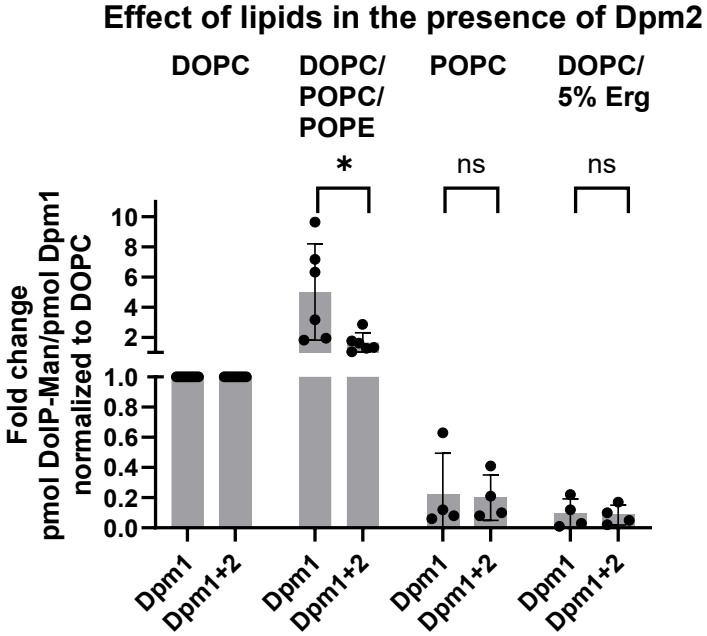


Figure 38 **Effect of lipids on Dpm1 activity in the absence and presence of Dpm2**
Data was normalized to the corresponding DOPC liposomes (either Dpm1 or Dpm1+2). Protein activity is presented as pmol DolP-Man formed within 1 min and was normalized to Dpm1 amount. Protein concentration of liposomes was calculated by western blot quantification using either anti-His or anti-Dpm1 antibody. Statistical analysis was performed for each lipid composition in the absence and presence of the Dpm2, by performing a paired t-test (GraphPad Prism, 9.5.1) n=4-6

In POPC and DOPC/Erg liposomes, no difference in fold change between liposomes with and without Dpm2 was seen when the activity was normalized to the respective DOPC liposomes. The increase caused by lipid compositions remained the same. In liposomes composed of DOPC/POPC/POPE Dpm1 a decrease in fold change was observed, due to the lowered difference between DOPC Dpm1+2 and DOPC/POPC/POPE Dpm1+2 samples, even though activity was still slightly increased compared to DOPC liposomes. However, the increase was less pronounced in the presence of Dpm2, suggesting that Dpm1 was already highly activated and could not be further stimulated by the lipid environment.

In conclusion, I found that Dpm1 activity can be altered by both the lipid environment and the additional Dpm2 protein. Both effects are independent, as seen in Figure 37 for Dpm2 and Figure 38 for lipids. In DOPC/POPC/POPE high Dpm1 activity was seen even in the absence of Dpm2 and the addition of Dpm2 did not lead to the same fold change as in the other lipid compositions tested. Thus, suggesting that either v_{max} was already reached by the fluid lipid environment and could not be further increased by the addition of Dpm2 or there was no Dpm1-Dpm2 interaction.

1.1.1.1 Dpm2 mutants and CDG

Human and yeast Dpm2 (Yil102c-A) share a high degree of structural similarity, as both are relatively small proteins consisting essentially only of two transmembrane helices. Up to date, six CDG-patients harboring a genetic defect in the Dpm2 protein are described [113-115] (see also 1.3.2). Strikingly, 3 of these patients show a Y23C mutation resulting in severe symptoms caused by hypoglycosylation [113]. As this mutated tyrosine residue is also conserved in yeast, it might play an important role in mannosylation. Therefore, I decided also to test also the effect of this mutant (Y13C in yeast Dpm2) in the activity assay when compared to wild type Dpm2. In addition, I tested with a W17A construct another mutation of this membrane helix.

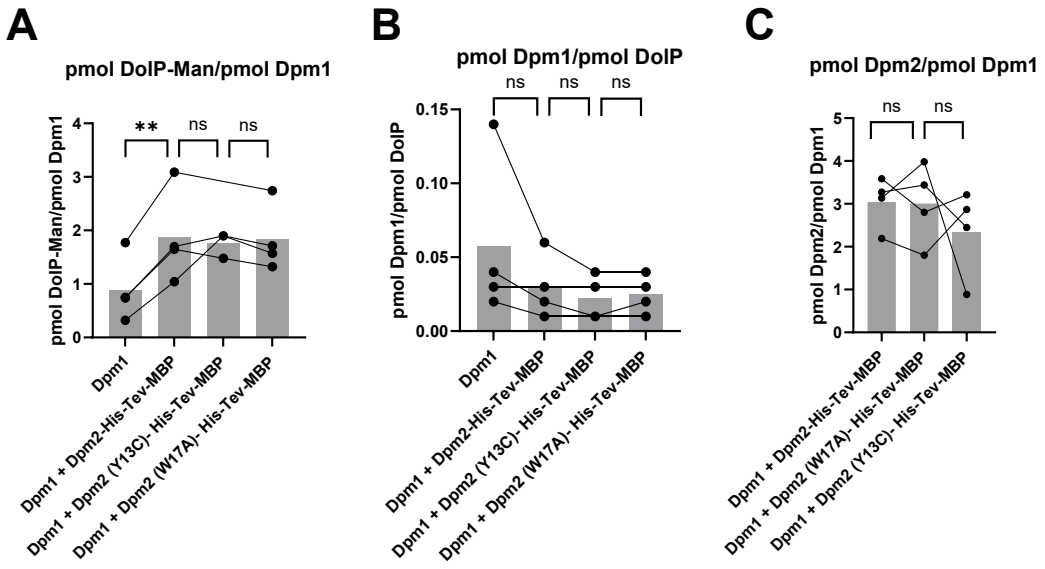


Figure 39 **Effect of Dpm2 mutants on Dpm1 activity**
Dpm1 and Dpm2 variants were co-reconstituted into DOPC liposomes. Activity of Dpm1 was measured as mannosyl transfer within 1 min (pmol DolP-Man. Protein concentrations were calculated by western blot quantification of blots that were developed with either anti-His or anti-Dpm1 antibody. DolP concentration was calculated after mass spectrometric analysis of PC lipids. n=3-4 Statistical analysis was performed using a paired t-test (GraphPad Prism, 9.5.1) **A** Activity of Dpm1 in the presence and absence of Dpm2, shown as pmol DolP-Man/pmol Dpm1; **B** Dpm1 reconstitution efficiency in liposomes, presented as pmol Dpm1/pmol DolP; **C** Dpm2/Dpm1 ratio in liposomes as pmol Dpm2/pmol Dpm1

As shown in Figure 39, the point mutations in Dpm2 did not lead to a reduction in Dpm1 activity compared to wild type Dpm2. Both mutants stimulated Dpm1 activity as more product was formed compared to liposomes lacking Dpm2. This effect was comparable to wtDpm2 protein. The Dpm1/Dpm2 ratio was found to be similar for all Dpm2 constructs, thus excluding differences due to protein amount. Reconstitution efficiency of Dpm1 (pmol Dpm1/pmol DolP) after flotation was slightly, but not statistically significant, elevated in the absence of Dpm2.

In conclusion, these data show that the CDG-like mutations of yeast Dpm2 did not impair Dpm1-Dpm2 interaction *in vitro* and did not cause a reduction in yeast Dpm1 activity. Thus, no CDG-like effect could be seen in the in *vitro assay.*

2.5.2 Reconstitution of the O-mannosylation pathway by the addition of Pmt4

DolP-Man, formed by DPMS, serves as a substrate for all mannosylation reactions within the ER. How the shuttling of DolP-Man into the different glycosylation pathways is regulated is yet not understood. An *in vitro* reconstitution system of the different glycosylation pathways would allow the study of the glycosyltransferase under defined conditions, to gain better understanding of the interplay between the different glycosylation routes. As a first step, I aimed to reconstitute the O-mannosylation pathway, by co-reconstituting Pmt4 and Dpm1.

2.5.2.1 Co-reconstitution of Dpm1 and Pmt4

The reconstitution protocol, developed for the reconstitution of Dpm1, proved to be also suitable for co-reconstitution of different proteins. Pmt4 was successfully co-reconstituted with Dpm1 by adding both proteins during proteoliposome preparation. Purified His-tagged *Sc*Pmt4 and *Ct*Pmt4 were kindly provided by Melanie McDowell and Antonella Chiapparino, as detergent solubilized protein purifications in 0.01% LMNG. The reconstitution was verified by flotation of liposomes reconstituted with Pmt4 and Dpm1 in a sucrose gradient. The analysis of floated liposomes by western blotting showed successful co-reconstitution of Dpm1 and Pmt4 (Figure 40).

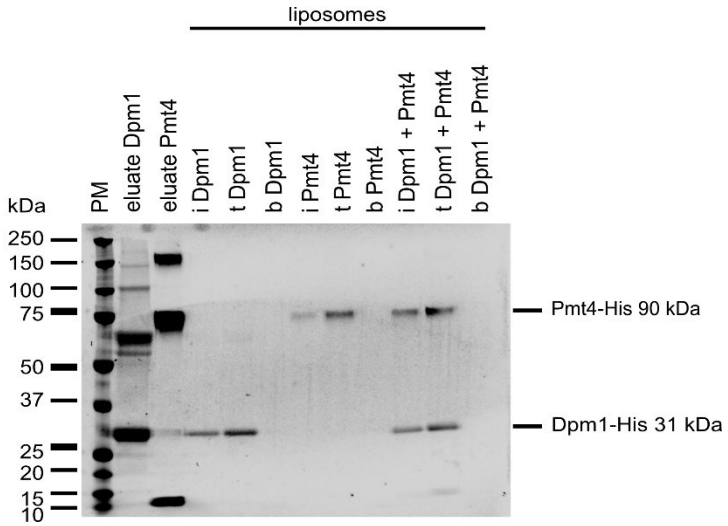


Figure 40 **Co-reconstitution of Dpm1 and Pmt4**
Successful reconstitution of CtPmt4 in the presence and absence of Dpm1. Dpm1 and CtPmt4 were co-reconstituted using the established reconstitution protocol. Liposomes were floated on a sucrose gradient and the presence of protein. Different fractions were checked for the presence of protein by western blotting, using anti-His antibody. i…input, t…top fraction, b…bottom fraction

2.5.2.2 Successful reconstitution of O-manosylation reaction

To analyze Pmt4 activity, I measured the transfer of mannose from DolP-Man onto a peptide substrate. An adapted protocol of Bausewein et.al [21] was used. Proteoliposomes were incubated with GDP-Man and a biotinylated α-Dystroclycan peptide (pαDG). The radioactivity, recovered with the peptide after biotin pull-down, was used to calculate the extent of O-mannosylation. (Figure 41 A). As seen in Figure 41 B, I could successfully monitor the transfer of mannose from GDP-Man, via DolP-Man onto pαDG. Radioactivity was only bound to the peptide in the presence of DolP, active Dpm1, active Pmt4 and the pαDG.

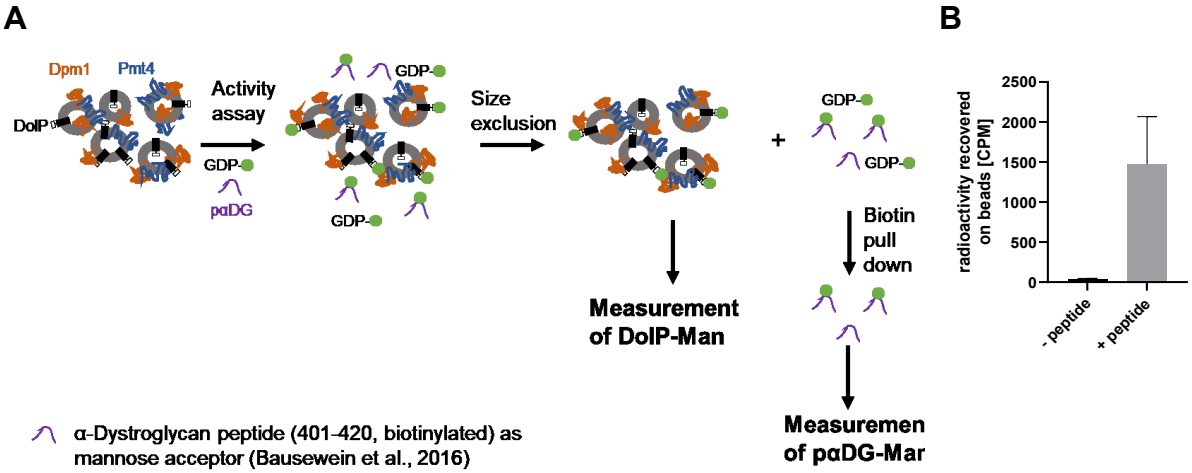


Figure 41 **Reconstitution of O-mannosylation reaction**
A Schematic workflow of the Pmt4 activity assay. Proteoliposomes with reconstituted Dpm1 and Pmt4 were incubated with tritiated GDP-Man and a biotinylated α-dystroglycan peptide (pαDG) to initiate mannosyl transfer. After the reaction, excess of GDP-Man and pαDG-Man was separated from liposomes using size exclusion columns. The enzyme free GDP-Man fraction was incubated with neutravidin beads for biotin pull down of the mannosylated peptide and the radioactivity found on the beads was used to quantify Pmt4 activity. Radioactivity found in the liposomal fraction corresponded to DolP-Man, formed by Dpm1. **B** Pmt4 activity is only seen in the presence of acceptor peptide pαDG

Surprisingly, the amount of DolP-Man measured was comparable between samples with and without pαDG, despite the fact that in the presence of pαDG DolP-Man gets partly consumed by the transfer of the mannose to the peptide. This may indicate that under the chosen assay conditions, the rate of DolP-Man formation was much faster compared to the mannosylation of pαDG. Whereas Dpm1 activity was already seen within minutes, at least 15 min were required to find mannosylated pαDG (see also Figure 43). As the catalytic domains of both proteins are facing the outside of the liposomes, theoretically, the released DolP (if not flipped during mannose transfer) could be reused by Dpm1, thus allowing more transferred mannose molecules than DolP present. However, in none of the experiments that I performed the amount of

radioactivity found on the peptides exceeded the amount of DolP-Man found in the absence of the peptide.

2.5.2.3 Activity of Pmt4 in different lipid environments

Next, I tested whether the lipid composition has an effect on Pmt4 activity and its interaction with Dpm1 or the DolP-Man substrate. Thus, I co-reconstituted Dpm1 and Pmt4 into liposomes with different lipid compositions. Different DOPC/DOPE ratios were tested, as for Dpm1 an increase in activity was seen in the presence of PE lipids. To ensure comparability between different lipid compositions, reconstitution efficiency and lipid recovery was analyzed.

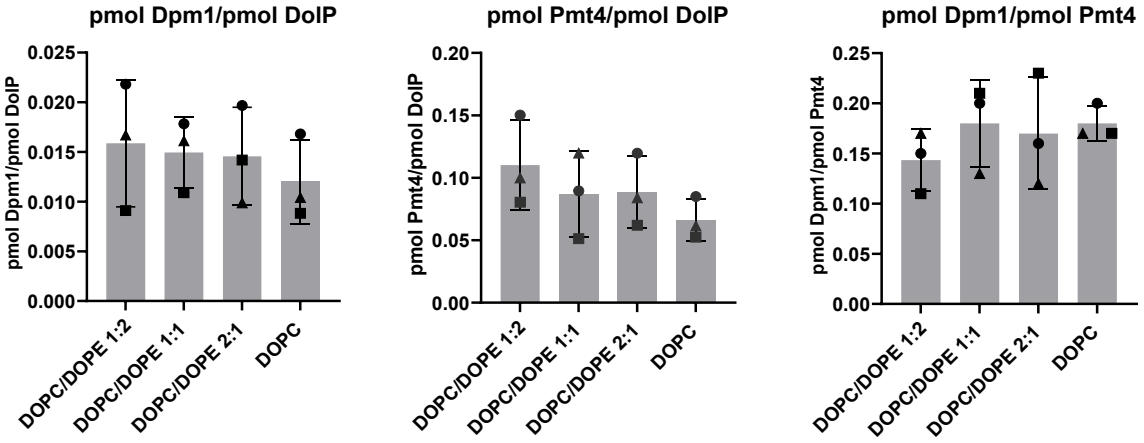


Figure 42 **Efficiency of Pmt4 and Dpm1 co-reconstitution**
Results are presented as pmol protein/pmol DolP. Protein amount was calculated using western blot quantification. Blots were developed against anti-Dpm1 for Dpm1 quantification and anti-His for Pmt4 quantification. DolP concentration was indirectly quantified by MS-measurement of PC content of the liposomes. Symbols represent liposome batches. Statistical analysis was performed for each lipid composition in comparison to DOPC liposomes, showing no significant differences between lipid compositions. A paired ratio t-test was used (GraphPad Prism, 9.5.1)

I found reconstitution and protein content to be comparable for all lipid compositions, as seen in Figure 42. In addition, the Dpm1-Pmt4 ration was constant under the different conditions, thus ensuring comparable reaction conditions with respect to protein stoichiometries.

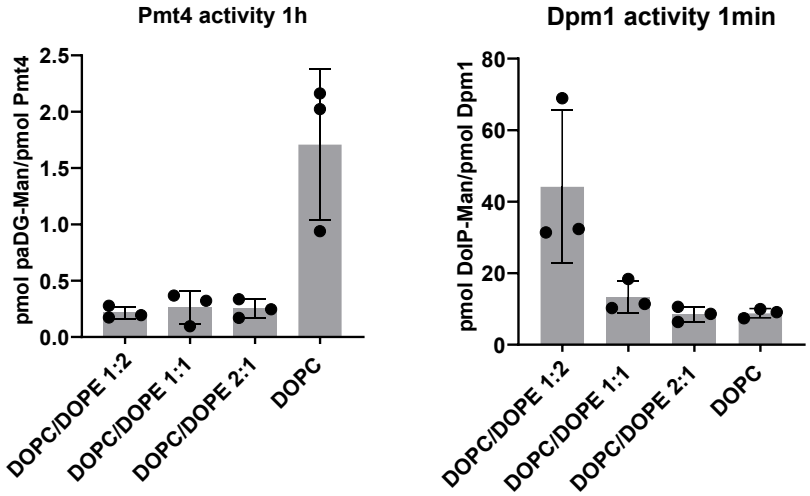


Figure 43 **Pmt4 and Dpm1 activity in different lipid environments**
Pmt4 and Dpm1 show inversed activity response to the addition of PE lipids. Dpm1 and Pmt4 were co-reconstituted into liposomes with different lipid compositions. Mannosyl transfer by Dpm1 and Pmt4 was started by addition of GDP-Man and acceptor peptide and quantified by radioactivity found bound to the peptide after 1h. Dpm1 activity was measured in the absence of pαDG and after 1 min incubation with GDP-Man, n =3

Liposomes were tested for Dpm1 and Pmt4 activity and results are shown in Figure 43. Also, in the presence of Pmt4, Dpm1 activity was increased by the addition of PE as seen before. Increasing amounts of DolP-Man were seen with an increase in PE content. Interestingly, Pmt4 showed opposite activity in the lipid environments tested. Highest Pmt4 activity was measured in DOPC liposomes, whereas the addition of PE lead to a decrease in pαDG-Man. This is especially striking, as due to the higher Dpm1 activity in DOPC/DOPE 1:2 liposomes also more DolP-Man substrate was available for Pmt4.

Thus, these findings demonstrate that both enzymes can be modified by their lipid environment. The fact that the enzymatic activity of Dpm1 and Pmt4 was not stimulated by the same lipids, might have an interesting regulating implication *in vivo*.

These first experiments were performed with *Ct*Pmt4, thus enzymes from two different species are present in the liposomes. As *Chaetomium thermopihilium* prefers higher temperatures of about 50-55 °C to grow [213], also the natural lipidome might be adapted to these conditions *in vivo*. Addition of PE to membranes increases fluidity and might not be optimal with respect to stabilization of the dimeric *Ct*Pmt4. Even though, the assay was carried out at 25 °C, the lipid preference might be due to the different species preferences. Experiments should be repeated with the yeast protein, to see whether this preference is also found in *S. cerevisiae.* The investigation of Dpm1,

Dpm2 and Pmt4 from the same species will help to clarify whether the lipids play a role in modulation of the interaction of glycosylation enzymes.

2.5.2.4 Activity of different Pmt4 from different species

As the experiments presented above were performed with Pmt4 from C. *thermopihilium* (*Ct*Pmt4) whereas the Dpm1 was from *S. cerevisiae,* I was interested to also test the activity of Pmt4 from *S. cerevisiae* (*Sc*Pmt4). Therefore, liposomes containing Dpm1 and *Sc*Pmt4 were analyzed and compared to liposomes with Dpm1 and *Ct*Pmt4. Reconstitution efficiency of for Pmt4 of both species was comparable, as seen in Figure 44 (pmol Pmt4/pmol DolP). However, more Dpm1 was reconstituted compared to Pmt4.

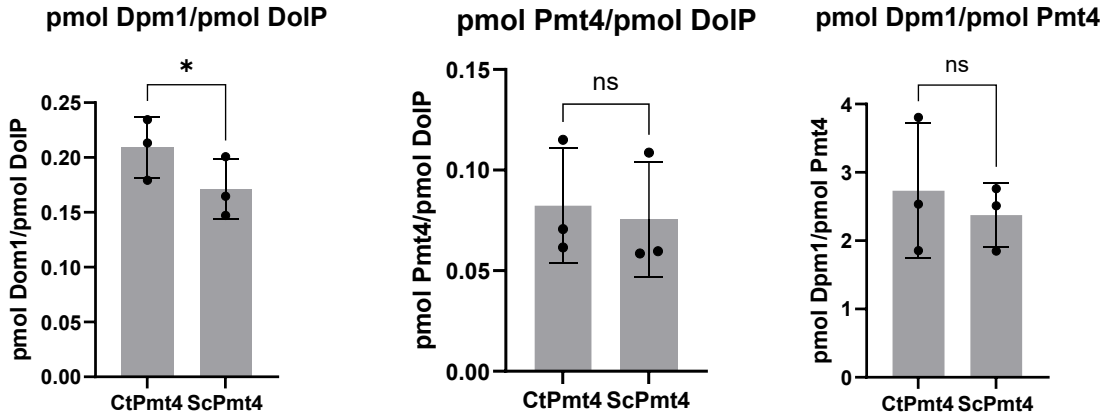


Figure 44 **Protein reconstitution in Pmt4 liposomes**
Dpm1 was co-reconstituted with either *Ct*Pmt4 or *Sc*Pmt4 into DOPC liposomes. The protein concentration was quantified using western blot analysis (anti-Dpm1 for Dpm1 and anti-His for Pmt4). The DolP concentration was calculated from PC concentration, that was quantified using mass spectrometry. Reconstitution was comparable for both Pmt4 species and Dpm1. About 2x more Dpm1 was reconstituted compared to Pmt4, as seen by the ratio of Dpm1 and Pmt4. Statistical analysis was done using a ratio-paired t-test (GraphPad Prism, 9.5.1).

To compare the efficiency of O-mannosylation, both Pmt4 enzymes analyzed. As usually, reactions were performed at 25°C. In addition, *Ct*Pmt4 liposomes were measured at 30°C, in order to see if the mannosylation reaction could be boosted by increased temperatures to match the one of *Sc*Pmt4.

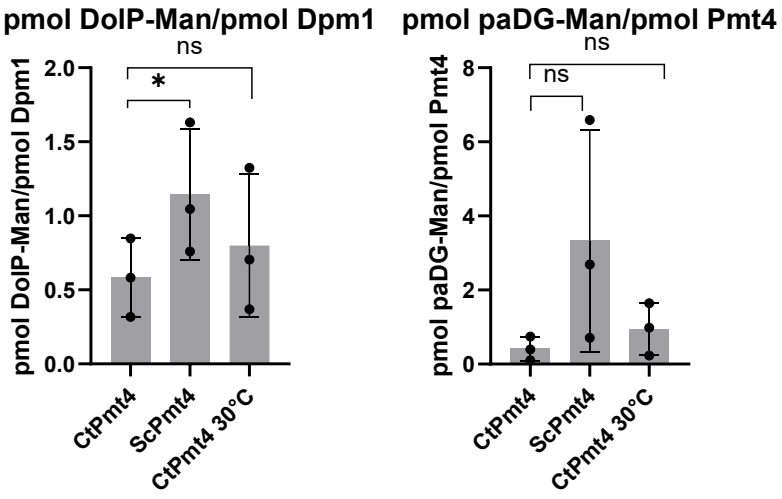


Figure 45 **Mannosylation of DolP and peptide in Dpm1-Pmt4 liposomes normalized to protein**
The activity of co-reconstituted Dpm1 and Pmt4 (either CtPmt4 or ScPmt4) was analyzed using radiolabeled GDP-Man. The amount of product was calculated by the radioactivity recovered in the liposomal fraction (DolP-Man) or the was recovered after biotin pull-down of the biotinylated pαDG (pαDG-Man). Activity was normalized to protein concentration, quantified using western blot analysis with anti-Dpm1 (Dpm1) or anti-His antibody (Pmt4). Statistical analysis was performed using a ratio paired t-test (GraphPad Prism, 9.5.1)

Both enzymes were able to use the DolP-Man, that was formed by yeast Dpm1 as a substrate. As shown in Figure 45, the activity of *Sc*Pmt4 was higher compared to *Ct*Pmt4. Also, Dpm1 activity was significantly increased in the presence of *Sc*Pmt4. The increase of temperature to 30°C during the reaction, also slightly increased Pmt4 activity, but *Ct*Pmt4 was still less active compared to *Sc*Pmt4.

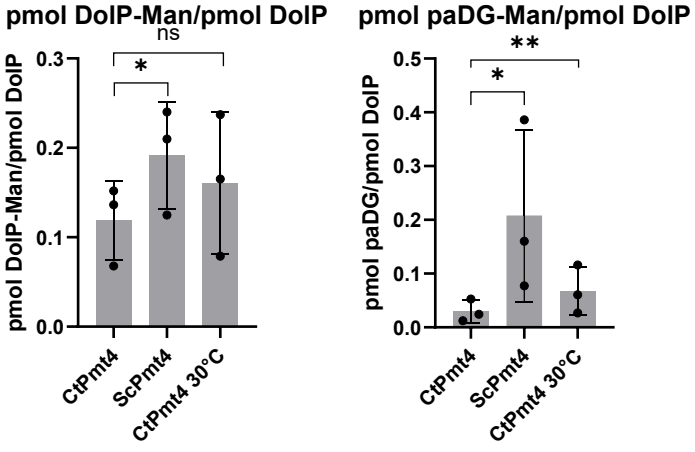


Figure 46 **Mannosylation of DolP and peptide in Dpm1-Pmt4 liposomes normalized to lipid**
The activity of co-reconstituted Dpm1 and Pmt4 (either *Ct*Pmt4 or *Sc*Pmt4) was analyzed using radiolabeled GDP-Man. The amount of product was calculated by the radioactivity recovered in the liposomal fraction (DolP-Man) or the was recovered after biotin pull-down of the biotinylated pαDG (pαDG-Man). Activity was normalized to DolP concentration, calculated from PC content quantified using MS. Statistical analysis was performed using a ratio paired t-test (GraphPad Prism, 9.5.1)

Differences were even more significant, when normalizing to liposomes by DolP concentration (Figure 46).

In conclusion, these results indicate that *Sc*Pmt4 shows higher activity at RT compared to *Ct*Pmt4, which might be due to temperature preferences of the respective organism. However, also Dpm1 activity was increased in the presence of *Sc*Pmt4. Whether this is due to increased turnover of DolP-Man or due to stimulation of yeast Dpm1 by interaction with *Sc*Pmt4 has to be studied in more detail in further experiments.

3 Discussion

Dpm1 is a central enzyme in glycosylation, by providing the DolP-Man as a sugar donor for all mannosylation reactions within the ER. It is an essential protein and mutations lead to severe CDGs usually due to hypoglycosylation. Like most glycosylation enzymes, DPMS is embedded into a lipid membrane. This embedding in the ER is important for the correct localization and conformation of the protein as well as for the spatial compartmentalization of glycosylation reactions. Through the membrane anchor glycosyltransferases such as DPMS could also sense changes in the lipid environment and activity could even be regulated by these alterations. In addition, DPMS could also interact directly with specific membrane lipids as well as with its lipid substrate DolP, which is essential for enzyme activity.

The aim of this thesis was to study the connection of the lipid environment and lipid homeostasis with glycosylation processes. Due to the membrane localization of the enzymes and the formation of lipid anchored sugar donors like the LLO, DolP-Man or DolP-Glc, lipids and glycosylation are closely linked. While the glycosylation pathway and the interaction of glycosylation enzymes have been extensively studied in the past, little is known about the contribution of lipids to glycosylation. Thus, with this work I aimed to gain a better understanding of the role of lipids in glycosylation processes. The results are discussed in the following section.

Part I Method development

3.1 Assay development and optimization

Membrane proteins are naturally embedded in chemically diverse membranes, composed of many different lipid species. Changes in the lipid environment *in vivo* are difficult to pursue and interpretations are challenging, because changes in lipid homeostasis have a global effect on the cell. Thus, to study the role of lipids for a specific protein, *in vitro* systems are more suitable. These systems allow the investigation of specific proteins in a minimal, chemically well-defined environment. For the work of this thesis I used a liposomal reconstitution system to study Dpm1 and the effect of the membrane environment its enzyme activity. Proteoliposomes consisted of Dpm1, the lipids of interest, the DolP substrate as well as additional proteins to be

studied. This simplified membrane system allowed me to directly study the effect of different lipids and proteins on Dpm1 activity. I used the yeast Dpm1 protein as a model protein to investigate the role of lipids on glycosylation. Within the work of this thesis, I therefore established a protocol to purify Dpm1, optimized reconstitution into liposomes and established a new activity assay readout for a fast and easy measurement of Dpm1 activity *in vitro*. The results are discussed in the following sections.

3.1.1 Purification of enzymatically active Dpm1

Membrane proteins are not easy to purify, due to their hydrophobic nature. They are mostly insoluble in aqueous solutions, and detergents must be used to mask the hydrophobic patches of the TMDs to bring the proteins into solution. This solubilization is often accompanied by protein denaturation and conformational changes due to the loss of the stabilizing membrane environment. Yeast Dpm1 protein is a type-IV transmembrane protein with a single C-terminal TMD. Thus, the protein itself is not soluble in aqueous solutions. Before I could study the activity of Dpm1 in different membrane environments, I had to develop a suitable purification and reconstitution protocol to obtain enzymatically active protein in a liposomal membrane environment.

Previous studies of Dpm1

DPMS activity was studied before in several different species including rat [8, 105, 193, 207, 208, 214-217], human [105, 207, 216, 217], *Trichoderma reesei* [97, 218], *Pyrococcus furiosus* [99] and *Saccharomyces cerevisiae* [90, 191, 208, 219-222]. In *S. cerevisiae* previous studies used partly purified or detergent enriched membrane fractions of either native yeast or overexpressed protein. Activity of partly purified native yeast Dpm1 was studied in the presence of 0.1% sodium deoxycholate Babczinski et al. [191] or in enzyme enriched triton extracts [192]. In addition, extracts of recombinantly expressed Dpm1 protein solubilized in NP-40 and NP40-SDS were studied [103, 193, 208,]. However, all purifications involved a multistep solubilization protocol to obtain suitable amounts of protein to study enzyme activity. Thus, I decided to optimize the purification. Dpm1 activity of yeast has been studied previously, mostly in the presence of non-ionic detergents using either crude lysates or detergent enriched membrane fractions [90, 103, 191, 192, 222]. Assays in the presence of lipids were performed only for rat [190] and yeast [193] Dpm1 and reconstitution was performed by incubation of detergent enriched protein extracts

with lipid vesicles. However, reconstitution efficiency, membrane insertion and liposomal size were not controlled in these assays. For the work of this thesis, the reconstitution into liposomes was optimized and, unlike previous studies, protein reconstitution, lipid concentration as well as liposomal size were carefully analyzed for the assay used.

Choice of tagging and expression host

In order to purify Dpm1 to high purity, I decided to work with a 6xHis tagged construct and to avoid interference with the catalytic domain the tag was placed on the C-terminus of the protein. This allowed me to purify the protein by Ni-affinity chromatography in good yield and high purity, as demonstrated in 2.1. For all experiments in this thesis, I used *E. coli* as host for protein expression. Expression in *S. cerevisiae* was tested but did not result in good purification efficiency.

Choice of Sarkosyl as a detergent

One major obstacle was to find a suitable detergent for the purification of Dpm1. The detergent had to meet several requirements. Firstly, high solubilization efficiency and good protein recovery was needed. This was necessary to obtain enough protein for the reconstitution experiments. However, there is no universal detergent suitable for the extraction of all membrane proteins. Hence, I performed an initial detergent screen to find detergents with good solubilization efficiency for yeast Dpm1 (2.1.1). Detergents are amphiphilic compounds, that can act like lipids and provide a membrane like environment for proteins. However, in contrast to lipids, detergents form monolayered micelles in aqueous environments, thus not resembling a membrane bilayer. Detergents are generally classified into three groups. Ionic detergents such as SDS have a negative (anionic) or positive (cationic) charge. They often show good solubilization efficiency, but interfering with inter- and intra-protein-protein interactions, they also denature the protein in many cases. Thus, non-ionic detergents such as Triton X-100, NP-40, DDM or OG are commonly used for the purification of membrane proteins as they act less aggressive and usually only disrupt protein-lipid interactions. Zwitterionic detergents like CHAPS or Fos-Cholines show intermediate solubilization strength [223, 224]. To find a suitable detergent for the purification of yeast Dpm1, different detergents including non-ionic detergents like Triton-X100, NP-40, DDM, OG as well as ionic SDS and Sarkosyl and zwitterionic LDAO were tested to solubilize Dpm1 from *E.coli* cell lysate after recombinant expression.

Triton and NP-40, as previously used to solubilize yeast Dpm1 [191, 193, 208], showed only low solubilization efficiency in the screen (2.1.1) with a single solubilization step. However, in these studies multi-step purifications with sequential solubilization steps were used. My aim was to reduce solubilization steps by finding a detergent that better solubilized the protein. As shown in Figure 10, Sarkosyl showed good solubilization efficiency of yeast Dpm1 expressed in *E. coli*. Expression and purification could be easily upscaled to obtain protein in mg amount. Purifications were stable in the presence of 0.75% Sarkosyl when stored at -80°C. Only in the presence of Mg^{2+} in the buffer Sarkosyl-Mg, crystals did form over time, leading to precipitation of Dpm1 (2.1.1), which was seen as white pellet after centrifugation. The crystals could not be removed from the protein or dissolved again, which prevented a recovery of the solubilized protein. Omitting Mg^{2+} during purification and reconstitution completely solved the problem of precipitation. In summary, using Sarkosyl in the absence of Mg^{2+} allowed me to purify sufficient protein for method development and reconstitution experiments. As Mg^{2+} was found to be required for optimal Dpm1 activity, I added $MgCl_2$ after detergent removal to the activity assay.

In addition to good solubilization efficiency, the detergent had to be compatible with a reconstitution protocol to allow the insertion of purified protein into the liposomal membrane. Lastly, Dpm1 had to be recovered in an active form after reconstitution. Sarkosyl met all these requirements as shown in the results and discussed in the following sections.

3.1.2 Protein reconstitution into liposomes

Solubilization with Sarkosyl extracted the protein from its native lipid environment and kept it in solution. However, my aim was to study Dpm1 activity in a detergent free membrane environment. Thus, I had to re-insert Dpm1 into artificial model membranes to analyze protein activity in a defined lipid environment.

Choice of liposomal reconstitution system

I chose to use liposomes for protein reconstitution. Liposomes consist of a spherical double-membrane that can provide a lipid environment for membrane proteins. As the liposomal membrane can be easily adapted by the choice of lipid during the preparation, it offers a good basis for studying the effect of specific lipids and membrane compositions on Dpm1 activity. In addition, the chosen liposome size was

large enough to accommodate a sufficient amount of DolP acceptor substrate. Due to the presence of an inner and outer leaflet also potential flipping reactions can be studied by the use of proteoliposomes. However, the membrane curvature might also lead to conformational restrictions. Alternative reconstitution into nanodiscs could provide a planar lipid and less restrictive membrane environment, but the smaller membrane area makes studies on protein-protein interactions more difficult.

Liposome preparation

Liposomes were pre-formed before the protein was reconstituted. This resulted in a more homogeneous proteoliposome preparation. Different methods for the preparation of liposomes are commonly used, as presented in the introduction (1.6. I chose to use the hydration method with subsequent reduction of the liposomal size. To produce more uniform liposomes, different methods were tested as presented in 2.2.1. Sonication in a water bath, as often used, resulted in very inconsistent liposome batches. The sonication efficiency of the water bath sonicator was not equally distributed which resulted in inhomogeneous liposome preparations with the average liposomal size depending on the positioning of the samples. It resulted in an inhomogeneous liposome preparation containing even liposomes with an average diameter smaller than 100 nm. Therefore, I tested other methods. In the end, the optimized liposomes preparation protocol started with an extensive drying step of the lipids under a stream of N_2 as well as under reduced pressure, to ensure complete removal of residual organic solvents. This was followed by suspension of the lipid film in buffer. Suspension was enhanced by slightly elevating the temperature to 40 °C. This heating greatly increased the recovery of lipids. In addition, I subjected the suspension to 10 freeze-thaw cycles to reduce the average particle size. To produce even more homogeneous liposome preparations, lipid suspensions were further extruded using an Avanti Mini Extruder as shown in 2.2.1. The freeze-thaw step also increased lipid recovery after extrusion and lipid solutions were easier to pass through the filter. Using an extruder system, homogeneous liposome preparations can be achieved. Liposomes of different size can be formed, depending on the filter chosen. For my experiments, a 100 nm filter membrane was used, to produce uniform liposomes solutions with unilamellar LUVs (according to manufacturer protocol). This method resulted in liposomes preparations with an average size of 140 nm (Figure 18), as previously observed using this extrusion technique [225].

Protein reconstitution and detergent removal

To insert Dpm1 into the pre-formed liposomes I chose a detergent-based method. In this technique, liposomes are destabilized by the addition of detergent to allow for an efficient reconstitution. Then the protein is added, and after equilibration, the detergent is removed. A critical issue is the integrity of the liposomes during reconstitution. The addition of detergent must not lead to complete disassembly of the liposomal membrane. On the other hand, enough detergent must be added to sufficiently destabilize the liposomal membrane and to keep the protein in solution until reconstitution. To check for detergent stability of liposomes, I measured the liposomal size upon addition of detergent (2.2.2.1). A slight increase in liposomal size can be seen upon incubation with Sarkosyl. This is due to the insertion of detergent molecules into the liposomal membrane and can be reversed by removing the detergent. If the detergent concentration is too high, the liposomes become instable and dissolve, resulting in an inhomogeneous polydisperse lipid solution. I found that the liposomal membrane is stable in the presence of 0.75% Sarkosyl. Thus, I used this concentration not only for protein purification, but also for protein reconstitution.

To reconstitute the protein into the liposomal membrane, the detergent has to be removed. Removal of detergent results in a transfer of the protein from the detergent micelles to the membrane, as hydration of the hydrophobic patch caused by the lack of detergent micelles, is less favorable. Dialysis and Bio-Beads are commonly used for detergent removal. Whereas dialysis is time consuming, Bio-Beads provide a faster way to remove detergents. Sarkosyl was found to be strongly absorbed by the hydrophobic Bio-Beads, and the removal could be detected by changes in the UV absorption, as presented in 2.2.2.2. The detergent was removed after a 2.5 h incubation with Bio-Beads at RT by addition of two sequential batches of beads. The removal of detergents with Bio-Beads was less efficient when performed in the cold (data not shown). In conclusion, the beads offered a fast and simple way for detergent removal. Alternative detergent removal by e.g. dialysis was not tested, as the beads allowed for an efficient removal of detergent and reconstitution. Resulting proteoliposome solutions could be directly used to measure enzyme activity.

Flotation

Flotation on a sucrose gradient was used to monitor protein reconstitution into liposomes. After high speed centrifugation, the proteoliposomes floated to the top fraction, whereas the non-reconstituted protein remained at the bottom of the tube. As shown in Figure 17, Dpm1 was found in the top fraction upon Bio-Bead incubation of the protein-liposomes mixture. In the presence of detergent, the protein was found only in the bottom fraction of the tube. A small amount of protein was also reconstituted without detergent destabilization of the liposomes. This could even be enhanced by the fact, that liposomes were snap frozen and stored at -80 °C before flotation. The best reconstitution efficiencies were observed when the protein was reconstituted in the presence of detergent, followed by detergent removal by the incubation with Bio-Beads (2.2.2.2). Thus, with this protocol I succeeded in reconstituting Dpm1 into liposomal membranes. Also, other proteins could be co-reconstituted with Dpm1, as shown by flotation for Pmt4 and Dpm2.

Quantification of absolute amount of reconstituted protein was difficult due to variations in recovery of the floated material. Both, lipid and protein recovery, depended on the lipid composition and also varied between replicates. Thus, the protein amount was normalized to the lipid concentration and Dpm1 to DolP ratios before and after flotation were used to compare reconstitution efficiencies of different preparations. The flotation step was used to remove eventually precipitated and non-reconstituted protein. As presented in 2.4.3.3, the Dpm1 to lipid ratio did not change before and after flotation for the lipid compositions tested. Thus, non-reconstituted protein was not found in proteoliposome preparations and all protein detected was reconstituted. The ratio was also comparable between different lipid compositions when liposomes were prepared on the same day. However, I observed differences between batches, indicating that absolute reconstitution varied between batches. Most likely, some protein precipitated upon detergent removal and was pelleted and removed when samples were shortly centrifuged to remove the supernatant from the Bio-Beads, leading to different reconstitution efficiencies. To ensure comparability, protein and lipid concentrations were measured for all liposome preparations and were used to normalize the data.

3.1.3 A new Dpm1 activity assay

To study the effect of the lipid environment on the protein, I compared the activity of Dpm1 in different membrane environments. Dpm1 catalyzes the transfer mannose

from GDP-Man onto the lipid anchor DolP and the so formed DolP-Man serves as the mannose donor for all mannosylation reactions within the ER *in vivo*. In the developed *in vitro* assay, the mannose acceptor DolP was embedded together with the protein in the liposomal membrane. The assay was started by the addition of GDP-Man and product amount formed during a defined period of time was used as a readout of the enzyme activity.
Previous studies showed, that DPMS required the presence of a divalent cation. Human DPMS was found to function best with Mn^{2+}, whereas yeast Dpm1 required Mg^{2+} for optimal activity [91]. Thus, I performed the assays in the presence of 5 mM $MgCl_2$.

The activity of Dpm1 can be followed either directly by quantification of the product DolP-Man or indirectly by measuring the decrease of one of the substrates or the formation of GDP as a biproduct. To unambiguously identify and quantify enzyme activity, I chose to directly quantify DolP-Man. In previous studies, radio labelled GDP-Man substrate was mostly used to study Dpm1 activity and in this thesis, most of the assays were also performed using tritiated GDP-Man. Radiolabeling offers a sensitive readout allowing the detection of even low product amounts. To measure the labelled DolP-Man product, lipids were extracted with organic solvents and DolP-Man was identified by its Rf on a TLC (e.g. 0.7 in $CHCl_3$/MeOH/H_2O 10:10:3 [216]). This method allowed identification of the DolP-Man product. Thus, I used it for initial experiments to check for Dpm1 activity of reconstituted protein (2.3.1.1). However, quantification was rather difficult due to the lack of internal standard to compensate for lipid loss during extraction. In addition, the method required the use of harmful organic solvents such as $CHCl_3$ for the extraction of lipids. In other experiments, DolP-Man was separated from the reaction mix using a biphasic scintillation mix. Detection was based on the partitioning of the DolP-Man product into the organic phase. This allowed a fast readout without the need for additional extraction. However, both methods used a phase separation for the isolation of DolP-Man and different DolPs might be extracted with different efficiency due to their different hydrophobicity.

Method development using columns

I started by testing size exclusion chromatography for the separation of GDP-Man from DolP-Man-containing liposomes. As presented in 2.3.1.2, the use of size exclusion columns provided a good way to separate substrate from product. The elution profile

was reproducible and only minimal background signal was observed in the absence of DolP or active enzyme. No organic solvent was required in this setup and the liposomes remained intact. The latter fact showed to be useful for decoupling the readout of Dpm1 and Pmt4 activity after co-reconstitution of the O-mannosylation pathway.

DolP-Man analysis by MS

Alternatively, DolP-Man can be analyzed by mass spectrometry. Mass spectrometry offers a sensitive method for detection and quantification of low abundant DolP-Man species as well as the DolP substrate. However, the analysis is also challenging due to the very low abundance of Dol-derived compounds compared to other membrane constituents. Additionally, the diversity of Dol species further reduces the abundance of individual DolP and DolP-Man species. As presented in section 2.4.1, I was involved in the development of a liquid chromatography-coupled mass spectrometric (LC-MS) method to quantify DolPs from cells as well as from liposomes. Derivatization of by the use of TMSD greatly enhanced the chromatographic behavior of DolP species and their ionization. DolP-Man detection was also improved by methylation, however quantification of DolP-Man was difficult due to the lack of commercially available standards. With a suitable standard, however, the method might be useful to quantify DolP and DolP-Man even in a single LC-MS run. For the work of this thesis, however, the mass spectrometric assay was used only for qualitative analysis of DolP-Man and DolP species distribution and not as a readout for the activity assay.

3.2 Limitations and potential of the assay

To detect changes in Dpm1 activity depending on different lipid compositions or the addition of other proteins, the liposomes must be comparable. The most important factors determining the activity of Dpm1 are the protein concentration and the availability of the substrates GDP-Man and DolP.

3.2.1 Enzyme concentration

In order to compare enzyme activities in different lipid environments, it is important to have the same enzyme concentration in all conditions, as the amount of DolP-Man formed is proportional to the enzyme concentration. Alternatively, the product amount has to be normalized to enzyme concentration, to compensate for differences and to

make the readout comparable. Therefore, same amounts of protein were used for reconstitution to keep the preparations as comparable as possible. Dpm1 concentration in the proteoliposomes were quantified using western blot. I found that reconstitution was relatively comparable between preparations of the same day, even for liposomes with different lipid compositions as shown in 2.4.3.1. However, I observed a huge bath to batch variation. Thus, Dpm1 concentration was used for normalization of the data, to allow the comparison of enzymatic activity between different batches. To monitor the efficiency of protein reconstitution into liposomes, a flotation step was included for some preparations. As discussed before (3.1.2), the ratio between liposomes and Dpm1 remained constant for a given sample before and after flotation. This was true for all lipid compositions, even though the absolute amount of recovered protein varied a lot due to different proteoliposome recoveries. Flotation efficiency and recovery depended a lot on the lipid composition used as well as on the presences of co-reconstituted proteins.

A factor that could further influence the high variance between different batches, but is not easily controlled, is the distribution of Dpm1 within the liposome population. This is especially important as DolP is part of the liposomal membrane and its availability per enzyme changes drastically with the amount of enzyme per liposome. Thus, the available DolP may vary due to inhomogeneous reconstitution. Therefore, it is important to have a sufficient large amount of DolP substrate in the membrane to ensure that the concentration of free DolP remains comparable over time even with different enzyme concentrations. Theoretical enzyme to liposome ratios were calculated to be about 50 protein molecules per liposome, but the exact numbers were not determined.

In addition, co-reconstituted proteins such as Pmt4 or Dpm2 can affect the distribution of Dpm1 in the liposomes. Inhomogeneous protein reconstitution may also lead to liposomes without protein. As DolP-Man is part of the membrane and presumably only DolP within the same liposome and leaflet can be used by Dpm1, this would lead to differences in the maximal amount of DolP-Man that can be formed. For the experiments presented in this thesis, empty liposomes were not separated from protein containing ones. To reduce the effect of differences in protein distribution, the reaction was stopped after 1 min and before the maximal DolP-Man concentration was reached.

3.2.2 Protein orientation

Another factor that is difficult to control is the orientation of Dpm1. *In vivo,* the large N-terminal catalytic domain is facing the cytosolic side of the ER [214]. In the liposomal system, Dpm1 presumably prefers to face outwards, due to liposomal curvature and steric hindrance within the luminal space of liposomes. However, the topology of Dpm1 after reconstitution might be altered by the lipid composition, as shown for other proteins like lactose permease [226]. Since only the outward-facing enzyme has access to GDP-Man and can perform the synthesis of DolP-Man, the enzymatically active pool of Dpm1 depends on the orientation of the enzyme. I performed initial experiments with selective protein digest of liposomes to investigate the orientation of Dpm1 in the liposomal membrane (data not shown). Liposomes were incubated with proteinase K to selectively digest outwards facing protein and digest was checked by western blot. After digestion, reconstituted Dpm1 was no longer detectable, suggesting an exclusively outwards facing orientation. However, due to the low protein concentration it was not possible to exclude the presence of a small amount of inwards facing protein. In addition, it cannot be excluded that liposomes were leaky under the chosen digesting conditions. Here additional control experiments are required. Thus, these preliminary data should be taken with caution. For this work, I assumed a similar orientation preference for Dpm1 in all liposome conditions.

When Dpm1 is co-reconstituted together with other proteins the relative topological orientation to each other is also important. However, this is difficult to control in the assay. In co-reconstitution experiments with Pmt4, both proteins presumably show an orientation that does not reflect the *in vivo* situation. *In vivo,* mannose is transferred to DolP by Dpm1 on the cytosolic side of the ER, whereas the O-mannosylation reaction takes place on the luminal side after flipping of DolP-Man. In the *in vitro* reconstitution system, only outwards facing Dpm1 is active, as GDP-Man is added from the outside and is not accessible to Dpm1 which is oriented to the luminal side of the liposomal membrane. Likewise, also the catalytic site of Pmt4 must face outwards as the acceptor peptide is also added from the outside. Since both enzymes showed catalytic activity, at least the enzymatically active portion of proteins is oriented differently than *in vivo*. As the orientation of both proteins was not actively controlled, in this assay enzymatically inactive Pmt4 might still interact with Dpm1 in a physiological manner and stabilize the protein. However, Dpm1 activity was similar in the absence and

presence of Pmt4. The ratio of inwards and outwards facing protein was assumed to be constant for all experiments, also in the presence of other proteins.

Likewise, the orientation of Dpm2 cannot be readily determined in the assay. C-terminally tagged Dpm2 enhanced Dpm1 activity, suggesting that this is due to its interaction with Dpm1. This interaction is presumably hindered when the tag is placed at the N-terminus, as no enhancement is seen with the N-terminal tag positioning (for a more detailed discussion see 3.4.1.1). Whether this is also the case *in vivo*, needs to be investigated in future experiments. In addition, the preferred orientation of Dpm1 could be altered in the presence of Dpm2. Thus, the change in DolP-Man formation could also be due to more active, outwards facing Dpm1 rather than higher individual enzyme activity. There is currently no possibility to selectively inhibit Dpm1-Dpm2 interaction. Thus, different preparations, with possibly different Dpm1 orientation, are needed to compare Dpm1 activity with or without Dpm2 interaction. However, when co-reconstituting N-terminally tagged Dpm2, Dpm1 did not show altered enzyme kinetics, thus making it more unlikely that Dpm2 drastically affects Dpm1 orientation during reconstitution.

3.2.3 Substrate availability

Substrate concentrations must be carefully to allow for comparison of Dpm1 activities in different liposomes. The mannosyl donor GDP-Man can be adjusted over a broad range of concentration. As it is a soluble substrate and is added just before the reaction starts, its concentration can be easily controlled.

On the other hand, the concentration of DolP is fixed by the amount added during liposomes preparation. As it is part of the membrane, it is not possible to manipulate its concentration after protein reconstitution. In previous studies, detergent solubilized DolP was added exogenously to study the activity of Dpm1 [90, 191]. Because we were interested in studying the lipids environment, this was not an option. Thus, it was important to keep lipid loss minimal and comparable during preparation. As shown in 2.4.3.1 the Dpm1 to DolP ratio remained comparable, independent of the lipid compositions used for liposome preparations. Another factor to consider is, that DolP does not flip spontaneously between the inner and the outer leaflet. Thus, only “outward-facing” DolP can be used as a substrate and the expected maximal DolP-Man amount is only half of the total DolP used for the preparation of liposomes. In addition,

empty liposomes from which DolP most likely cannot be used *in trans* for DolP-Man synthesis also reduce the amount of product to be formed. Furthermore, the number of DolP molecules per liposome and enzyme is limited by the liposome size. For the study, I assumed that DolP was equally distributed between the leaflets and that the orientation was the same for all liposomes tested. Liposomes of same size were prepared and initial protein-lipid ratio was kept constant to ensure comparability.

3.2.4 Dpm1 and kinetics

As discussed above, the GDP-Man concentration used in the assay can be well controlled. The kinetic parameters for GDP-Man in DOPC liposomes containing 1% DolP were found to be similar to previous findings [191] (see 2.3.2). In contrast, the DolP concentration is more difficult to modulate as it is part of the liposomal membrane. For kinetic studies, substrates must be added in excess and remain relatively constant over the time measured. This might not be possible in regard to DolP, as its overall or local concentration might be too less. In the assay, I mostly added 1% DolP of total lipids. This is a concentration higher than naturally found in eukaryotes, where the total Dol pool is found to be about 0.1% [52]. In addition, strong increase in DolP concentration might also alter membrane properties. The total DolP concentration is limited by the liposomal membrane and its size and its decrease depends on enzyme concentration and enzymatic rate. Due a limited amount of DolP available in liposomes, the enzyme concentration in the liposomes cannot be drastically increased. However, if the protein concentration is too low, the product amount is also low and may not even be detectable. The immediate DolP substrate availability depends on the size of the liposomes, as larger liposomes contain more substrate that can be mannosylated compared to smaller ones. Thus, the DolP pool in smaller liposomes is consumed faster compared to larger ones with the same number of enzymes reconstituted and the DolP concentration decreases faster in these liposomes. Consequently, the linear range of the kinetic reaction is also smaller in these liposomes. This is problematic if there is a wide distribution of liposome size within a batch as this leads to mixed reaction kinetics at later time points. In addition, the number of enzymes per liposome determines the rate in which the local DolP concentration decreases. If the DolP to Dpm1 ratio differs within the liposome population or between conditions, the resulting difference in product may not only depend on the enzymatic speed but also on differences in DolP availability. To avoid that differences in enzyme kinetics were due

to different DolP concentrations of different liposome types or batches, the DolP percentage membrane was kept the same for all preparations. In addition, an extrusion step was inserted into the liposome preparation to ensure similar size distribution of liposomes. Furthermore, the reaction time was kept as short as possible and stopped before reaching the endpoint of the reaction to ensure DolP availability during the reaction. Other parameters such as pH, temperature and GDP-Man concentration were kept constant as well. Thus, kinetic changes are indeed due to differences in the lipid compositions or the presence of co-reconstituted proteins. However, it is impossible to control the local DolP concentration in the assay. Different liposomal membranes may also affect lateral diffusion, orientation and DolP availability. Hence, differences seen can be either due to altered Dpm1 kinetic activity or due to changes in the DolP availability. As DolP is part of the membrane itself, these parameters cannot be decoupled and increase the difficulty of kinetic studies and interpretation of the results. Kinetic studies for DolP would also require individual liposome preparations as the DolP concentration cannot be changed after liposome formation. Thus, kinetic curve might be noisier. In addition, the range of DolP concentration that can be used is limited by the assay sensitivity and speed as well as by the liposomal membrane. In general, a range between 1-10% of total phospholipids can be used, to ensure enough substrate and prevent a dominating effect on membrane properties. But no kinetic studies of lipid dependent DolP binding were done. The dependency of Dpm1 activity on DolP concentration was measured only in DOPC liposomes and activity was compared after 1 min for 1%, 2% and 4% DolP (2.4.2). As expected, more product was formed at higher substrate concentrations.

Part II Activity of Dpm1 in different membrane environments

3.3 Dpm1 activity depends on its lipid environment

As discussed above, the established assay was robust, reproducible and showed to be a suitable method to investigate Dpm1 activity. A major interest was to study the effect of the membrane lipid composition on Dpm1 activity and protein-protein interaction. Thus, I decided to use artificial lipid compositions and exchange lipid classes and lipid species to alter the overall membrane properties and analyze the effect on Dpm1 activity. DOPC liposomes were used as a reference model membrane. In DOPC membranes, Dpm1 was moderately active and the reaction was slow enough

to follow the kinetic reaction. In addition to changing the lipid composition of the liposomal membranes, the role of the DolP lipid and its isoprene chain composition was investigated. Since the mannose acceptor substrate of Dpm1 itself is a lipid that is embedded in the membrane, it could also affect membrane properties or the protein itself.

3.3.1 Dpm1 and DolP

DolP chemical diversity

The DolP substrate is composed of a polyprenol chain, which is saturated at the α-end, and a phosphate group, as acceptor of the mannose residue. MDS predicts that the phosphate group is localized on the membrane surface, whereas the isoprene chain moves rather flexibly within and between both leaflets (collaboration with Rainer Beck, Fabio Lolicato and Walter Nickel, unpublished results). The number of isoprene units of the polyprenol tail varies within and between species. In the liposomal assay, a commercially available DolP mixture composed of DolP chain lengths ranging from C65 to C105 was used, as presented in 2.2.3.2.3. The main species in the mixture were C85 and C90 DolP. In contrast, the predominant DolP species in yeast are DolP C75 and DolP C80, accounting together for about 75% of the total DolP pool (compare Kale et al. [53]). Hence, it was possible that these chain lengths were getting preferentially or exclusively mannosylated in the reconstitution system. However, mass spectrometric analysis of chain length distribution of DolP and DolP-Man revealed that there was no preference for specific DolP species i.e. DolPs of all chain lengths were used as substrates by yeast Dpm1 (2.4.1). Thus, the DolP chain length is not important for substrate recognition by Dpm1 and Dpm1 activity is not affected by the DolP species profile *in vitro*. Nonetheless, there may be regulation and DolP species preferences *in vivo*, which could also contribute to shuttling of the DolP-Man into different pathways. Further experiments need to be performed, i.e. using the LC-MS method to analyze the DolP-Man species distribution *in vivo* and to detect presumable chain length preferences and changes under different conditions.

I also performed additional experiments to test, whether the isoprene chain itself has an effect on enzyme activity. As the DolP substrate is embedded into the membrane by its isoprene tail, it could interact with the TMD of Dpm1 and change the enzymatic activity. In addition, DolP could change local membrane properties of the lipid

environment and regulate Dpm1 activity. Hence, proteoliposomes containing Dol in addition to DolP were prepared. Dol has a similar structure as DolP, but by lacking the phosphate group it does not serve as substrate for the mannosylation reaction. Thus, changes in Dpm1 activity can be attributed to the effect of the isoprene moiety. However, no difference in Dpm1 activity was seen compared to proteoliposomes that only contained DolP. Product formation did not change in the presence of Dol. As expected for enzymatic reactions, activity was enhanced in the presence of higher DolP concentrations (2.4.2). In conclusion, these results demonstrate, that the isoprene chain has little to no effect on the *in vitro* activity of Dpm1.

Previous studies on DolP species as substrates for Dpm1

Eukaryotic DPMS specifically mannosylates DolP *in vivo*. However, it is not clear how the substrate is recognized by the enzyme. In earlier studies on yeast Dpm1 activity, a DolP recognition sequence, found by sequence comparison of the TMD of different glycosylation enzymes that were all using Dol derivatives as substrate, was postulated [227]. However, this idea was later discarded as mutations in the region had little effect on enzyme activity. In addition, not all glycosyltransferases interacting with Dol derivatives share this sequence and the sequence was not required for yeast Dpm1 activity *in vitro* and *in vivo* [228]. Previous studies also tested DPMS activity with different DolP species as well as unnatural and chemically modified DolP as substrates. A long isoprene anchor of DolP does not seem to be required for substrate recognition, as even very shot DolP mimics such as citronellyl phosphate (CitP) are mannosylated [58]. However, a reduction in Dpm1 activity is seen for these short DolP species compared to longer ones [58]. Thus, a certain length of the isoprene tail is needed for maximal activity. In these experiments, detergent solubilized DolP and CitP were added exogenously [58 209]. It is not clear whether the substrate was mannosylated in solution and whether the lipid linked mannose was bound to the membrane after the reaction. However, using a clickable CitP-analogue and proteomic analysis of the clicked proteins revealed that the unnatural substrates are recognized by Dpm1 and other downstream glycosylation enzymes [58]. Examples for other proteins interacting with CitP analogues are Pmt1 and Pmt2, Alg12 and OST2 that were all found to be clicked to PAL-CitP [58]. In addition, Alg5, the enzyme that is catalyzing DolP-Glc was found to bind to the DolP analogue [58]. Thus, it is likely that also these other enzymes have low requirements for the isoprene tail lengths. Fluorescent DolP analogues, with fluorophores attached to the ω-end of the isoprene chain, were also found to be

suitable acceptor substrates for Dpm1 [229] [100, 221]. As in many previous studies, the substrate was solubilized in detergent before exogenous addition and not embedded in a membrane. Sprung et al. [230] could even show that acceptor substrate, that was tail anchored on beads, could be mannosylated by Dpm1 in yeast microsomes. This finding underlines the minor role of the ω-end of the isoprene chain for the enzymatic activity of Dpm1. Wilson et. al [209] showed, that also a phytanyl phosphate (a C20 polyprenol with only saturated isoprene units) could serve as a substrate for Dpm1. However, the mannosylation rate was only 60-70% when compared to DolP substrate. This shows, that the chemical structure of the isoprene chain, specifically the α-saturation, plays an important role in substrate binding and Dpm1 activity. In this study [209], also S-3-methyloctadecanyl phosphate, but not the linear tetradecanyl phosphate was found to be mannosylated. Thus, the methyl group at the C3 position seems to be a key feature for substrate recognition.

In summary, these studies indicate that DolP chains plays only a minor role in the Dpm1 activity under *in vitro* conditions. The only necessary structural elements for substrate recognition of eukaryotic DPMS are the phosphate group, the α-saturation of the isoprene chain and the methyl group of the first isoprene unit. Similar results were observed within the work of this thesis by using the liposomal reconstitution assay. All DolP species were used as acceptor substrate. No preference for chain length of DolP was observed for Dpm1 as the species profile of DolP-Man resembled the one of the DolP substrate (2.4.1). In contrast to eukaryotes, bacteria do not require the α-saturation of DolP and use polyprenol phosphate (PolP) as a lipid carrier [52]. Interestingly, the chain length distribution of polyprenol phosphate in bacteria seems to be less divers [231]. C55 was found as the major PolP species in most bacteria, nonetheless, also other chain lengths were found in some species [231]. The reason for the large natural diversity in DolP species is still not understood. A certain chain length seems to be needed for efficient anchoring of the DolP-Man in the membrane, but a huge diversity of acceptor substrates is recognized by Dpm1. Thus, there is still need to understand the reasons for DolP diversity, not only between but also within species. The newly developed LC-MS method will help to study and compare DolPs from different species and to clarify whether the DolP or DolP-Man species distribution changes under temperature stress or altered growth conditions. The chain length distribution of DolP could have an effect on lateral membrane diffusion, could be

matched to membrane thickness, could be required for flipping of DolP-Man or could mediate shuttling of DolP-Man into various pathways.

3.3.2 Dpm1 activity is influenced by the lipid environment

Dpm1 of *S. cerevisiae* is embedded into the membrane by its C-terminal TMD domain (predicted aa 239-259, based on sequence analysis, UniProt database for P14020). This TMD is surrounded by membrane lipids and could sense changes in membrane properties or directly interact with specific lipids. To analyze the effect of lipids on Dpm1 activity, I reconstituted Dpm1 into liposomes with different lipid compositions with reduced lipid diversity compared to the *in vivo* membrane. Therefore, all compositions tested did not resemble the natural composition in its complexity. By reducing the lipid complexity of the membrane to specific lipid species, effects can be better interpreted and assigned to specific properties.

The assay was developed and optimized using DOPC liposomes. PC is the major lipid species of the ER in *S. cerevisiae*, making almost 40% of the total phospholipids. Oleic acid (18:1) is the predominant fatty acid (FA) in yeast, followed by palmitoleic acid (16:1) [135]. In addition, all liposomes contained DolP as a substrate (1% unless otherwise stated) and 0.2% Rh-PE. Rh-PE, a fluorescent analogue of PE, was used to visualize the liposomes during preparation and could be used to assess lipid loss (2.2.3.2.1). Dpm1 showed activity in DOPC liposomes and the results were robust and reproducible. Therefore, this composition was used as a reference and included in all preparations.

To investigate the effect of FA saturation, I compared Dpm1 activity in liposomes containing PC species with different fatty acyl compositions. As presented in 2.4.3.2, Dpm1 activity was reduced in SOPC (18:0/18:1) and POPC (16:0/18:1) liposomes compared to DOPC (18:1/18:1) liposomes. Thus, saturation of 50% of the side chains was less favorable than di-monounsaturated fatty acyl moieties as present in DOPC. However, I found that Dpm1 activity was increased in DOPC/POPC 1:1 liposomes compared to DOPC only. Hence, a certain degree of saturation in the acyl chains can also promote Dpm1 activity. In the tested mixture, the resulting overall fatty acyl saturation was 25%. *In vivo*, the degree of saturation depends on growth conditions, but about 20% fatty acyl moieties of glycerophospholipids are saturated under standard conditions [135]. Thus, the ratio of saturated to unsaturated fatty acyl species

tested in the activity assay closely resembled the natural saturation ratio when using DOPC/POPC 1:1 liposomes. There could be an optimal ratio of saturation to unsaturation for Dpm1 activity that would have to be determined in further experiments. Saturation of FAs leads to a tighter lipid packing whereas unsaturated lipids form more fluid membranes [134]. Thus, changing the degree of lipid saturation is a fast way to modulate membrane fluidity. An example how cells use this modulation of membrane properties is the change in lipid saturation in response to temperature changes [156, 232]. A change in lipid saturation allows the cells to maintain fluidity and compensate for temperature-induced fluidity changes.

By comparing proteoliposomes containing either POPC (16:0/18:1) or SOPC (18:0/18:1), the contribution of fatty acyl chain length to Dpm1 activity was investigated. The shortening of the acyl chains reduces membrane thickness and makes membranes stiffer. However, when comparing POPC and SOPC liposomes, I did not observe a significant difference in Dpm1 activity. Thus, this suggests that differences in Dpm1 activity in POPC liposomes compared to DOPC were due to the FA saturation rather than the FA chain length. However, under the tested conditions the acyl chains differed only by two carbon atoms. Thus, they may not be ideal choices to study the effect of FA chain length on Dpm1 activity, as the changes may be too subtle to be visible in the assay. To study the modulation of Dpm1 by FA chain length, the experiments could be repeated using PC species with significantly shorter or longer FA moieties. However, both, C18 and C16 are the most common chain lengths in yeast [135] and there are studies showing a homeoviscous adaption of yeast to different growth temperatures by changing the C16 to C18 ratio [233]. An increase in saturation and a shortening of chain lengths in other lipid classes like PE was found as a response to PC depletion in yeast [234], suggesting this adaptation is a general mechanism to compensate for changes in membrane fluidity. In summary, changes in FA chain length from C16 of C18 were not affecting enzymatic activity of yeast Dpm1 *in vivo* under the conditions tested.

I also tested the effect of PE on Dpm1 activity. PE is another highly abundant phospholipid species in yeast ER membranes [135]. PE has a smaller headgroup compared to PC, thus, it can form tighter packed membranes on the membrane surface [157]. However, due to the size of the headgroup, the lipid is inverted-conically shaped with higher width at the acyl tail [157]. Thus, PE tends to form hexagonal phases rather

than lamellar membranes and therefore is known to induce negative curvature stress in PC based membranes [235]. This induction of packing defect is more pronounced in the presence of unsaturated fatty acids that take up more space compared to straight and saturated lipid chains. The PC to PE ratio was found to change during cellular in growth in yeast, with more PE found in the lipidome during the growth phase [236]. In addition, PE was found to be a key regulator of membrane fluidity in eukaryotic cells [157] and thus adaption in the PE concentration can regulate important membrane properties like curvature and the lateral pressure profile [233]. In previous *in vitro* studies, the activity of Dpm1 from rat and yeast has been reported to be greatly enhanced in the presence of PE [190, 193, 237]. Similarly, in my experiment's liposomes composed of DOPC/POPC/POPE (5:1:4) showed the highest activity of all liposomes tested, including DOPC/POPC (1:1) liposomes. Thus, these data indicate that PE has a positive effect on Dpm1 activity in the liposomal assay.

Another interesting lipid that I tested was ceramide, which is the precursor of sphingolipids. Ceramides are known for their impact on lipid ordering and domain formation in the plasma membrane and raft like microdomains were also suggested to occur in ER membranes [211]. Thus, the presence of ceramides could also affect Dpm1 activity. In the reconstitution assay, Dpm1 activity was found to be significantly reduced in the presence of 5% ceramide. Ceramides and ceramide microdomains thus may also regulate Dpm1 activity *in vivo*. The lipid phosphatase Sac1, that was found to bind to Dpm1 in the ER under growth conditions [238], was also found to interact with ORM proteins [239, 240]. These ORM proteins are negative regulators of Cer and SL synthesis, by inhibiting serine palmitoyl transferase activity (SPT) [240]. SPT, ORM and Sac1, were found to form the "SPOTS" complex in yeast, and to regulate the first step of Cer synthesis [239, 240]. The association of Dpm1 with Sac1 and presumably also to the SPOT complex, could present a way how Dpm1 activity might be coupled to the ceramide and SL synthesis.

Ergosterol is the predominant sterol species in yeast [135]. Like ceramide, it is synthesized in the ER and its concentration increases progressively along the secretory pathway towards the plasma membrane where most sterols are located [135]. The addition of sterols increases membrane rigidity, mainly by ordering and condensing saturated lipids. In contrast to cholesterol, ergosterol was found to slightly thinning lipid bilayers containing unsaturated lipids [241]. In my reconstitution assay, the

addition of either 20% or 5% ergosterol strongly inhibited Dpm1 activity. This is an interesting observation, as ergosterol is also present in the ER. However, due to domain formation the local concentration could vary, and thus Dpm1 could be active *in vivo* also at higher global ergosterol concentrations. In addition, other lipids not present in the assay could compensate for the induced rigidity of the membrane *in vivo.* Also, de novo sterol synthesis is also linked to DolP synthesis by the mevalonate pathway by requiring the same FPP substrate (1.1.5.2.2). Thus, there might be a crosstalk between ergosterol synthesis and Dol-based glycosylation.

The effect of lipids on the enzymatic activity of Dpm1 was studied by comparing to its basal activity in DOPC liposomes. Liposomal membranes composed of PC and DolP were sufficient to support enzyme activity and no additional lipid was required for basal Dpm1 activity. However, other lipid species were found to enhance or dampen Dpm1 activity *in vitro*, as discussed above. The low requirement regarding lipids for basal activity was also indicated by the fact the Dpm1 was found to be active also in the presence of detergent [90, 209] (also shown in 2.3.1.1). Thus, lipids might rather affect Dpm1 activity as a bulk and through changing the overall membrane properties like fluidity, thickness or domain formation instead of specific interactions with annular or non-annular lipid species. The results of the tested lipid compositions indicate that the major influencing factor on Dpm1 activity are changes in fluidity. Increased fluidity by the presence of the non-bilayer forming lipid PE [242] greatly increased Dpm1 activity. PE can cause packing defects and membrane curvature stress and therefore leads to increased fluidity of membranes [242]. Dpm1 appears to more active in more fluid membranes, but a certain rigidity is needed to stabilize the protein and liposomes. Dpm1 activity was increased in the presence of more unsaturated FA and thus more fluid membranes, however the best activity was seen at a saturation/unsaturation ratio of 1 to 3. The addition of membrane domain-forming lipids ergosterol and ceramides drastically decreased Dpm1 activity to near background levels.

It is difficult to translate these findings to the *in vivo* situation, as membrane properties can be achieved by different lipid combinations and lipids can compensate for each other. The complexity of lipids and membrane properties is not reflected in the reconstitution assay. However, the results show that membrane proteins such as Dpm1 and Pmt4 are directly affected by their lipid environment. It also shows a possible connection between glycosylation and membrane homeostasis and that glycosylation

could be modulated by the lipid composition of the membrane. Also, raft-like domain formation in membranes, as observed in the plasma membrane and also suggested for the ER [211], can contribute to protein localization and activity. Super resolution microscopy with could be used to study the nanodomains and the preferential localization of proteins into certain membrane areas, to get a better understanding on the dynamics of lipid membranes [243].

Both, synthesis of lipids and glycosylation occur in the ER and are tightly regulated during cellular growth. An example for the interconnection of cellular glycosylation and lipid synthesis is the interaction of Sac1 and Dpm1 [126, 238]. In co-immunoprecipitation experiments, Dpm1 was found to associate with Sac1 during exponential growth of *S. cerevisiae*, Sac 1 localized in the ER. *In vivo*, this interaction is needed for efficient N-glycosylation [238]. This might be due to altered Dpm1 activity in the presence of Sac1. Sac1 is a phosphatase and converts PI(4)P into PI in the ER [126, 238] and it reversibly cycles between ER and Golgi, in a growth dependent manner [126]. Under high proliferation conditions, Sac1 was found to be associated with Dpm1 in the ER membrane, whereas under starvation the enzyme was translocated to the Golgi. In both organelles, the phosphatase regulates the presence of PI and PI(4)P species. Co-reconstitution experiments of Sac1 and Dpm1 in the presence and absence of Dpm2 and PI lipid species could be used to further study the interaction and regulation of these enzymes. Due to the close proximity to Dpm1, Sac1 and lipid substrate and product also effects the immediate lipid environment of the glycosylation enzyme. In a preliminary experiment, however, no difference in Dpm1 activity was seen between liposomes containing 5% PI(4)P or 5% PI. Thus, Sac1 most likely does not regulate Dpm1 activity by changing the PI(4)P concentration in the immediate lipid environment. However, localization of Sac1 to the ER is also connected to the lipid transporter Osh4p. Osh4p was found to transport sterol from the ER to *trans* Golgi, by counter transporting PI(4)P to the ER[244] that is then readily hydrolyzed by Sac1. Indeed, the hydrolysis of PI(4)P by Sac1 was proposed to be the driving force for ergosterol export of the ER. Thus, association of Dpm1 with Sac1 might be also be beneficial due to the reduction of sterol content in the immediate enzyme environment. Sac1 was further be found to be part as the SPOTS complex, that regulates ceramide synthesis in the ER, as discussed earlier. In conclusion, Sac1 is associated with the regulation of Cer and Erg levels in the ER and its interaction with Dpm1 could form a link between glycosylation and lipids.

3.4 Interaction of Dpm1 with other glycosylation proteins

Dpm1 is a central hub in cellular glycosylation, as it provides the mannosyl donor of for all mannosylation reactions within the ER. Thus, all downstream enzymes involved in mannosylation compete for the DolP-Man substrate. The regulation of the shuttling of DolP-Man into different glycosylation pathways is not yet understood. This channeling of DolP-Man could be regulated by spatial distribution of DolP-Man and downstream mannosyl transferases or by direct interaction of other proteins with Dpm1, both ways potentially regulated by the lipid composition of the membrane. As a first step towards understanding this connection between glycosylation routes, I co-reconstituted the O-mannosyl transferase Pmt4 with Dpm1. In addition, Dpm1 itself competes with other enzymes for its own substrates. GDP-Man is also used for the formation of the lipid linked N-glycan precursor on the outside of the ER (e.g. by Alg1, Alg2, Alg11). Increased GDP-Man concentration by overexpression of GDP-mannose pyrophosphorylase could restore glycosylation defects in Alg1 and Dpm1 defect yeast cells [245]. Further, Dpm1 and Alg5 are both using DolP as a substrate to form mannosyl and glucosyl donors for ER glycosylation. In addition, DPAGT1 requires DolP to form DolPP-GlcNAc as a first step of LLO synthesis [246]. These upstream regulations are not addressed within this thesis but are potential targets to modulate protein glycosylation.

Besides the possible interaction with other glycosyltransferases, the catalytically active Dpm1 in human was found to require interaction with Dpm2 and Dpm3 to form an active DPMS complex and for its correct intracellular localization. The C-terminally anchored Dpm1 of *S. cerevisiae* can catalyze the formation of DolP-Man *in vitro* without interacting with additional proteins. However, Piłsyk et al. reported in 2020 that Yil102c-A fulfils the function of Dpm2 in *S. cerevisiae* [122]. In their study, a deletion of Yil102c-A in yeast was lethal and could be rescued by the *dpm2* gene from *Trichoderma reesei,* suggesting Yil102c-A has essential Dpm2 functionalities in yeast. In addition, reduced DolP-Man formation was observed in Yil102c-A knockout cells [122]. Thus, I tested the effect of Yil102c-A (Dpm2) on the enzymatic activity of Dpm1 using my reconstitution assay.

3.4.1 Dpm1 and its interaction with Dpm2

In contrast to the human trimeric DPMS complex, consisting of soluble Dpm1 and the two small membrane proteins Dpm2 and Dpm3, it was believed that in yeast only the

C-terminally anchored transmembrane protein Dpm1 is needed for full DPMS activity (compare introduction 1.3.1). In agreement with this, I observed Dpm1 activity also in the absence of Dpm2 (Yil102c-A) in the *in vitro* reconstitution system. Nonetheless, the presence of Dpm2 could increase Dpm1 activity by stabilizing the enzyme, helping to position the DolP substrate and/or by interacting with other glycosyl transferases. Thus, I co-reconstituted Dpm2 with Dpm1 to study its effect on Dpm1 activity *in vitro*.

3.4.1.1 Positioning of the affinity tag affects the Dpm1-Dpm2 interaction

As presented in in 2.5.1.2, I recombinantly expressed and purified a tagged construct of Yil102c-A (Dpm2) from *E. coli*. Different tagging strategies were tested, as an initial expression with an 8xHis-tag (either N- or C-terminally) only resulted in a very low yield of protein. Eventually, Dpm2 could be successfully expressed and purified as a Dpm2-His-Tev-MBP or MBP-Tev-His-Dpm2 construct in sufficient yields. The initially planned enzyme mediated Tev-cleavage, to reduce the size of the tag after purification, was unsuccessful. This was most likely due to inactivity of the protease in the presence of Sarkosyl (27 mM). However, proteolytical removal of the tag was not required for interaction of Dpm2 with Dpm1 when using the C-terminally tagged protein, as shown in 2.5.1.2. Thus, the constructs were used without Tev-mediated removal of the tag.

I successfully co-reconstituted both N- and C-terminally tagged Dpm2 with Dpm1 in liposomes, as presented in 2.5.1.1. In both cases, the addition of Dpm2 did not change the reconstitution efficiency of Dpm1. Thus, Dpm1 activity in both types of proteoliposomes could be directly compared. Nonetheless, to reduce batch to batch variations, Dpm1 activities were normalized to the Dpm1 amount in the respective proteoliposomes. Dpm2 reconstitution into liposomes was lower when the N-terminally tagged Dpm2 was used, and the protein showed a degradation band on the western blot. Nonetheless, also N-terminally tagged protein with the correct size was reconstituted in sufficient amounts to be detected by western blot. An increase of Dpm1 activity was observed only for the C-terminally tagged Dpm2, but not for the N-terminally tagged Dpm2 (2.5.1.2.). These findings are in line with the work of Piłsyk et.al who could co-purify Dpm1 and Dpm2 only when the myc tag was introduced at the C-terminus of Dpm2. Together with the results of the *in vitro* activity assay, these data suggest that the N-terminal TMD is required for interaction of Dpm2 with Dpm1, which is hindered in the presence of a tag. Thus, I continued working with C-terminally Dpm2-His-Tev-MBP tagged constructs.

3.4.1.2 Dpm1 activity is enhanced in the presence of Dpm2

In the liposomal assay, DolP-Man formation by Dpm1 was found to be increased by about 3-fold when co-reconstituted with C-terminally tagged Dpm2 into DOPC liposomes. The reconstitution efficiency of Dpm1 was not significantly altered by Dpm2. Thus, Dpm2 was found to stimulate Dpm1 activity *in vitro*. In contrast to the *in vivo* situation, where Dpm2 was essential for Dpm1 activity, the additional protein was not needed for Dpm1 activity when reconstituted into liposomes.

3.4.1.3 The effect of lipids on Dpm1 and Dpm2

As Dpm1 was found to be regulated by the lipid environment (2.4.3), I was interested whether the interaction of Dpm1 with Dpm2 was also dependent on the lipid composition. The membrane lipid composition could assist in mediating Dpm1-Dpm2 interaction and thus could play a regulatory role in glycosylation processes. Therefore, I co-reconstituted Dpm1 and Dpm2 into liposomes with different lipid compositions and compared the activity to liposomes without Dpm2. I chose four different lipid compositions, namely DOPC, POPC, DOPC/Erg 95:5 and DOPC/POPC/POPE 5:1:4. As presented in 2.5.1.3, the presence of Dpm2 enhanced the activity of Dpm1 in all lipid compositions except from DOPC/POPC/POPE 5:1:4, where the Dpm1 activity was already high in the absence of Dpm2. In DOPC liposomes, Dpm1 activity could be boosted more by the presence of Dpm2 than in POPC liposomes, but both increases were significant. The increase of Dpm1 activity in DOPC/Erg liposomes by the addition of Dpm2 was not statistically significant, as the overall activity remained close to background even in the presence of Dpm2. The fold change increase of Dpm1 activity due to the presence of Dpm2 (Dpm1+2/Dpm1) was not significantly altered by the lipid composition. The only exception was the activity of Dpm1 in DOPC/POPC/POPE 5:1:4 liposomes, that was already high in the absence of Dpm2 and was not further increased by Dpm2. Whether this was due to disturbed a Dpm1-Dpm2 interaction in the presence of PE or whether the enzyme already reached its maximal speed by the lipid environment has to be tested in further experiments. Further conditions should be included to test whether the Dpm1-Dpm2 interaction is modulated by the lipid environment.

The lipid composition of liposomes had a similar effect on Dpm1 activity in the presence and absence of Dpm2. The fold change of DolP-Man formation due to the lipid

environment was found to be comparable in the presence and absence of Dpm2 for all tested lipid compositions, when the data was normalized to the respective DOPC liposomes. This finding suggests, that the positive effects of lipids and Dpm2 on Dpm1 activity are independent and do not act synergistically. It also shows, that the membrane properties are not significantly altered by the presence of Dpm2 and that the differences in Dpm1 activity were due to altered Dpm1-Dpm2 interaction.

In conclusion, I found that Dpm2 (Yil102c-A) significantly increased yeast Dpm1 activity, even though it is not essential for the mannosylation of DolP *in vitro*. Nonetheless, the Dpm1-Dpm2 interaction provides a mechanism to regulate Dpm1 activity *in vivo*. Whether this regulation can be stimulated by the lipid environment and whether Dpm2 helps in maintaining optimal Dpm1 activity even in less favorable lipid environments remains to be investigated. In addition, Dpm2 is an interesting target, to study the effect of CDG-like mutations *in vitro*, as it is not harboring the catalytically active site but activates Dpm1 and can therefore help in better understanding molecular mechanisms to regulate cellular glycosylation processes.

3.4.1.4 Dpm2 mutants and CDG

In human, Dpm2-CDG is a very rare genetic defect and only six cases have been described in literature [113, 115, 247]. Strikingly, the same Y13C point mutation in the N-terminally TMD was found in three of the cases. In all cases, this mutation resulted in drastically reduced Dpm1 activity causing hypoglycosylation. The associated decrease in DolP-Man lead to dystroglycanopathy and severe epilepsy and caused an early death of the patients. The other three patients, who had mutations in the C-terminal TMD (G58N or G66E) showed milder symptoms compared with the other patients [115, 247]. This might be due to the different localization of the mutation on the protein, which may result in different impacts on cellular function.

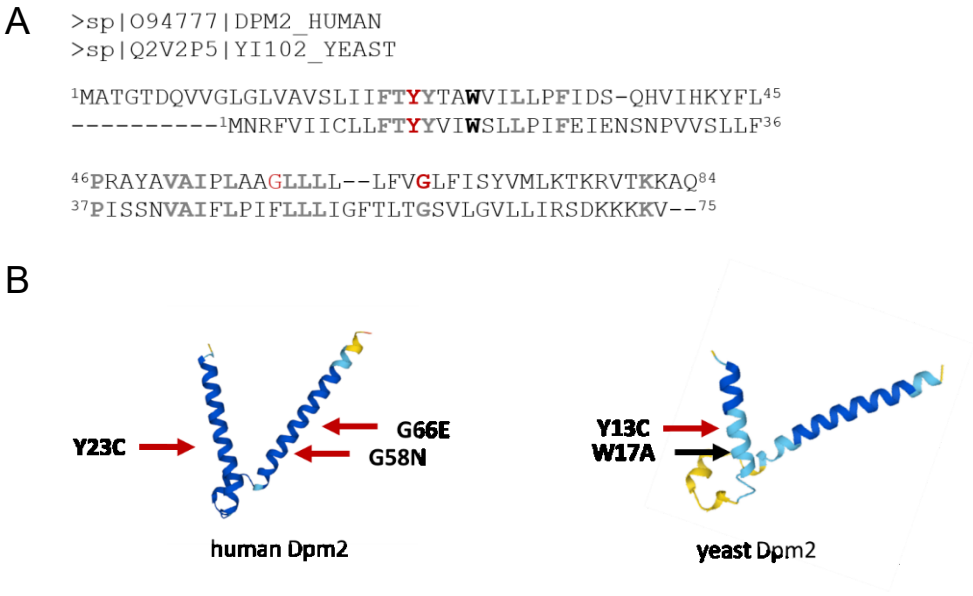


Figure 47 **Sequence alignment and structure comparison of Dpm2 from human and yeast**
A The sequence alignment shows conserved residues (bold) and the CDG-mutations found in human Dpm2 as well as the corresponding amino acids in yeast (red) and the additionally tested conserved tryptophane (black) Sequence alignment was performed using Clustal Omega program with the Uniprot FASTA files. **B** AlphaFold predicted structures of human Dpm2 and yeast Dpm2 (Yil102c-A). Arrows indicate the location of point mutations found in human Dpm2-CDG-patients (red) and the corresponding (red) and additionally tested (black) mutation in yeast Dpm2

Sequence comparison of the structurally similar human and yeast Dpm2 revealed that the mutated tyrosine residue was conserved (Figure 47). Thus, I was interested to see whether this mutation reduced Dpm1 activity *in vitro.* Hence, I tested two mutants of Dpm2, Y13C (Y corresponding to the Y23C DPM2-CDG mutation) and W17A (with the tryptophan at this position being conserved across all species analyzed) for their effect on Dpm1 activity and compared the results to wild type Dpm2 (1.1.1.1). Both mutations are located in the N-terminal TMD (Figure 47). As also the tagging at the N-terminus was disturbing Dpm1-Dpm2 interaction in yeast [122] (as discussed in 3.4.1.1), this mutation could lead to a similar loss of protein-protein interaction. However, in the liposomal *in vitro* system none of the mutations impaired Dpm1 activity. In fact, both mutant proteins could still stimulate Dpm1 and increase DolP-Man formation, similar to the wild type Dpm2. Therefore, the mutations of the yeast Dpm2 did not lead to a loss of the functional Dpm1-Dpm2 interaction, indicating that the mutated residues are not relevant for the enhancing effect of Dpm2 in yeast *in vitro.* Minor changes may not be visible using the reconstitution system, however a drastic reduction of Dpm1 activity as seen in the Dpm2-CDG patient fibroblasts [113] was not observed when using the yeast protein in the reconstitution system. More detailed interaction studies of Dpm1-Dpm2 interaction could be done using MDS, to get a better understanding on

important interaction sites and the relevance of certain residues for protein-protein interactions. Analyzing CDG mutations may give a better understanding on the mechanistic cause of the misfunction. Studying the *in vivo* interaction partners of the DPMS (e.g. by proteomics) and investigating changes in the CDG-mimicking situation could help in understanding the affected pathways and disturbed protein-protein interactions to better understand the role of Dpm2 in the glycosylation network.

It would be interesting to see, if the mutations have an effect on DolP-Man formation in yeast *in vivo*. If so, this would suggest a mechanism for regulation of mannosylation by Dpm1-Dpm2 interaction that may stabilize Dpm1 or contribute to correct localization of the enzyme. If no effect of the mutants is seen *in vivo* either, the mutation of Y23C may not be directly transferable to yeast. Even though human and yeast Dpm2 share structural features, the mode of action and effect on Dpm1 activity could be different. As Dpm1 from yeast and human differ by the presence of a C-terminal TMD in yeast, Dpm2 is not required for membrane anchoring of yeast Dpm1 and thus Dpm2 could mainly play a regulatory role.

3.4.2 Pmt4 and reconstitution of protein O-mannosylation

DolP-Man formed by Dpm1 is an important substrate for mannosylation reactions within the ER. It is used by ALG3, ALG9 and ALG12 to elongate the core structure of LLO that is then transferred onto asparagine residues for N-glycosylation [6]. In addition, DolP-Man is used by POMTs (PMTs in yeast) for O-mannosylation[6] and by the DPY family proteins for C-mannosylation [36]. DolP-Man is also required for the formation of the GPI-anchor [6]. Thus, all downstream mannosyl transferases within the ER require DolP-Man as a substrate. How the distribution of DolP-Man between the different glycosylation routes is regulated is not understood yet.

The mannosylation of a Ser or Thr residue in yeast is catalyzed by enzymes of the PMT family [17, 20]. PMTs show high similarity in their polytropic structure but differ in their substrate specificity. They can be grouped into three different families, namely Pmt1, Pmt2 and Pmt4 based on sequence homology. Whereas Pmt1 family members form heterodimers with members of Pmt2 family, Pmt4 members are found to form homodimers [17].

3.4.2.1 Successful *in vitro* reconstitution of the O-mannosylation pathway using Pmt4

The aim of this experiment was to co-reconstitute Pmt4 with Dpm1 in order to study sequential O-mannosylation starting from GDP-Man and DolP. As shown in 2.5.2.1, I could successfully co-reconstitute Dpm1 and Pmt4 into proteoliposomes. To test if the reconstituted enzymes were active, I performed an activity assay using GDP-Man as the mannose donor and a short peptide (pαDG), which was derived from α-Dystroclycan, as a final mannose acceptor [21]. The Dpm1 enzyme catalyzed the transfer of mannose from GDP-Man onto the membrane-embedded DolP acceptor and the so formed DolP-Man served as a substrate for Pmt4. As shown in 2.5.2.2, I could successfully reconstitute both proteins in their active form as seen by presence of radiolabeled mannose on the peptide. Thus, Pmt4 was able to use DolP-Man, that was formed by Dpm1, as a substrate, showing the successful reconstitution of protein O-mannosylation. The rate of mannose transfer was significantly slower for Pmt4 than for Dpm1. While DolP-Man was already detectable within seconds, peptide mannosylation was only found after approx. 15 min. No optimization regarding Pmt4 activity was done, as the assay already allowed comparison of Dpm1 and Pmt4 activity under different conditions. However, experiments could be repeated with liposomes containing more Pmt4 or more acceptor substrate to increase mannosylation of the peptide. As Pmt4 requires dimerization for full activity, an increase in Pmt4 concentration might help dimer formation. More detailed investigation on the dimerization state of Pmt4 and its dependence on DolP-Man, Dpm1 and the membrane lipid composition should be done. The optimization of enzyme ratios of Dpm1 and Pmt4 could increase mannosylation and help in understanding protein stoichiometries. Pmt4 activity may depend on a protein-protein interaction with Dpm1. In most of the assays, however, I mixed yeast Dpm1 with Pmt4 of *C. thermophilium*. For having optimal Pmt4 and Dpm1 activity and to study possible interactions, enzymes from the same species should be used. Further, different assay conditions such as temperature or lipid compositions could be tested for their effect on the enzymatic activity of the glycosylation enzymes.

DolP-Man is shuttled into all mannosylation reactions within the ER and thus there is a higher need in the enzymatic product of Dpm1. Hence, more Dpm1 is required or glycosylation enzymes may have different v_{max}. Kinetic studies of Pmt4 using different

pαDG and DolP concentrations could help in understanding whether this difference in activity could have a biological relevance. Also, in all assays presented within this thesis GDP-Man and the pαDG was added simultaneously to the same reaction mixture. Due to the different reaction rates it might be useful to decouple both reactions by sequentially adding GDP-Man and the acceptor peptide. This would also allow to study Pmt4 activity under more controlled substrate concentrations.

3.4.2.2 Orientation of Pmt4 and Dpm1

Dpm1 and Pmt4 are both ER-resident transmembrane proteins. *In vivo*, the catalytic domain of Dpm1 is facing the cytosol, whereas Pmt4 is catalyzing the transfer of mannose to the acceptor protein at the luminal side of the ER. Thus, *in vivo* the catalytic domains of the protein are facing opposite sides of the ER-membrane. However, in the liposomal reconstitution system the catalytic domains of at least the active pool of both enzymes are likely facing the same direction. This assumption is supported by the fact that both enzymes are active when presenting their soluble substrates on the outside of liposomes. This complicates the investigation of direct interactions between DPMS and Pmt4 using the liposomal assay, as the active enzymes are probably oriented in an unnatural way towards each other. Nonetheless, there could be also a population of Pmt4 oriented with its catalytic activity towards the lumen of the ER that interacts with Dpm1 and activates or stabilizes the protein. Initial experiments, however, did not indicate an enhanced activity of Dpm1 in the presence or absence of Pmt4 (data not shown). The different orientation also eliminates the need of a DolP-Man flippase in the assay. *In vivo*, to serve as a substrate for Pmt4, DolP-Man is flipped to the luminal side of the ER by a yet unidentified flippase. Theoretically flippase-activity could also be a moonlighting function of Dpm1 or Pmt4. Thus, it cannot be excluded that DolP-Man was partly flipped into the lumen of the liposomes. As in my assay both catalytic sites presumably have to face the outside of the liposomes to get access to their substrates, no translocation of DolP-Man is needed for enzymatic activity.

Similar to DolP-Man, DolP is not expected to spontaneously flip across membranes [68]. Thus, Dpm1 can only use DolP in the outer leaflet as mannosyl acceptor. In the liposomal assay, theoretically, the available DolP pool should be replenished after the Pmt4-catalyzed transfer of mannose from DolP-Man to the acceptor peptide. Thus, peptide mannosylation by Pmt4 could exceed the amount of available DolP, even though in all experiments the amount of pαDG-Man did not exceed the amount of

available DolP. This direct recycling of DolP is not possible *in vivo,* where the DolP has to be flipped back to the cytosolic side of the ER. The mechanism of DolP recycling *in vivo* is not yet understood. A flippase based mechanism is proposed for the translocation, but no flippase was identified yet [68]. Further development of the assay could be used, to test whether DolP is flipped after the transfer of the mannose by Pmt4 or if a specific flippase is required.

In conclusion, to study the sequential reaction of Dpm1 and Pmt4 in a physiological context, this reconstitution system should be further developed. Due to the unnatural orientation of at least a part of the enzymes, the “handing-over” of the DolP-Man by protein-protein interaction cannot be readily studied. However, the fact that both active sites are facing the same side also enabled me to study the transfer reaction uncoupled from DolP-Man flipping and allows further investigation of the roles of lipids in the sequential enzymatic reactions of Dpm1 and Pmt4.

3.4.2.3 Pmt4 activity in different lipid environments

As I succeeded in reconstituting Pmt4-catalyzed O-glycosylation, I was interested to investigate the role of lipids on the enzyme activity of co-reconstituted Dpm1 and Pmt4. Both proteins are resident in the ER membrane, but different spatiotemporal localization might also lead to a difference in the immediate lipid environment. In addition, lipids might regulate the interaction of Dpm1 and Pmt4, either with each other or with their respective substrates.

Hence, I prepared proteoliposomes with different lipid compositions. Since Dpm1 activity was enhanced in the presence of PE lipids, different PC:PE ratios were tested (PC:PE 1:0, 2:1, 1:1, 1:2). Proteoliposomes containing only PE were not analyzed, due to their instability and poor reproducibility. As expected from previous results (2.5.1.3) and as reported in literature [190, 193, 237], Dpm1 activity was found to be highest in liposomes composed of PC:PE 1:2. Interestingly, the opposite trend was observed for Pmt4. Highest activity of Pmt4 was seen in DOPC liposomes. This result is even more striking, considering that in this lipid composition Dpm1 activity, and thus also DolP-Man availability, was lowest. This pronounced difference in preferred membrane environment could indicate that *in vivo* both proteins are spatially separated and localized to different ER microdomains. Additionally, Pmt4 dimerization could be

enhanced in more rigid membranes compared to more fluid ones. Thus, increased stability of this complex might also lead to increased enzyme activity.

However, for these reconstitution experiments Pmt4 from *C. thermophilium* was used. Thus, differences roles of lipids in modulating the activity of Dpm1 and Pmt4 could also be caused by differences of ER lipid composition in *C. thermophilium* compared to *S. cerevisiae*. *C. thermophilium* prefers higher temperatures of approximately 50-55 °C for optimal growth [213]. Modification and adaption of the lipid membrane composition is an effective way to maintain membrane properties at altered temperatures [156, 232]. Increased saturation of fatty acid chains leads to a tighter packing and increases stability at higher temperatures. Lipidomic analysis of thermophilic fungi showed an increase in saturation of lipid chains compared to species that prefer lower temperatures [232]. Similarly, also changes in lipid saturation in plants were observed as adaption to cold temperatures [156, 248]. Here, unsaturated fatty acids were found more in winter to maintain fluidity of membranes even at lower temperatures. Also, a reduction fatty acid chain length was seen as a response to low temperatures in plants [249] and yeast [250, 251].

In summary, these findings show how proteins are differently affected by their lipid environment. Whether this is a method of the cell to regulate spatial distribution and activity of glycosylation enzymes *in vivo*, remains to be studied in further experiments.

3.4.2.4 *Sc*Pmt4 vs *Ct*Pmt4

Within this thesis, I used Pmt4 from two different species. On one hand, I used Pmt4 from *C. thermophilium*. This protein was used initially, as it showed good stability for purification. On the other hand, I used the protein from *S. cerevisiae*, that showed to be more difficult to purify as it was less stable. Both proteins were successfully reconstituted and showed activity in the presence of available DolP-Man and acceptor peptide, as presented in 2.5.2.4. Thus, both enzymes were able to use the DolP-Man as a substrate, provided by co-reconstituted Dpm1. Whether there was a chain length preference of Pmt4 for certain DolP-Man species was not analyzed. However, this would be interesting to study in future experiments, as the length of the isoprene chain could be involved in shuttling of DolP-Man into different glycosylation pathways. The developed reconstitution assay can be easily adapted to study this question. Paired with a mass spectrometric analysis of DolP-Man the assay could be used to analyze

the reduction of specific DolP-Man species. In addition, species-dependent chain length preferences could be analyzed. For this thesis, the effect of Pmt4 on Dpm1 activity was not tested, as liposomes containing only Dpm1 were not included in these initial batches. Additional experiments are required to test, whether the presence of Pmt4 effects Dpm1 activity and whether this changes by using Pmt4 of different species. Preliminary data, however, indicates that there is no enhancing effect by either *Sc*Pmt4 or *Ct*Pmt4 (data not shown).

3.4.2.5 Pmt4 and Dpm2

Besides its enhancing effect on Dpm1 activity, Dpm2 could also aid in protein glycosylation by “handing over” the DolP-Man substrate to mannosyl transferases or by stabilizing an interaction with these proteins. Thus, I started to co-reconstitute Pmt4 and Dpm1 together with Dpm2 and compared Pmt4 activity to liposomes without Dpm2. The aim of this experiment was to investigate, whether the addition of Dpm2 affects the mannosyl transfer of Pmt4 onto pαDG. Preliminary data shows that the mannosyl transfer by Pmt4 was indeed enhanced in the presence of Dpm2 (results not shown). CDG-like mutations of Dpm2 (Y13C) showed the same effect on Pmt4 activity as the wild type protein. As previously shown, also Dpm1 activity was enhanced in the presence of Dpm2 and thus more DolP-Man substrate was formed. As the enzymatic rate is also dependent on the substrate concentration, the increase in Pmt4-catalyzed peptide mannosylation could not only be due to a stabilizing effect of a potential Dpm1-Dpm2-Pmt4 complex, but also due to increased DolP-Man availability. To study the effect of DolP-Man concentration on Pmt4 activity in the presence of Dpm2, a different setup is needed. Since the DolP-Man substrate cannot be added additionally, as it is not commercially available, its availability is dependent on co-reconstituted Dpm1. Decoupling the two catalytic reactions could offer a possibility to study Pmt4-Dpm2 interaction independent from Dpm1 activity. Giving enough time, Dpm1 would mannosylate all available DolP independent of the Dpm1-catalyzed reaction rate in the membrane environment. Thus, at the endpoint of the Dpm1 reaction DolP-Man concentrations should be comparable. Dpm1 activity can be stopped by the removal of excess GDP-Man, and subsequently initiated Pmt4 activity is therefore independent from the rate of DolP-Man formation. This setup would allow the comparison of Pmt4 activity in the presence and absence of Dpm2.

In summary, the results show that the liposomal reconstitution assay is an expandable system. For the future, it would be interesting to also include other mannosyl transferases and study the effect of lipids on additional glycosylation enzymes and DolP-Man channeling into different pathways.

3.5 Clinical relevance and the interplay of glycosylation and lipids

One essential lipid class in glycosylation are the dolichol derivatives. DolP-Man and DolP-Glc are important sugar donors for glycosylation in the ER. In addition, DolPP serves as a membrane platform for LLO assembly required for N-glycosylation. The connection between dolichol synthesis and glycosylation defects is also recognized by genetic defects of dolichol biosynthesis, such as DHDDS-CDG, SRD5A3-CDG, NUS1-CDG or DOLK-CDG [252, 253]. Besides the specific interaction of glycosylation enzymes of the ER with dolichol species, also the bulk membrane lipids have an effect on the proteins. All glycosyltransferases are membrane resident proteins or form complexes localized at the membrane and thus can be affected by the lipid environment. Within this thesis, I specifically studied the role of lipids on Dpm1 activity, as a representative of glycosyltransferases. I could show, that Dpm1 can be directly affected and regulated by the immediate lipid environment. The results further indicate, that Dpm1 is regulated by the overall membrane properties, rather than by interaction with a specific lipid species. Thus, modulation of the membrane composition could also be used to improve glycosylation reactions *in vitro* and *in vivo*. In cell culture experiments using Dpm1-CDG fibroblasts, Dpm1 activity was found to be improved when DolP availability was increased by inhibiting squalene synthase using zaragozic acid [254]. In addition to increased DolP levels, this inhibition also leads to reduced cholesterol levels in human skin fibroblasts. As I found sterols being inhibitory for Dpm1 activity, thus, this *in vivo* change in membrane composition may increase Dpm1 activity. The membrane lipids might also regulate protein-protein interactions by spatiotemporal protein localization through nanodomain formation or by direct interaction with glycosylation complexes.

The findings of this work show the interconnection of glycosylation and lipid homeostasis and that the membrane resident glycosylation enzymes can be directly affected by the membrane lipid composition. Changes in the lipid compositions lead to a modulation of Dpm1 enzyme activity, thus underlining the interconnection of lipids and glycosylation. Therefore, not only defects in glycosylation enzymes, but potentially

also problems in maintaining lipid homeostasis could lead to hypoglycosylation. Further, glycosylation efficiency could be improved by modulating membrane composition. The results presented here are a first step towards understanding the role of lipids in glycosylation, which could eventually also help in identifying new approaches in treatment of CDG patients.

4 Materials and methods

4.1 List of chemicals

Abbreviation	Compound	Company
AcOH	Acetic acid	Honeywell, Morristown, USA
BSA	Albumin fraction V	Roth, Karlsruhe, Germany
NH_3	Ammonia	Honeywell, Morristown, USA
APS	Ammoniumperoxidisulfate	Roth, Karlsruhe, Germany
Amp	Ampicillin sodium salt	SigmaAldrich, Munich, Germany
	Bacto Tryptone	BD Biosciences, Franklin Lakes, USA
	Bacto Yeast Extract	BD Biosciences, Franklin Lakes, USA
	Bio-Beads SM2	Bio-Rad, Hercules, USA
	Blotting paper MN827B	Macherey Nagel, Germany
	Bromophenol blue	Waldeck, Muenster, Germany
$CHCl_3$	Chloroform	Honeywell, Morristown, USA
	cOmplete™, Mini, EDTA-freier Protease-Inhibitor-Cocktail	Roche, Rotkreuz, Switzerland
	Coomassie BrilliantBlue R250	Serva, Heidelberg, Germany
Na_2HPO_4 x$2H_20$	Disodium hydrogen phosphate dihydrate	Honeywell, Morristown, USA
DTT	1,4-Dithio-D,L-threitol	Gerbu, Heidelberg, Germany
SDS	Dodecylsulfate-Na-salt (pellets)	Serva, Heidelberg, Germany
EtOH	Ethanol	Honeywell, Morristown, USA
	Glycerol	SigmaAldrich, Munich, Germany
	Glycin	Labochem international
	Guanosine 5′-diphospho-D-mannose sodium salt from Saccharomyces cerevisiae	SigmaAldrich, Munich, Germany
	Guanosine diphosphate D-mannose [6-3H] (ART-0723)	Biotrend, Germany
	High performance Ni-Sepharose	GE Healthcare
	HPLC-grade water	FisherScientific
HCl	Hydrochloric acid	Honeywell, Morristown, USA
	illustra™ NICK™ columns	GE Healthcare
	Imidazole	Honeywell, Morristown, USA
	Immobilon-FL transfer membrane	MerckMillipore, Darmstadt, Germany
	InstantBlue (coomassie stain)	Expedeon, Swavesy, Cambridge
iProp	Isopropanol	Honeywell, Morristown, USA
IPTG	Isopropyl-β-D-thiogalactopyranosid	Gerbu, Heidelberg, Germany
Kan	Kanamycin sulfate	SigmaAldrich, Munich, Germany
LDAO	Lauryldimethylamine oxide	Cube Biotech, Germany
$MgCl_2$	Magnesium chloride hexahydrate	MerckMillipore, Darmstadt, Germany
	MES buffer	invitrogen
MeOH	Methanol	Honeywell, Morristown, USA
DDM	n-Dodecyl-β-Maltoside	Roth, Karlsruhe, Germany
	NICK column (Sephadex G-50 DNA Grade)	GE Healthcare

Sarkosyl	N-Lauroylsarcosin sodium salt	SigmaAldrich, Munich, Germany
	NUPAGE 4-12% BisTris gel from Life Technologies GmbH	Invitrogen, Waltham, USA
NP-40	Nonidet P-40 substitute	Roche, Rotkreuz, Switzerland
FosChol13	n-Tridecyl-phosphocholine	Serva, Heidelberg, Germany
OG	Octyl-β-D-glucopyranosid	Roth, Karlsruhe, Germany
	Powdered milk	Roth, Karlsruhe, Germany
	precast gels	invitrogen
PM	Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad, Hercules, USA
PM	protein marker	BioRad
	Revert™ 700 Total Protein Stain	LI-COR Biosciences, Lincoln, USA
	ROTIPHORESE®Gel 30 (37,5:1)	Roth, Karlsruhe, Germany
	Saccharose (=Sucrose)	Roth, Karlsruhe, Germany
NaCl	Sodium chloride	Labochem international
	Sodium Deoxycholate	SigmaAldrich, Munich, Germany
NaH_2PO_4 xH_2O	Sodium dihydrogen phosphate monohydrate	Honeywell, Morristown, USA
NaOH	Sodium hydroxide	SigmaAldrich, Munich, Germany
TEMED	Tetramethylethylendiamin	Roth, Karlsruhe, Germany
	TLC Silica gel 60, alumninum backed	MerckMillipore, Darmstadt, Germany
TRIS	Tris-(hydroxymethyl)-aminomethan	Roth, Karlsruhe, Germany
	Triton X-100	MerckMillipore, Darmstadt, Germany
	Tween-20	Roth, Karlsruhe, Germany
	Ultima Gold™ Universal LSC-Cocktail	PerkinElmer, Waltham, USA
	Urea	SigmaAldrich, Munich, Germany

4.2 List of antibodies

Primary antibodies used for Western blot were diluted in PBS containing 3% (w/v) BSA, 0.1% (v/v) Tween20. Secondary antibodies were diluted 1:10000 in PBS containing 2% (w/v) BSA, 0.1% (w/v) Tween20. All antibody baths were stored at -20 °C and used up to 10 times.

Antibody	**Origin, clonality**	**Manufacturer**	**Order number**	**Dilution**
Anti-polyhistidine antibody	Mouse, mono	Sigma	H1029	1:1000
Anti-DPM1 antibody	Mouse, mono	Abcam	ab113686	1:500
Anti-MBP antibody	Mouse, mono	NEB	E8032	1:4000
Anti-mouse IgG (H+L), AlexaFluor® 680 Conjugate (WB)	Goat, poly	Thermo Fischer Scientific	A-21057	1:10000
Anti-mouse IgG (H+L), IRDye® 800CW Conjugate (WB)	Donkey, poly	Rockland	600-145-098	1:10000

4.3 List of lipids

Avanti/order no	Lipid	Abbreviation	M [g/mol]	c [mg/ml]	c [mM]
850375	1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 18:1 PC)	**DOPC**	786.1	25	31.80
850725	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1 18:1 PE)	**DOPE**	744.0	25	33.60
850467	1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (18:0 18:0 PC)	**SOPC**	788.1	25	31.72
850457	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine	**POPC**	760.1	25	32.89
850757	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine	**POPE**	718.0	25	34.82
860052	Ceramide (Brain, Porcine)	**Cer**	565.9	1	1.77
Fluka 45480	Ergosterol	**Erg**	396.6	10	25.21
810150	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)	**RhPE**	1301.7	1	0.77
Larodan	C75-Dolichyl monophosphate	**DolP C75**	1155.9	10	8.65
900201	Dolichol (13~21) phosphate	**DolP Mix**	Av.1286.5	1	0.78
900200	Dolichol (13~21)	**Dol Mix**	?	1	

4.4 Buffer and solutions

All buffers were prepared using ddH_2O.

Buffer	Composition
Blocking solution	5% milk in PBS-T
Coomassie stain	0.1% Coomassie, 40% MeOH, 10% AcOH
Elution buffer	20 mM Tris/HCl pH 7.4, 100 mM NaCl, 300 mM imidazole

5x Laemmli buffer	250 mM Tris/HCl pH 6.8, 8% SDS, 20% Glycerol, some bromophenolblue, 200 mM DTT (freshly added from 1M stock, frozen at -20 °C)
LB-media	10 g Tryptone, 5 g Yeast extract, 5 g NaCl per 1 l
Liposome buffer	20 mM Tris/HCl pH 7.4, 100 mM NaCl
Lysis buffer	20 mM Tris/HCl pH 7.4, 100 mM NaCl, 20 mM imidazole, 0.5 mM DTT
NuPAGE™ MES SDS-Laufpuffer (20x)	1x MES buffer: 50 mM MES, 50 mM TRIS, 0,1 % (w/v) SDS, 1 mM EDTA, pH 7.3
10x PBS	80 mM Na_2HPO_4 x $2H_2O$ (14.24 g), 20 mM NaH_2PO_4 x H_2O (3.12 g), 1.4M NaCl (81.9 g) pH 7,0 (per l H_2O)
PBS-T	1x PBS + 0.1% Tween
Sarkosyl stock	10% (w/v) in ddH_2O
1x SDS running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS
Separation buffer	1.5 M Tris/HCl pH 8.8, 0.4 % SDS
Stacking buffer	1.5 M Tris/HCl pH 6.8, 0.4 % SDS
Transfer buffer	25 mM Tris, 192 mM glycine in MeOH/H_2O 1:5

4.5 List of strains and plasmids

Cloning and transformation were performed by Alexia Herrmann (Dpm1) and Thomas Kupke (Dpm2).

Strain	**Plasmid**	**Protein**	**Resistance**
BL21	pet28a	ScDpm1-His	Kan
T7 Express	pETDuet1	Dpm2-His-TEV-MBP	Amp
T7 Express	pETDuet1	MBP-TEV-His-Dpm2	Amp
T7 Express	pETDuet1	Dpm2-W17A-His-Tev-MBP	Amp
T7 Express	pETDuet1	Dpm2-Y13C-His-Tev-MBP	Amp

4.6 Protein expression and purification

4.6.1.1 Expression of Dpm1 in *E.coli*

His tagged *S.cerivisiae* Dpm1 was over expressed in *E.coli*. In detail, an overnight culture in 50 ml LB-media, containing 5 mg/l Kanamycin was prepared. The next day, 2 l pre-warmed LB-media containing 5 mg/l Kanamycin were inoculated with 4 ml of cells from the overnight culture and cells were grown at 37 °C, shaking at 180 rpm for

approx. 3h. Protein expression was induced at OD 0.6-0.8 by the addition of 0.3 mM IPTG. After 3 h cells were harvested by centrifugation at 4000 rpm for 15 min. 2 l of culture usually resulted in 2-3 g of wet pellet. Cells were washed once with PBS before the pellet was snap frozen and stored at -80 °C.

4.6.1.2 Cell lysis

Cell pellet from 2 l expression was thawed in a RT water bath and suspended in 20 ml of cold lysis buffer and supplemented with 1 tablet of protease inhibitor (Roche cOmplete EDTA free). Cells were lysed either using a microfluidizer or by probe sonication. When using the microfluidizer, cells were lysed for approx. 10 cycles until the lysate was visibly clear. Instrument was flushed with lysis buffer to wash out all lysate. The final volume of the lysate was approx. 30 ml. Sonication was performed using a probe sonicator (Branson Sonifier 250; settings: Duty cycle %:40, Output control 5) 2x 5 min with 5 min cooling on ice in-between. The lysate was split into 2 parts and either used directly for purification or snap-frozen and stored at -80 °C.

4.6.1.3 Detergent screen for Dpm1

The solubility of Dpm1 in different detergents was tested, by mixing 150 µl of cell lysate with different detergents and lysis buffer to a final volume of 500 µl containing 1% of the detergent. Samples were incubated for 30 min rotating at RT before they were ultra-centrifuged at 153.700 xg for 45 min using a TLA-55 rotor in an Optima MAX-XP Ultracentrifuge (Beckman Coulter). The supernatant was taken and checked for the presence of Dpm1 by western blotting using anti-His antibody.

4.6.1.4 Optimization of detergent concentration for Dpm1 solubilization

150 µl of lysate were incubated with 0-2% Sarkosyl in a total volume of 200 µl. After incubation for 15 min at RT, samples were ultra-centrifuged at 153.700 xg for 45 min using a TLA-55 rotor in an Optima MAX-XP Ultracentrifuge (Beckman Coulter). The supernatant was taken and checked for the presence of Dpm1 by western blotting. Protein concentrations were compared by quantifying band intensities after development with anti-Dpm1 antibody.

4.6.1.5 Purification of Dpm1

Lysate of 1 l cell culture was adjusted to approx. 15 ml and 0.75% of Sarkosyl were added (from a 10% stock in H_2O). Proteins were solubilized for 15 min on ice and lysate was centrifuged at 35000 rpm (147.800xg) for 1 h at 4 °C. Supernatant was transferred onto 1 ml of Ni-Bead slurry that were prewashed 1x with 10 ml elution buffer and 3x with 10 ml of lysis buffer. Mixture was incubated for 1 h rotating in the cold before supernatant was removed from the beads after short centrifugation for 2 min at 2000 rpm (751xg). The beads were washed 3x batch-wise using 10 ml of lysis buffer containing 0.75% Sarkosyl before being transferred to an empty column. Beads were washed once more with 10 ml of lysis buffer containing 0.75% Sarkosyl before proteins were eluted by the addition of 2 ml of elution buffer containing 0.75% Sarkosyl. 8x 250 µl fractions were collected and tested for the presence of protein using Nanodrop measurement. Fractions containing protein were pooled, aliquoted and snap frozen and stored at -80 °C until further use.

4.6.1.6 Expression and Purification of Dpm2

Expression and purification of Dpm2-His-Tev-MBP and MBP-Tev-His-Dpm1 were done in the same way as for Dpm2. Only, 100 mg/l ampicillin was used instead of kanamycin.

4.6.2 Pmt4

His-tagged *Ct*Pmt4 and His-tagged *Sc*Pmt4 purifications in 0.01% LMNG (final buffer: 200 mM NaCl, 20 mM HEPES, pH 7.5, 0.01% LMNG) were kindly provided by Antonella Chiapparino and Melanie McDowell. Proteins were aliquoted and stored at -80 °C until use.

4.7 Liposome preparation and protein reconstitution

4.7.1 Liposome preparation

5 µmol of total lipids in $CHCl_3$ were mixed in a Wheaton glass tube in indicated ratios. In general, the amount of DolP (~1 mol%, 50 µg) and RhPE (0.2 mol%) was kept constant if not indicated differently. The solvent was evaporated under a stream of N_2. Lipids were further dried in an exicator under reduced pressure over night and then

stored at -20 °C until further use. For liposome preparation, dried lipids were hydrated with 200 µl of liposome buffer and vortexing. Suspended lipids were subjected to 10x freeze thaw cycles by alternative placing of the tube into liquid N_2 and a 40 °C water bath and short vortexing in-between. Liposome mixture was further extruded 21x through a 100 nm filter, using an Avanti Mini Extruder. The extruder was washed once with 200 µl of buffer and wash was added to extruded liposomes, yielding in 400 µl of 12.5 mM liposome solution.

4.7.2 Protein reconstitution

Extruded liposomes were split into 5 aliquots of 80 µl (each 1 µmol of total lipids). Liposomes were destabilized by the addition of 0.75% Sarkosyl (25.6 mM) and incubated for 30 min, shaking at 1000 rpm at 25 °C. Purified protein was added (~130 pmol) and mixture was further incubated for 30 min shaking. Liposome-detergent mixture was diluted under the CMC by addition of buffer to a final volume of 400 µl and samples were incubated additional 30 min, shaking at RT. Bio-Beads SM-2 were equilibrated by sonication for 5 min 1x in MeOH followed by 2x H_2O (equilibrated beads are sedimenting and can be stored in water for several weeks). 40 µl of Bio-Bead slurry were taken (using a 200 µl pipette tip that was cut at “50 µl”) and the supernatant was removed. Samples were added onto beads and detergent was removed for 1h, shaking (1000 rpm) at RT. Samples were shortly centrifuged (2 min, 14000 rpm) and the supernatant was taken and added onto a fresh aliquot of beads and further detergent removal for 1.5 h. Samples were shortly centrifuged (2 min, 14000 rpm) and the supernatant containing proteoliposomes was taken. Liposomes were split into aliquots, snap frozen in liquid nitrogen and stored at -80 °C until further use.

4.7.3 Sucrose gradient floatation

250 µl of liposomes were mixed with 190 µl of 75% sucrose in liposome buffer. Sample was filled into a 0.7 ml centrifuge tube (Herolab, 252020) and overlayed with 200 µl of 25% sucrose in liposome buffer and 50 µl of liposome buffer. Samples were centrifuged in a swing bucket rotor (Beckman SW60) at 164.800 xg for 1.5 h. 50 µl of the top fraction and the bottom fraction were collected.

4.8 Method optimization and quality control

4.8.1 Size of liposomes

The size of liposomes was measured by DLS using a DynaPro NanoStar instrument (Wyatt). Acquisition was done using Dynamics software (Version 7.9.05) with standard settings in a range of 0.5-10000 nm, allowing polydispersity. Acquisition was set to 5 s and 15 acquisitions per sample. Temperature was 25 °C. A globular model was chosen and buffer density dn/dc was customized to 0.9793 ml/g (values were calculated using SEDNTERP 3 Version 3.0.3)

4.8.2 SDS-PAGE and Western blot

In general, samples were mixed prior loading with 5x Laemmli buffer containing to 1x concentration and heated prior loading to 95 °C for 5 min.

Proteins were separated by SDS-PAGE, using either a 12% self-casted SDS-gel or a NUPAGE 4-12% BisTris gel from Life Technologies GmbH. The composition of self-casted gels can be found in Table 4.

Table 4 **Composition of SDS-gel**

separation gel			**stacking gel**		
reagent	**volume**	**final conc.**	**reagent**	**volume**	**final conc.**
H_2O	3.2 ml		H_2O	1.8 ml	
1.5 M Tris/HCl pH 8.8	2.6 ml	0.4 M	1.5 M Tris/HCl pH 6.8	0.75 ml	0.4 M
with 0.4 % SDS		0.1%	with 0.4 % SDS		0.1%
Rotipherose 30%	4.2 ml	12.5%	Rotipherose 30%	0.75 ml	4.4%
APS 10%	0.1 ml		APS 10%	0.05 ml	
TEMED	0.02 ml		TEMED	0.005 ml	

Pre-stained marker (Precision Plus Protein All Blue Prestained) was loaded as reference. After loading of the samples, gels were run at 180 V for approx. 40 min. Pre-casted gels were run in MES buffer using a XCell SureLock Mini-Cell electrophoresis system. Self-casted gels were run using a Mini-PROTEAN® Tetra System in 1x SDS buffer.

To detect proteins from purifications and to check for impurities, gels were stained with Coomassie for about 2h at RT. Unbound stain was removed by incubation in 5% AcOH

in H_20 overnight and after short wash with H_2O the gel was scanned using a commercial scanner.

To check for protein in liposome preparations, separated proteins were blotted onto PVDF membranes using wet tank blotting (Mini-PROTEAN® Tetra System). In short, membranes were activated for 30 s in MeOH and a “blotting-sandwich” was assembled (2x blotting paper – gel – membrane – 2x blotting paper). The sandwich was placed into the chamber containing transfer buffer and an ice block was added. Proteins were transferred either at 100 V for 1.5h or at 30 V overnight in the cold. Membranes were blocked in skimmed milk (5% (w/v) in PBS-T for 45 min at RT. Incubation with primary and secondary antibody was performed for 1h at RT or at 4 °C overnight. Membranes were washed after blocking and incubation with each antibody thoroughly 3x with PBS-T for 10 min. To detect protein bands, membrane was scanned using LiCor Odyssey CLx with standard settings.

4.8.3 Mass spectrometric detection of Dpm1

The presence of Dpm1 in the eluate fraction was confirmed by mass spectrometry, performed by the Core Facility for Mass Spectrometry & Proteomics (CFMP) at the ZMBH. Proteins were digested with trypsin and analyzed by LC-MS. Protein was identified using Proteome Discoverer 2.3.0.523.

4.8.4 DolP quantification

4.8.4.1 Rh-PE fluorescence

A calibration curve was prepared in duplicates (5 points, final concentration 0-5 µM), by pipetting Rhodamine-PE from $CHCl_3$-stocks into 1.5 ml Eppendorf tubes. Lipids were dried under reduced pressure and resuspended in 50 µl of liposome buffer containing 1% OG by gentle shaking. To determine the lipid content of different liposome preparations, 10 µl of liposomes were mixed with 5 µl of 10% OG in liposome buffer and 35 µl of liposome buffer. All solutions were transferred into a Grainer black well plate and fluorescence was measured using a SpectraMax M5 (molecular devices) plate reader. Software: SoftMaxPro Parameters set: λExt = 550 nm, λEm = 590 nm, 25 °C.

4.8.4.2 Lipidomics analysis of PC

Liposomes were diluted 1:500 in ammonium bicarbonate buffer pH 7.5 and subjected to acidic Bligh&Dyer (SBD) lipid extractions in the presence of internal PC lipid standards (phosphatidylcholine, 13:0/13:0, 14:0/14:0, 20:0/20:0; 21:0/21:0, Avanti Polar Lipids). Lipids recovered in the organic phase were dried under a stream of nitrogen. The dried lipids were dissolved in 10 mM ammonium acetate in methanol and transferred to a 96-well plate (Eppendorf twintec plate 96). Mass spectrometric measurements were performed in positive ion mode on an AB SCIEX QTRAP 6500+ mass spectrometer, equipped with chip-based (HD-D ESI Chip, Advion Biosciences) nano-electrospray infusion and ionization (Triversa Nanomate, Advion Biosciences) as described. PC was detected by precursor ion scanning of +184. Data was analyzed using LipidView (ABSciex). The amount of DolP was calculated as 1% of the PC content.

4.8.4.3 DolP analysis by mass spec

50 µl of proteoliposomes preparation was mixed with 50 µl of buffer and subsequently diluted by 1:1 by the addition of MeOH. 40-80 µl of the mixture were taken and DolP was extracted in the presence of 10 pmol PolP C60 as internal standard and 10 pmol DolP C55 as internal control using Bligh&Dyer (BD) lipid extraction. In short, 1.9 µl of $CHCl_3$/MeOH 1:2 were added to the sample. After quick vortexing, phase separation was induced by the sequential addition of 0.5 ml of $CHCl_3$ and 0.5 ml of H_2O. Samples were vigorously shaken for 10 min, centrifuged for 2 min at 2000 rpm and the organic phase was transferred to a fresh tube. The organic phase was washed once by the addition of 0.5 µl H_2O. To recover more lipids, the initial aqueous phase was reextracted by the addition of 0.5 ml of $CHCl_3$, washed once more and the combined organic phases were dried under a stream of N_2.

Methylation of extracted lipids with TMSD and LC-MS analysis was performed as published in Kale et al. [53]

4.8.5 Theoretical calculations of lipid molecules per liposome

The theoretical number of liposomes were calculated using the following equation.

$$lipids\ per\ liposome = \frac{4\left[\frac{d}{2}\right]^2 \pi + 4\left[\frac{d-2m}{2}\right]^2 \pi}{a_{lipid}}$$

With d being the average liposomal diameter, m being the membrane thickness and a_{lipid} being the surface area per lipid.

4.9 Activity assay

4.9.1.1 DolP-Man extraction, TLC and ß-imager

Membrane fractions (prepared by Alexia Hermann)

Yeast strain SEY 6210, overexpressing flag-tagged Dpm1 was used. Yeast was grown in 100 ml YPD media at 30 °C, shaking at 180 rpm overnight. The cells were harvested by centrifugation at 4000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 50 ml PBS containing proteases inhibitor. Cells were lysed using 25 ml of glass beads (0.5 mm) in a Precellys instrument (2x 20 s at 5500 rpm). Membranes were pelleted by centrifuging at 500 g for 10 min and the supernatant was discarded. The pellet was resuspended in 1 ml of PBS + 0.5% Triton for 15 min at RT before 0.18% deoxycholate were added and membranes were further solubilized for another 15 min at RT. Samples were centrifuged at 100000 g at RT for 45 min and the supernatant was aliquoted and snap frozen as solubilized membrane fractions.

Assay

For inactivation of endogenous enzyme activity, membrane fractions were heated to 70 °C for 10 min in a heating block. 100 µl yeast membrane fractions either active or inactivated, 4-16 µl Dpm1 eluates, 20 µl liposomes and detergent (0.5%) were mixed in indicated ratios and filled to a total volume of 135 µl by addition of H_2O. A 10x GDP-Man master mix was prepared, containing 50 mM Tris/HCl pH 7.4, 50 mM $MnCl_2$, 70 mM $MgCl_2$, 250 nM GDP-Man (40 Ci/mmol). The assay was started by the addition of 15 µl GDP-Man master mix and performed at RT for 20 min. Final reaction conditions: 5 mM Tris/HCl pH 7.4, 5 mM $MnCl_2$, 7 mM $MgCl_2$, 25 nM GDP-Man (0.15 µCi/sample). The enzymatic reaction was stopped by addition of 1 ml $CHCl_3$-MeOH (2:1). DolP-Man product was extracted into the organic phase after the addition

of 500 µl H2O and votexing for 30 s. Samples were centrifuged at 4 °C for 5 min and the organic phase was collected. Sample was extracted a second time into 1 ml $CHCl_3$-MeOH (2:1) and the combined organic phases were washed once with 2 ml of H_2O. The organic phase, containing the extracted product, was dried under a stream of N_2. The dried extract was resuspended in 10 µl $CHCl_3$-MeOH (2:1) and spotted on a TLC (silica 60, 20x20 cm, aluminum). TLC was developed in $CHCl_3$/MeOH/H_2O/AcOH (65:35:4:1). After drying, TLC was imaged using a ß-imager 2000 (Biospace, Paris, France). Default manufacturer settings were chosen, with ^3H as measured isotope, full field scan and 24 h acquisition time.

4.9.1.2 Size exclusion column and scintillation counter

Usually, 20-50 µl of liposome preparations were analyzed in a total reaction volume of 100 µl. Liposomes were diluted to 60 µl in liposome buffer and supplemented with 5 mM $MgCl_2$. A 2.5x GDP-Man master mix was prepared, containing 50 µM GDP-Man (spiked with 0.3% of tritiated GDP-Man) in 20 mM Tris/HCl pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$.

NICK columns were equilibrated with 2 column volumes of buffer. Liposomes and GDP-Man master mix were equilibrated at 25 °C in a thermoblock. The reaction was started by adding 40 µl of mastermix to samples. Samples were shortly mixed by pipetting up and down and the reaction was carried out in a thermoblock at 25 °C. After t (usually 1 min), 90 µl of the reaction mixture were taken and applied onto a NICK-column. Liposomes were immediately eluted by the addition of 770 µl of liposome buffer. Excess of GDP-Man was eluted in a second fraction by the addition of 1.7 ml of liposome buffer.

To measure transfer of radioactivity, 700 µl of each fraction and 5 µl of input with 695 µl of buffer were mixed with 5 ml of scintillation cocktail and radioactivity was measured using a Scintillation counter (LS-6000TA Beckmann Coulter, Brea, USA). Measurements were set to 1.5 min and to 3H isotope. The resulting average CPM were used for calculation of mannose transfer.

To calculate the mannose transfer, CPM of the liposomal fraction were compared to CPM of either input or the total eluted radioactivity (sum CPM of liposomal and GDP-Man fraction). In general, both calculation methods gave the same results. The

pmol-values were calculated as % of the total GDP-Man input using the following formulas:

$$\%\ transfer = \frac{CPM_{liposomes} * \frac{860}{700}}{CPM_{input} * \frac{100}{5}}$$

$$\%\ transfer = \frac{CPM_{liposomes} * \frac{860}{700}}{CPM_{liposomes} * \frac{860}{700} + CPM_{GDP-Man} * \frac{1700}{700}}$$

$$pmol_{DolPMan} = \%transfer * pmol_{GDP-Man\ input}$$

4.9.1.3 Activity of Pmt4

To analyze Pmt4 activity, an adapted protocol of Bausewein et al. 2016 [21] was used. Dpm1 and Pmt4 were co-reconstituted and the assay was carried out as described in 4.9.1.2, with the difference that 40 µM pαDG (biotinylated α-dystroglycan peptide (401-420), from a 4 mM stock in ddH_2O) was added to the reaction mixture. Reactions were carried out for a defined time (at least 15 min). Mannosylated peptide was only found in the GDP-Man fraction after size exclusion column. It was recovered by incubating the GDP-Man fraction with a two times buffer washed 20 µl slurry aliquot of High Capacity Neutravidin agarose (Thermo Scientific) for 1 h shaking at 1000 rpm at 4 °C. After binding, the beads were washed two times with liposome buffer plus 1% Triton X-100 and two times with liposome buffer. The supernatant was removed after each step and centrifugation at 20000xg for 5 min. After removal of the final supernatant, the beads were slurred in 700 µl of liposome buffer and transferred to a scintillation vial containing 5 ml of scintillation mix. Incorporated radioactivity was measured by liquid scintillation for 1.5 min.

4.9.1.4 DolP-Man detection by mass spectrometry

Mass spectrometric analysis of DolP-Man was performed as described for DolP (4.8.4.3), with the difference that liposomes were incubated in with 40 µM GDP-Man at RT for 1h before the reaction was stopped by the addition of the same volume of MeOH.

4.10 Normalization and data analysis

The amount of transferred mannose (pmol) was calculated using the concentration of GDP-Man in the assay and the % of radioactivity found associated with the liposomal fraction as described in 4.9.1.2The amount of DolP was determined indirectly by quantifying the PC content of the liposomes via mass spec (4.8.4.2) and calculating the DolP concentration as 1% of total lipids. The protein concentration was calculated using western blot quantification by comparison of band intensities of samples to protein standard (eluate) of known concentration. Blots were analyzed using Image Studio Lite Ver 5.2. As the reconstitution efficiency was not the comparable between batches, the enzyme activity was normalized to the protein amount (pmol DolP-Man/pmol Dpm1).

Calculations were performed using Microsoft Excel 2019.

Statistical analysis was performed in GaphPad Prism 9.5.1 or 10.0.2. Outliers were removed using the inbuilt outlier test. Statistical analysis was performed using a paired ratio t-test or paired t-test.

5 References

[1] Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in Cellular Mechanisms of Health and Disease, *Cell 126*, 855-867.

[2] Flynn, R. A., Pedram, K., Malaker, S. A., Batista, P. J., Smith, B. A. H., Johnson, A. G., George, B. M., Majzoub, K., Villalta, P. W., Carette, J. E., and Bertozzi, C. R. (2021) Small RNAs are modified with N-glycans and displayed on the surface of living cells, *Cell 184*, 3109-3124.e3122.

[3] Varki, A. (2017) Biological roles of glycans, *Glycobiology 27*, 3-49.

[4] Schjoldager, K. T., Narimatsu, Y., Joshi, H. J., and Clausen, H. (2020) Global view of human protein glycosylation pathways and functions, *Nature Reviews Molecular Cell Biology 21*, 729-749.

[5] Albesa-Jové, D., Cifuente, J. O., Trastoy, B., and Guerin, M. E. (2019) Chapter Fifteen - Quick-soaking of crystals reveals unprecedented insights into the catalytic mechanism of glycosyltransferases, In *Methods in Enzymology* (Shukla, A. K., Ed.), pp 261-279, Academic Press.

[6] Maeda, Y., and Kinoshita, T. (2008) Dolichol-phosphate mannose synthase: Structure, function and regulation, *Biochimica et Biophysica Acta (BBA) - General Subjects 1780*, 861-868.

[7] Lehle, L., Strahl, S., and Tanner, W. (2006) Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases, *Angew Chem Int Ed Engl 45*, 6802-6818.

[8] Schwarz, F., and Aebi, M. (2011) Mechanisms and principles of N-linked protein glycosylation, *Curr Opin Struct Biol 21*, 576-582.

[9] Breitling, J., and Aebi, M. (2013) N-linked protein glycosylation in the endoplasmic reticulum, *Cold Spring Harb Perspect Biol 5*, a013359.

[10] Helenius, J., Ng, D. T., Marolda, C. L., Walter, P., Valvano, M. A., and Aebi, M. (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein, *Nature 415*, 447-450.

[11] Kaplan, H. A., Welply, J. K., and Lennarz, W. J. (1987) Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly, *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes 906*, 161-173.

[12] Marshall, R. D. (1974) The nature and metabolism of the carbohydrate-peptide linkages of glycoproteins, *Biochem Soc Symp*, 17-26.

[13] Petrescu, A.-J., Milac, A.-L., Petrescu, S. M., Dwek, R. A., and Wormald, M. R. (2004) Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding, *Glycobiology 14*, 103-114.

[14] Kellokumpu, S., Hassinen, A., and Glumoff, T. (2016) Glycosyltransferase complexes in eukaryotes: long-known, prevalent but still unrecognized, *Cellular and Molecular Life Sciences 73*, 305-325.

[15] Leroy, J. G. (2006) Congenital Disorders of N-Glycosylation Including Diseases Associated With O- as Well as N-Glycosylation Defects, *Pediatric Research 60*, 643-656.

[16] Kelleher, D. J., Karaoglu, D., Mandon, E. C., and Gilmore, R. (2003) Oligosaccharyltransferase Isoforms that Contain Different Catalytic STT3 Subunits Have Distinct Enzymatic Properties, *Molecular Cell 12*, 101-111.

[17] Neubert, P., and Strahl, S. (2016) Protein O-mannosylation in the early secretory pathway, *Curr Opin Cell Biol 41*, 100-108.

[18] Neubert, P., Halim, A., Zauser, M., Essig, A., Joshi, H. J., Zatorska, E., Larsen, I. S. B., Loibl, M., Castells-Ballester, J., Aebi, M., Clausen, H., and Strahl, S. (2016) Mapping the O-Mannose Glycoproteome in Saccharomyces cerevisiae, *Molecular & Cellular Proteomics 15*, 1323-1337.

[19] Klis, F. M., Boorsma, A., and De Groot, P. W. J. (2006) Cell wall construction in Saccharomyces cerevisiae, *Yeast 23*, 185-202.

[20] Girrbach, V., and Strahl, S. (2003) Members of the Evolutionarily Conserved PMT Family of ProteinO-Mannosyltransferases Form Distinct Protein Complexes among Themselves*, *Journal of Biological Chemistry 278*, 12554-12562.

[21] Bausewein, D., Engel, J., Jank, T., Schoedl, M., and Strahl, S. (2016) Functional Similarities between the Protein O-Mannosyltransferases Pmt4 from Bakers' Yeast and Human POMT1, *The Journal of biological chemistry 291*, 18006-18015.

[22] Gentzsch, M., and Tanner, W. (1997) Protein-O-glycosylation in yeast: protein-specific mannosyltransferases, *Glycobiology 7*, 481-486.

[23] Hutzler, J., Schmid, M., Bernard, T., Henrissat, B., and Strahl, S. (2007) Membrane association is a determinant for substrate recognition by PMT4 protein O-mannosyltransferases, *Proc Natl Acad Sci U S A 104*, 7827-7832.

[24] Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity, *Proc Natl Acad Sci U S A 101*, 500-505.

[25] Barresi, R., and Campbell, K. P. (2006) Dystroglycan: from biosynthesis to pathogenesis of human disease, *Journal of Cell Science 119*, 199-207.

[26] Ervasti, J. M., and Campbell, K. P. (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin, *J Cell Biol 122*, 809-823.

[27] Godfrey, C., Foley, A. R., Clement, E., and Muntoni, F. (2011) Dystroglycanopathies: coming into focus, *Current Opinion in Genetics & Development 21*, 278-285.

[28] Larsen, I. S. B., Narimatsu, Y., Joshi, H. J., Yang, Z., Harrison, O. J., Brasch, J., Shapiro, L., Honig, B., Vakhrushev, S. Y., Clausen, H., and Halim, A. (2017) Mammalian O-mannosylation of cadherins and plexins is independent of protein O-mannosyltransferases 1 and 2, *Journal of Biological Chemistry 292*, 11586-11598.

[29] de Beer, T., Vliegenthart, J. F. G., Loeffler, A., and Hofsteenge, J. (1995) The Hexopyranosyl Residue That Is C-Glycosidically Linked to the Side Chain of Tryptophan-7 in Human RNase Us Is .alpha.-Mannopyranose, *Biochemistry 34*, 11785-11789.

[30] Hofsteenge, J., Mueller, D. R., de Beer, T., Loeffler, A., Richter, W. J., and Vliegenthart, J. F. G. (1994) New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase Us, *Biochemistry 33*, 13524-13530.

[31] Crine, S. L., and Acharya, K. R. (2022) Molecular basis of C-mannosylation – a structural perspective, *The FEBS Journal 289*, 7670-7687.

[32] Minakata, S., Manabe, S., Inai, Y., Ikezaki, M., Nishitsuji, K., Ito, Y., and Ihara, Y. (2021) Protein C-Mannosylation and C-Mannosyl Tryptophan in Chemical Biology and Medicine, *Molecules 26*.

[33] Shcherbakova, A., Preller, M., Taft, M. H., Pujols, J., Ventura, S., Tiemann, B., Buettner, F. F. R., and Bakker, H. (2019) C-mannosylation supports folding and enhances stability of thrombospondin repeats, *eLife 8*, e52978.

[34] Frank, M., Beccati, D., Leeflang, B. R., and Vliegenthart, J. F. G. (2020) C-Mannosylation Enhances the Structural Stability of Human RNase 2, *iScience 23*, 101371.

[35] Doucey, M. A., Hess, D., Cacan, R., and Hofsteenge, J. (1998) Protein C-mannosylation is enzyme-catalysed and uses dolichyl-phosphate-mannose as a precursor, *Mol Biol Cell 9*, 291-300.

[36] Buettner, Falk F. R., Ashikov, A., Tiemann, B., Lehle, L., and Bakker, H. (2013) C. elegans DPY-19 Is a C-Mannosyltransferase Glycosylating Thrombospondin Repeats, *Molecular Cell 50*, 295-302.

[37] Harbuz, R., Zouari, R., Pierre, V., Ben Khelifa, M., Kharouf, M., Coutton, C., Merdassi, G., Abada, F., Escoffier, J., Nikas, Y., Vialard, F., Koscinski, I., Triki, C., Sermondade, N., Schweitzer, T., Zhioua, A., Zhioua, F., Latrous, H., Halouani, L., Ouafi, M., Makni, M., Jouk, P. S., Sèle, B., Hennebicq, S., Satre, V., Viville, S., Arnoult, C., Lunardi, J., and Ray, P. F. (2011) A recurrent deletion of DPY19L2 causes infertility in man by blocking sperm head elongation and acrosome formation, *Am J Hum Genet 88*, 351-361.

[38] Koscinski, I., Elinati, E., Fossard, C., Redin, C., Muller, J., Velez de la Calle, J., Schmitt, F., Ben Khelifa, M., Ray, P. F., Kilani, Z., Barratt, C. L., and Viville, S. (2011) DPY19L2 deletion as a major cause of globozoospermia, *Am J Hum Genet 88*, 344-350.

[39] Shcherbakova, A., Tiemann, B., Buettner, F. F., and Bakker, H. (2017) Distinct C-mannosylation of netrin receptor thrombospondin type 1 repeats by mammalian DPY19L1 and DPY19L3, *Proc Natl Acad Sci U S A 114*, 2574-2579.

[40] Julenius, K. (2007) NetCGlyc 1.0: prediction of mammalian C-mannosylation sites, *Glycobiology 17*, 868-876.

[41] Kinoshita, T. (2020) Biosynthesis and biology of mammalian GPI-anchored proteins, *Open Biology 10*, 190290.

[42] Watanabe, R., Murakami, Y., Marmor, M. D., Inoue, N., Maeda, Y., Hino, J., Kangawa, K., Julius, M., and Kinoshita, T. (2000) Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2, *The EMBO Journal 19*, 4402-4411.

[43] Kinoshita, T., and Fujita, M. (2016) Thematic Review Series: Glycosylphosphatidylinositol (GPI) Anchors: Biochemistry and Cell Biology Biosynthesis of GPI-anchored proteins: special emphasis on GPI lipid remodeling, *Journal of Lipid Research 57*, 6-24.

[44] Kanzawa, N., Maeda, Y., Ogiso, H., Murakami, Y., Taguchi, R., and Kinoshita, T. (2009) Peroxisome dependency of alkyl-containing GPI-anchor biosynthesis in the endoplasmic reticulum, *Proc Natl Acad Sci U S A 106*, 17711-17716.

[45] Pittet, M., and Conzelmann, A. (2007) Biosynthesis and function of GPI proteins in the yeast Saccharomyces cerevisiae, *Biochim Biophys Acta 1771*, 405-420.

[46] Freeze, H. H., Boyce, M., Zachara, N. E., Hart, G. W., and Schnaar, R. L. (2022) Glycosylation Precursors, In *Essentials of Glycobiology* (Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Mohnen, D., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., and Seeberger, P. H., Eds.), pp 53-66, Cold Spring Harbor Laboratory Press

[47] Kean, E. L., Münster-Kühnel, A. K., and Gerardy-Schahn, R. (2004) CMP-sialic acid synthetase of the nucleus, *Biochimica et Biophysica Acta (BBA) - General Subjects 1673*, 56-65.

[48] Hennet, T., and Cabalzar, J. (2015) Congenital disorders of glycosylation: a concise chart of glycocalyx dysfunction, *Trends in Biochemical Sciences 40*, 377-384.

[49] Parker, J. L., and Newstead, S. (2017) Structural basis of nucleotide sugar transport across the Golgi membrane, *Nature 551*, 521-524.

[50] Hadley, B., Litfin, T., Day, C. J., Haselhorst, T., Zhou, Y., and Tiralongo, J. (2019) Nucleotide Sugar Transporter SLC35 Family Structure and Function, *Computational and Structural Biotechnology Journal 17*, 1123-1134.

[51] Hadley, B., Maggioni, A., Ashikov, A., Day, C. J., Haselhorst, T., and Tiralongo, J. (2014) Structure and function of nucleotide sugar transporters: Current progress, *Computational and Structural Biotechnology Journal 10*, 23-32.

[52] Hartley, M. D., and Imperiali, B. (2012) At the membrane frontier: A prospectus on the remarkable evolutionary conservation of polyprenols and polyprenyl-phosphates, *Arch. Biochem. Biophys. 517*, 83-97.

[53] Kale, D., Kikul, F., Phapale, P., Beedgen, L., Thiel, C., and Brügger, B. (2023) Quantification of Dolichyl Phosphates Using Phosphate Methylation and Reverse-Phase Liquid Chromatography-High Resolution Mass Spectrometry, *Anal Chem 95*, 3210-3217.

[54] Swiezewska, E., Sasak, W., Mańkowski, T., Jankowski, W., Vogtman, T., Krajewska, I., Hertel, J., Skoczylas, E., and Chojnacki, T. (1994) The search for plant polyprenols, *Acta biochimica Polonica 41*, 221-260.

[55] Szkopińska, A., Swiezewska, E., and Chojnacki, T. (1992) On the specificity of dolichol kinase and DolPMan synthase towards isoprenoid alcohols of different chain length in rat liver microsomal membrane, *Int J Biochem 24*, 1151-1157.

[56] Swiezewska, E., and Danikiewicz, W. (2005) Polyisoprenoids: structure, biosynthesis and function, *Prog Lipid Res 44*, 235-258.

[57] Rush, J. S., Shelling, J. G., Zingg, N. S., Ray, P. H., and Waechter, C. J. (1993) Mannosylphosphoryldolichol-mediated reactions in oligosaccharide-P-P-dolichol biosynthesis. Recognition of the saturated alpha-isoprene unit of the mannosyl donor by pig brain mannosyltransferases, *Journal of Biological Chemistry 268*, 13110-13117.

[58] Rush, J. S., Subramanian, T., Subramanian, K. L., Onono, F. O., Waechter, C. J., and Spielmann, H. P. (2015) Novel Citronellyl-Based Photoprobes Designed to Identify ER Proteins Interacting with Dolichyl Phosphate in Yeast and Mammalian Cells, *Current chemical biology 9*, 123-141.

[59] Lemoine, H., Raud, L., Foulquier, F., Sayer, J. A., Lambert, B., Olinger, E., Lefèvre, S., Knebelmann, B., Harris, P. C., Trouvé, P., Després, A., Duneau, G., Matignon, M., Poyet, A., Jourde-Chiche, N., Guerrot, D., Lemoine, S., Seret, G., Barroso-Gil, M., Bingham, C., Gilbert, R., Le Meur, Y., Audrézet, M. P., and Cornec-Le Gall, E. (2022) Monoallelic pathogenic ALG5 variants cause atypical polycystic kidney disease and interstitial fibrosis, *Am J Hum Genet 109*, 1484-1499.

[60] Cantagrel, V., and Lefeber, D. J. (2011) From glycosylation disorders to dolichol biosynthesis defects: a new class of metabolic diseases, *J Inherit Metab Dis 34*, 859-867.

[61] Grabowska, D., Karst, F., and Szkopińska, A. (1998) Effect of squalene synthase gene disruption on synthesis of polyprenols in Saccharomyces cerevisiae, *FEBS Letters 434*, 406-408.

[62] Park, E. J., Grabińska, K. A., Guan, Z., Stránecký, V., Hartmannová, H., Hodaňová, K., Barešová, V., Sovová, J., Jozsef, L., Ondrušková, N., Hansíková, H., Honzík, T., Zeman, J., Hůlková, H., Wen, R., Kmoch, S., and Sessa, W. C. (2014) Mutation of Nogo-B receptor, a subunit of cis-prenyltransferase, causes a congenital disorder of glycosylation, *Cell Metab 20*, 448-457.

[63] Sato, M., Sato, K., Nishikawa, S., Hirata, A., Kato, J., and Nakano, A. (1999) The yeast RER2 gene, identified by endoplasmic reticulum protein localization mutations, encodes cis-prenyltransferase, a key enzyme in dolichol synthesis, *Molecular and cellular biology 19*, 471-483.

[64] Grabińska, K. A., Park, E. J., and Sessa, W. C. (2016) cis-Prenyltransferase: New Insights into Protein Glycosylation, Rubber Synthesis, and Human Diseases, *The Journal of biological chemistry 291*, 18582-18590.

[65] Rush, J. S., Matveev, S., Guan, Z., Raetz, C. R. H., and Waechter, C. J. (2010) Expression of functional bacterial undecaprenyl pyrophosphate synthase in the yeast rer2{Delta} mutant and CHO cells, *Glycobiology 20*, 1585-1593.

[66] Sagami, H., Kurisaki, A., and Ogura, K. (1993) Formation of dolichol from dehydrodolichol is catalyzed by NADPH-dependent reductase localized in microsomes of rat liver, *The Journal of biological chemistry 268*, 10109-10113.

[67] Shridas, P., and Waechter, C. J. (2006) Human Dolichol Kinase, a Polytopic Endoplasmic Reticulum Membrane Protein with a Cytoplasmically Oriented CTP-binding Site*, *Journal of Biological Chemistry 281*, 31696-31704.

[68] Rush, J. S. (2015) Role of Flippases in Protein Glycosylation in the Endoplasmic Reticulum, *Lipid Insights 8*, 45-53.

[69] Frank, C. G., Sanyal, S., Rush, J. S., Waechter, C. J., and Menon, A. K. (2008) Does Rft1 flip an N-glycan lipid precursor?, *Nature 454*, E3-E4.

[70] Sanyal, S., and Menon, A. K. (2009) Specific transbilayer translocation of dolichol-linked oligosaccharides by an endoplasmic reticulum flippase, *Proc Natl Acad Sci U S A 106*, 767-772.

[71] Rush, J. S., Gao, N., Lehrman, M. A., Matveev, S., and Waechter, C. J. (2009) Suppression of Rft1 Expression Does Not Impair the Transbilayer Movement of Man5GlcNAc2-P-P-Dolichol in Sealed Microsomes from Yeast*, *Journal of Biological Chemistry 284*, 19835-19842.

[72] Rush, J. S., and Waechter, C. J. (1995) Transmembrane movement of a water-soluble analogue of mannosylphosphoryldolichol is mediated by an endoplasmic reticulum protein, *Journal of Cell Biology 130*, 529-536.

[73] Rush, J. S., van Leyen, K., Ouerfelli, O., Wolucka, B., and Waechter, C. J. (1998) Transbilayer movement of Glc-P-dolichol and its function as a glucosyl donor: protein-mediated transport of a water-soluble analog into sealed ER vesicles from pig brain, *Glycobiology 8*, 1195-1205.

[74] Rush, J. S., and Waechter, C. J. (2004) Functional Reconstitution into Proteoliposomes and Partial Purification of a Rat Liver ER Transport System for a Water-Soluble Analogue of Mannosylphosphoryldolichol, *Biochemistry 43*, 7643-7652.

[75] Sanyal, S., and Menon, A. K. (2010) Stereoselective transbilayer translocation of mannosyl phosphoryl dolichol by an endoplasmic reticulum flippase, *Proceedings of the National Academy of Sciences of the United States of America 107*, 11289-11294.

[76] Anand, M., Rush, J. S., Ray, S., Doucey, M. A., Weik, J., Ware, F. E., Hofsteenge, J., Waechter, C. J., and Lehrman, M. A. (2001) Requirement of the Lec35 gene for all known classes of monosaccharide-P-dolichol-dependent glycosyltransferase reactions in mammals, *Mol Biol Cell 12*, 487-501.

[77] Schenk, B., Imbach, T., Frank, C. G., Grubenmann, C. E., Raymond, G. V., Hurvitz, H., Korn-Lubetzki, I., Revel-Vik, S., Raas-Rotschild, A., Luder, A. S., Jaeken, J., Berger, E. G., Matthijs, G., Hennet, T., and Aebi, M. (2001) MPDU1 mutations underlie a novel human congenital disorder of glycosylation, designated type If, *The Journal of clinical investigation 108*, 1687-1695.

[78] Péanne, R., de Lonlay, P., Foulquier, F., Kornak, U., Lefeber, D. J., Morava, E., Pérez, B., Seta, N., Thiel, C., Van Schaftingen, E., Matthijs, G., and Jaeken, J. (2018) Congenital disorders of glycosylation (CDG): Quo vadis?, *Eur J Med Genet 61*, 643-663.

[79] Francisco, R., Marques-da-Silva, D., Brasil, S., Pascoal, C., dos Reis Ferreira, V., Morava, E., and Jaeken, J. (2019) The challenge of CDG diagnosis, *Molecular Genetics and Metabolism 126*, 1-5.

[80] Jaeken, J., Hennet, T., Matthijs, G., and Freeze, H. H. (2009) CDG nomenclature: Time for a change!, *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1792*, 825-826.

[81] Cylwik, B., Naklicki, M., Chrostek, L., and Gruszewska, E. (2013) Congenital disorders of glycosylation. Part I. Defects of protein N-glycosylation, *Acta biochimica Polonica 60*, 151-161.

[82] Babovic-Vuksanovic, D., and O'Brien, J. F. (2007) Laboratory Diagnosis of Congenital Disorders of Glycosylation Type I by Analysis of Transferrin Glycoforms, *Molecular Diagnosis & Therapy 11*, 303-311.

[83] Lefeber, D. J., Morava, E., and Jaeken, J. (2011) How to find and diagnose a CDG due to defective N-glycosylation, *J Inherit Metab Dis 34*, 849-852.

[84] Verheijen, J., Tahata, S., Kozicz, T., Witters, P., and Morava, E. (2020) Therapeutic approaches in Congenital Disorders of Glycosylation (CDG) involving N-linked glycosylation: an update, *Genetics in Medicine 22*, 268-279.

[85] Park, J. H., and Marquardt, T. (2021) Treatment Options in Congenital Disorders of Glycosylation, *Front Genet 12*, 735348.

[86] Thiel, C., and Körner, C. (2013) Therapies and therapeutic approaches in Congenital Disorders of Glycosylation, *Glycoconjugate journal 30*, 77-84.

[87] Feichtinger, R. G., Hüllen, A., Koller, A., Kotzot, D., Grote, V., Rapp, E., Hofbauer, P., Brugger, K., Thiel, C., Mayr, J. A., and Wortmann, S. B. (2021) A spoonful of L-fucose-an efficient therapy for GFUS-CDG, a new glycosylation disorder, *EMBO Mol Med 13*, e14332.

[88] Himmelreich, N., Kikul, F., Zdrazilova, L., Honzik, T., Hecker, A., Poschet, G., Lüchtenborg, C., Brügger, B., Strahl, S., Bürger, F., Okun, J. G., Hansikova, H., and Thiel, C. (2023) Complex metabolic disharmony in PMM2-CDG paves the way to new therapeutic approaches, *Molecular Genetics and Metabolism 139*, 107610.

[89] Tanner, W., Jung, P., and Behrens, N. H. (1971) Dolicholmonophosphates: Mannosyl acceptors in a particulate in vitro system of S. cerevisiae, *FEBS Letters 16*, 245-248.

[90] Orlean, P., Albright, C., and Robbins, P. W. (1988) Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein, *The Journal of biological chemistry 263*, 17499-17507.

[91] Banerjee, D. K., Zhang, Z., Baksi, K., and Serrano-Negron, J. E. (2017) Dolichol phosphate mannose synthase: a Glycosyltransferase with Unity in molecular diversities, *Glycoconjugate journal 34*, 467-479.

[92] Mazhari-Tabrizi, R., Eckert, V., Blank, M., Müller, R., Mumberg, D., Funk, M., and Schwarz, R. T. (1996) Cloning and functional expression of glycosyltransferases from parasitic protozoans by heterologous complementation in yeast: the dolichol phosphate mannose synthase from Trypanosoma brucei brucei, *Biochem J 316 (Pt 3)*, 853-858.

[93] Zimmerman, J. W., Specht, C. A., Cazares, B. X., and Robbins, P. W. (1996) The isolation of a Dol-P-Man synthase from Ustilago maydis that functions in Saccharomyces cerevisiae, *Yeast 12*, 765-771.

[94] Ilgoutz, S. C., Zawadzki, J. L., Ralton, J. E., and McConville, M. J. (1999) Evidence that free GPI glycolipids are essential for growth of Leishmania mexicana, *Embo j 18*, 2746-2755.

[95] Villagomez-Castro, J. C., Calvo-Mendez, C., Flores-Carreon, A., and Lopez-Romero, E. (2000) Partial purification and characterization of dolichol phosphate mannose synthase from Entamoeba histolytica, *Glycobiology 10*, 1311-1316.

[96] Colussi, P. A., Taron, C. H., Mack, J. C., and Orlean, P. (1997) Human and Saccharomyces cerevisiae dolichol phosphate mannose synthases represent two classes of the enzyme, but both function in Schizosaccharomyces pombe, *Proc Natl Acad Sci U S A 94*, 7873-7878.

[97] Kruszewska, J. S., Saloheimo, M., Migdalski, A., Orlean, P., Penttilä, M., and Palamarczyk, G. (2000) Dolichol phosphate mannose synthase from the filamentous fungus Trichoderma reesei belongs to the human and Schizosaccharomyces pombe class of the enzyme, *Glycobiology 10*, 983-991.

[98] Maeda, Y., Tanaka, S., Hino, J., Kangawa, K., and Kinoshita, T. (2000) Human dolichol-phosphate-mannose synthase consists of three subunits, DPM1, DPM2 and DPM3, *The EMBO Journal 19*, 2475-2482.

[99] Gandini, R., Reichenbach, T., Tan, T.-C., and Divne, C. (2017) Structural basis for dolichylphosphate mannose biosynthesis, *Nature Communications 8*, 120.

[100] Lamani, E., Mewbourne, R. B., Fletcher, D. S., Maltsev, S. D., Danilov, L. L., Veselovsky, V. V., Lozanova, A. V., Grigorieva, N. Y., Pinsker, O. A., Xing, J., Forsee, W. T., Cheung, H. C., Schutzbach, J. S., Shibaev, V. N., and Jedrzejas, M. J. (2006) Structural studies and mechanism of Saccharomyces cerevisiae dolichyl-phosphate-mannose synthase: insights into the initial step of synthesis of dolichyl-phosphate-linked oligosaccharide chains in membranes of endoplasmic reticulum, *Glycobiology 16*, 666-678.

[101] Breton, C., Šnajdrová, L., Jeanneau, C., Koča, J., and Imberty, A. (2005) Structures and mechanisms of glycosyltransferases, *Glycobiology 16*, 29R-37R.

[102] Eichler, J., and Imperiali, B. (2018) Stereochemical Divergence of Polyprenol Phosphate Glycosyltransferases, *Trends in Biochemical Sciences 43*, 10-17.

[103] Banerjee, D. K., Carrasquillo, E. A., Hughey, P., Schutzbach, J. S., Martínez, J. A., and Baksi, K. (2005) In Vitro Phosphorylation by cAMP-dependent Protein Kinase Up-regulates Recombinant Saccharomyces cerevisiae Mannosylphosphodolichol Synthase, *Journal of Biological Chemistry 280*, 4174-4181.

[104] Baksi, K., Zhang, Z., Banerjee, A., and Banerjee, D. K. (2009) Cloning and expression of mannosylphospho dolichol synthase from bovine adrenal medullary capillary endothelial cells, *Glycoconjugate journal 26*, 635-645.

[105] Kim, S., Westphal, V., Srikrishna, G., Mehta, D. P., Peterson, S., Filiano, J., Karnes, P. S., Patterson, M. C., and Freeze, H. H. (2000) Dolichol phosphate mannose synthase (DPM1) mutations define congenital disorder of glycosylation Ie (CDG-Ie), *The Journal of clinical investigation 105*, 191-198.

[106] Imbach, T., Schenk, B., Schollen, E., Burda, P., Stutz, A., Grunewald, S., Bailie, N. M., King, M. D., Jaeken, J., Matthijs, G., Berger, E. G., Aebi, M., and Hennet, T. (2000) Deficiency of dolichol-phosphate-mannose synthase-1 causes congenital disorder of glycosylation type Ie, *The Journal of clinical investigation 105*, 233-239.

[107] García-Silva, M. T., Matthijs, G., Schollen, E., Cabrera, J. C., del Pozo, J. S., Herreros, M. M., Simón, R., Maties, M., Hernández, E. M., Hennet, T., and Briones, P. (2004) Congenital disorder of glycosylation (CDG) type Ie. A new patient, *Journal of Inherited Metabolic Disease 27*, 591-600.

[108] Dancourt, J., Vuillaumier-Barrot, S., de Baulny, H. O., Sfaello, I., Barnier, A., le Bizec, C., Dupre, T., Durand, G., Seta, N., and Moore, S. E. (2006) A new intronic mutation in the DPM1 gene is associated with a milder form of CDG Ie in two French siblings, *Pediatr Res 59*, 835-839.

[109] Yang, A. C., Ng, B. G., Moore, S. A., Rush, J., Waechter, C. J., Raymond, K. M., Willer, T., Campbell, K. P., Freeze, H. H., and Mehta, L. (2013) Congenital disorder of glycosylation due to DPM1 mutations presenting with dystroglycanopathy-type congenital muscular dystrophy, *Molecular Genetics and Metabolism 110*, 345-351.

[110] Bursle, C., Brown, D., Cardinal, J., Connor, F., Calvert, S., and Coman, D. (2017) DMP1-CDG (CDG1e) with Significant Gastrointestinal Manifestations; Phenotype and Genotype Expansion, *JIMD Rep 34*, 27-32.

[111] Medrano, C., Vega, A., Navarrete, R., Ecay, M. J., Calvo, R., Pascual, S. I., Ruiz-Pons, M., Toledo, L., García-Jiménez, I., Arroyo, I., Campo, A., Couce, M. L., Domingo-Jiménez, M. R., García-Silva, M. T., González-Gutiérrez-Solana, L., Hierro, L., Martín-Hernández, E., Martínez-Pardo, M., Roldán, S., Tomás, M., Cabrera, J. C., Mártinez-Bugallo, F., Martín-Viota, L., Vitoria-Miñana, I., Lefeber, D. J., Girós, M. L., Serrano Gimare, M., Ugarte, M., Pérez, B., and Pérez-Cerdá, C. (2019) Clinical and molecular diagnosis of non-phosphomannomutase 2 N-linked congenital disorders of glycosylation in Spain, *Clinical Genetics 95*, 615-626.

[112] Lausmann, H., Zacharias, M., Neuhann, T. M., Locher, M. K., and Schettler, K. F. (2022) Case Report: DPM1-CDG: Novel Variant with Severe Phenotype and Literature Review, *Front Genet 13*, 889829.

[113] Barone, R., Aiello, C., Race, V., Morava, E., Foulquier, F., Riemersma, M., Passarelli, C., Concolino, D., Carella, M., Santorelli, F., Vleugels, W., Mercuri, E., Garozzo, D., Sturiale, L., Messina, S., Jaeken, J., Fiumara, A., Wevers, R. A., Bertini, E., Matthijs, G., and Lefeber, D. J. (2012) DPM2-CDG: a muscular dystrophy-dystroglycanopathy syndrome with severe epilepsy, *Ann Neurol 72*, 550-558.

[114] Radenkovic, S., Fitzpatrick-Schmidt, T., Byeon, S. K., Madugundu, A. K., Saraswat, M., Lichty, A., Wong, S. Y. W., McGee, S., Kubiak, K., Ligezka, A., Ranatunga, W., Zhang, Y., Wood, T., Friez, M. J., Clarkson, K., Pandey, A., Jones, J. R., and Morava, E. (2021) Expanding the clinical and metabolic phenotype of DPM2 deficient congenital disorders of glycosylation, *Molecular genetics and metabolism 132*, 27-37.

[115] Zhao, P., Hu, Y., Hu, J., Li, C., Huang, Y., Zhang, L., Luo, S., Zhu, H., Jiang, J., and He, X. (2023) Identification and characterization of a new variation in DPM2 gene in two Chinese siblings with mild intellectual impairment, *Front Genet 14*, 930692.

[116] Lefeber, D. J., Schönberger, J., Morava, E., Guillard, M., Huyben, K. M., Verrijp, K., Grafakou, O., Evangeliou, A., Preijers, F. W., Manta, P., Yildiz, J., Grünewald, S., Spilioti, M., van den Elzen, C., Klein, D., Hess, D., Ashida, H., Hofsteenge, J., Maeda, Y., van den Heuvel, L., Lammens, M., Lehle, L., and Wevers, R. A. (2009) Deficiency of Dol-P-Man Synthase Subunit DPM3 Bridges the Congenital Disorders of Glycosylation with the Dystroglycanopathies, *The American Journal of Human Genetics 85*, 76-86.

[117] Van den Bergh, P. Y. K., Sznajer, Y., Van Parys, V., van Tol, W., Wevers, R. A., Lefeber, D. J., Xu, L., Lek, M., MacArthur, D. G., Johnson, K., Phillips, L., Töpf, A., and Straub, V. (2017) A homozygous DPM3 mutation in a patient with alpha-dystroglycan-related limb girdle muscular dystrophy, *Neuromuscular Disorders 27*, 1043-1046.

[118] Svahn, J., Laforêt, P., Vial, C., Streichenberger, N., Romero, N., Bouchet-Séraphin, C., Bruneel, A., Dupré, T., Seta, N., Menassa, R., Michel-Calemard, L., and Stojkovic, T. (2019) Dilated cardiomyopathy and limb-girdle muscular dystrophy-dystroglycanopathy due to novel pathogenic variants in the DPM3 gene, *Neuromuscul Disord 29*, 497-502.

[119] van Tol, W., Michelakakis, H., Georgiadou, E., van den Bergh, P., Moraitou, M., Papadimas, G. K., Papadopoulos, C., Huijben, K., Alsady, M., Willemsen, M. A., and Lefeber, D. J. (2019) Toward understanding tissue-specific symptoms in dolichol-phosphate-mannose synthesis disorders; insight from DPM3-CDG, *Journal of Inherited Metabolic Disease 42*, 984-992.

[120] Fu, J., Ma, M., Song, J., Pang, M., Yang, L., Li, G., and Zhang, J. (2019) Novel mutations in DPM3 cause dystroglycanopathy with central nervous system involvement, *Clinical Genetics 96*, 590-591.

[121] Nagy, S., Lau, T., Alavi, S., Karimiani, E. G., Vallian, J., Ng, B. G., Noroozi Asl, S., Akhondian, J., Bahreini, A., Yaghini, O., Uapinyoying, P., Bonnemann, C., Freeze, H. H., Dissanayake, V. H. W., Sirisena, N. D., Schmidts, M., Houlden, H., Moreno-De-Luca, A., and Maroofian, R. (2022) A recurrent homozygous missense DPM3 variant leads to muscle and brain disease, *Clin Genet 102*, 530-536.

[122] Piłsyk, S., Perlinska-Lenart, U., Janik, A., Gryz, E., Ajchler-Adamska, M., and Kruszewska, J. S. (2020) Yil102c-A is a Functional Homologue of the DPMII Subunit of Dolichyl Phosphate Mannose Synthase in Saccharomyces cerevisiae, *Int J Mol Sci 21*.

[123] Yadav, B., Bhatnagar, S., Ahmad, M. F., Jain, P., Pratyusha, V. A., Kumar, P., and Komath, S. S. (2014) First step of glycosylphosphatidylinositol (GPI) biosynthesis cross-talks with ergosterol biosynthesis and Ras signaling in Candida albicans, *The Journal of biological chemistry 289*, 3365-3382.

[124] Banerjee, D. K. (2012) N-glycans in cell survival and death: Cross-talk between glycosyltransferases, *Biochimica et Biophysica Acta (BBA) - General Subjects 1820*, 1338-1346.

[125] Kean, E. L., Wei, Z., Anderson, V. E., Zhang, N., and Sayre, L. M. (1999) Regulation of the Biosynthesis ofN-Acetylglucosaminylpyrophosphoryldolichol, Feedback and Product Inhibition*, *Journal of Biological Chemistry 274*, 34072-34082.

[126] Faulhammer, F., Konrad, G., Brankatschk, B., Tahirovic, S., Knödler, A., and Mayinger, P. (2005) Cell growth-dependent coordination of lipid signaling and glycosylation is mediated by interactions between Sac1p and Dpm1p, *The Journal of cell biology 168*, 185-191.

[127] Faulhammer, F., Kanjilal-Kolar, S., Knödler, A., Lo, J., Lee, Y., Konrad, G., and Mayinger, P. (2007) Growth control of Golgi phosphoinositides by reciprocal localization of sac1 lipid phosphatase and pik1 4-kinase, *Traffic 8*, 1554-1567.

[128] Mesmin, B., Bigay, J., Moser von Filseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013) A Four-Step Cycle Driven by PI(4)P Hydrolysis Directs Sterol/PI(4)P Exchange by the ER-Golgi Tether OSBP, *Cell 155*, 830-843.

[129] Davis, D., Kannan, M., and Wattenberg, B. (2018) Orm/ORMDL proteins: Gate guardians and master regulators, *Adv Biol Regul 70*, 3-18.

[130] Breslow, D. K., Collins, S. R., Bodenmiller, B., Aebersold, R., Simons, K., Shevchenko, A., Ejsing, C. S., and Weissman, J. S. (2010) Orm family proteins mediate sphingolipid homeostasis, *Nature 463*, 1048-1053.

[131] Brice, S. E., Alford, C. W., and Cowart, L. A. (2009) Modulation of Sphingolipid Metabolism by the Phosphatidylinositol-4-phosphate Phosphatase Sac1p through Regulation of Phosphatidylinositol in Saccharomyces cerevisiae*, *Journal of Biological Chemistry 284*, 7588-7596.

[132] van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nature reviews. Molecular cell biology 9*, 112-124.

[133] Resh, M. D. (2016) Fatty acylation of proteins: The long and the short of it, *Progress in Lipid Research 63*, 120-131.

[134] Harayama, T., and Riezman, H. (2018) Understanding the diversity of membrane lipid composition, *Nature reviews. Molecular cell biology 19*, 281-296.

[135] Klug, L., and Daum, G. (2014) Yeast lipid metabolism at a glance, *FEMS yeast research 14*, 369-388.

[136] Gault, C. R., Obeid, L. M., and Hannun, Y. A. (2010) An overview of sphingolipid metabolism: from synthesis to breakdown, *Adv Exp Med Biol 688*, 1-23.

[137] Rego, A., Trindade, D., Chaves, S. R., Manon, S., Costa, V., Sousa, M. J., and Côrte-Real, M. (2014) The yeast model system as a tool towards the understanding of apoptosis regulation by sphingolipids, *FEMS yeast research 14*, 160-178.

[138] Russo, D., Parashuraman, S., and D'Angelo, G. (2016) Glycosphingolipid-Protein Interaction in Signal Transduction, *Int J Mol Sci 17*.

[139] Dufourc, E. J. (2008) Sterols and membrane dynamics, *J Chem Biol 1*, 63-77.

[140] Simons, K., and Ehehalt, R. (2002) Cholesterol, lipid rafts, and disease, *The Journal of clinical investigation 110*, 597-603.

[141] Lingwood, D., and Simons, K. (2010) Lipid rafts as a membrane-organizing principle, *Science 327*, 46-50.

[142] Pichler, H., and Emmerstorfer-Augustin, A. (2018) Modification of membrane lipid compositions in single-celled organisms – From basics to applications, *Methods 147*, 50-65.

[143] Wong, L. H., Gatta, A. T., and Levine, T. P. (2019) Lipid transfer proteins: the lipid commute via shuttles, bridges and tubes, *Nature Reviews Molecular Cell Biology 20*, 85-101.

[144] Buas, M. F., Drescher, C. W., Urban, N., Li, C. I., Bettcher, L., Hait, N. C., Moysich, K. B., Odunsi, K., Raftery, D., and Yan, L. (2021) Quantitative global lipidomics analysis of patients with ovarian cancer versus benign adnexal mass, *Scientific Reports 11*, 18156.

[145] Wolrab, D., Jirásko, R., Cífková, E., Höring, M., Mei, D., Chocholoušková, M., Peterka, O., Idkowiak, J., Hrnčiarová, T., Kuchař, L., Ahrends, R., Brumarová, R., Friedecký, D., Vivo-Truyols, G., Škrha, P., Škrha, J., Kučera, R., Melichar, B., Liebisch, G., Burkhardt, R., Wenk, M. R., Cazenave-Gassiot, A., Karásek, P., Novotný, I., Greplová, K., Hrstka, R., and Holčapek, M. (2022) Lipidomic profiling of human serum enables detection of pancreatic cancer, *Nature Communications 13*, 124.

[146] Radic Shechter, K., Kafkia, E., Zirngibl, K., Gawrzak, S., Alladin, A., Machado, D., Lüchtenborg, C., Sévin, D. C., Brügger, B., Patil, K. R., and Jechlinger, M. (2021) Metabolic memory underlying minimal residual disease in breast cancer, *Molecular Systems Biology 17*, e10141.

[147] Suvitaival, T., Bondia-Pons, I., Yetukuri, L., Pöhö, P., Nolan, J. J., Hyötyläinen, T., Kuusisto, J., and Orešič, M. (2018) Lipidome as a predictive tool in progression to type 2 diabetes in Finnish men, *Metabolism 78*, 1-12.

[148] Kao, Y. C., Ho, P. C., Tu, Y. K., Jou, I. M., and Tsai, K. J. (2020) Lipids and Alzheimer's Disease, *Int J Mol Sci 21*.

[149] Klose, C., Surma, M. A., Gerl, M. J., Meyenhofer, F., Shevchenko, A., and Simons, K. (2012) Flexibility of a eukaryotic lipidome--insights from yeast lipidomics, *PLoS One 7*, e35063.

[150] Schneiter, R., Brügger, B., Sandhoff, R., Zellnig, G., Leber, A., Lampl, M., Athenstaedt, K., Hrastnik, C., Eder, S., Daum, G., Paltauf, F., Wieland, F. T., and Kohlwein, S. D. (1999) Electrospray Ionization Tandem Mass Spectrometry (Esi-Ms/Ms) Analysis of the Lipid Molecular Species Composition of Yeast Subcellular Membranes Reveals Acyl Chain-Based Sorting/Remodeling of Distinct Molecular Species En Route to the Plasma Membrane, *Journal of Cell Biology 146*, 741-754.

[151] Sanyal, S., and Menon, A. K. (2009) Flipping lipids: why an' what's the reason for?, *ACS Chem Biol 4*, 895-909.

[152] Fadeel, B., and Xue, D. (2009) The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease, *Crit Rev Biochem Mol Biol 44*, 264-277.

[153] Fan, W., and Evans, Ronald M. (2015) Turning Up the Heat on Membrane Fluidity, *Cell 161*, 962-963.

[154] Klose, C., Surma, M. A., Gerl, M. J., Meyenhofer, F., Shevchenko, A., and Simons, K. (2012) Flexibility of a Eukaryotic Lipidome – Insights from Yeast Lipidomics, *PLOS ONE 7*, e35063.

[155] Ikonen, E. (2008) Cellular cholesterol trafficking and compartmentalization, *Nature Reviews Molecular Cell Biology 9*, 125-138.

[156] Barrero-Sicilia, C., Silvestre, S., Haslam, R. P., and Michaelson, L. V. (2017) Lipid remodelling: Unravelling the response to cold stress in Arabidopsis and its extremophile relative Eutrema salsugineum, *Plant Sci 263*, 194-200.

[157] Dawaliby, R., Trubbia, C., Delporte, C., Noyon, C., Ruysschaert, J.-M., Van Antwerpen, P., and Govaerts, C. (2016) Phosphatidylethanolamine Is a Key Regulator of Membrane Fluidity in Eukaryotic Cells*, *Journal of Biological Chemistry 291*, 3658-3667.

[158] McMahon, H. T., and Boucrot, E. (2015) Membrane curvature at a glance, *Journal of Cell Science 128*, 1065-1070.

[159] Coskun, Ü., Grzybek, M., Drechsel, D., and Simons, K. (2011) Regulation of human EGF receptor by lipids, *Proceedings of the National Academy of Sciences 108*, 9044-9048.

[160] Contreras, F. X., Ernst, A. M., Haberkant, P., Björkholm, P., Lindahl, E., Gönen, B., Tischer, C., Elofsson, A., von Heijne, G., Thiele, C., Pepperkok, R., Wieland, F., and Brügger, B. (2012) Molecular recognition of a single sphingolipid species by a protein's transmembrane domain, *Nature 481*, 525-529.

[161] Pannwitt, S., Stangl, M., and Schneider, D. (2019) Lipid Binding Controls Dimerization of the Coat Protein p24 Transmembrane Helix, *Biophysical Journal 117*, 1554-1562.

[162] Contreras, F. X., Ernst, A. M., Wieland, F., and Brügger, B. (2011) Specificity of intramembrane protein-lipid interactions, *Cold Spring Harb Perspect Biol 3*.

[163] Lee, A. G. (2004) How lipids affect the activities of integral membrane proteins, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1666*, 62-87.

[164] Alam, A., Kowal, J., Broude, E., Roninson, I., and Locher, K. P. (2019) Structural insight into substrate and inhibitor discrimination by human P-glycoprotein, *Science 363*, 753-756.

[165] Pyle, E., Kalli, A. C., Amillis, S., Hall, Z., Lau, A. M., Hanyaloglu, A. C., Diallinas, G., Byrne, B., and Politis, A. (2018) Structural Lipids Enable the Formation of Functional Oligomers of the Eukaryotic Purine Symporter UapA, *Cell Chem Biol 25*, 840-848.e844.

[166] Gomez, B., Jr., and Robinson, N. C. (1999) Phospholipase digestion of bound cardiolipin reversibly inactivates bovine cytochrome bc1, *Biochemistry 38*, 9031-9038.

[167] Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome bc1 complex structure, *Embo j 20*, 6591-6600.

[168] Seddon, A. M., Curnow, P., and Booth, P. J. (2004) Membrane proteins, lipids and detergents: not just a soap opera, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1666*, 105-117.

[169] Mozafari, M. R., Mazaheri, E., and Dormiani, K. (2021) Simple Equations Pertaining to the Particle Number and Surface Area of Metallic, Polymeric, Lipidic and Vesicular Nanocarriers, *Scientia Pharmaceutica 89*, 15.

[170] Skrzypek, R., Iqbal, S., and Callaghan, R. (2018) Methods of reconstitution to investigate membrane protein function, *Methods 147*, 126-141.

[171] Lira, R. B., and Dimova, R. (2019) Chapter Six - Fusion assays for model membranes: a critical review, In *Advances in Biomembranes and Lipid Self-Assembly* (Lipowsky, R., Ed.), pp 229-270, Academic Press.

[172] Rigaud, J.-L., and Lévy, D. (2003) Reconstitution of Membrane Proteins into Liposomes, In *Methods in Enzymology*, pp 65-86, Academic Press.

[173] MacDonald, R. C., Jones, F. D., and Qiu, R. (1994) Fragmentation into small vesicles of dioleoylphosphatidylcholine bilayers during freezing and thawing, *Biochim Biophys Acta 1191*, 362-370.

[174] Scott, H. L., Skinkle, A., Kelley, E. G., Waxham, M. N., Levental, I., and Heberle, F. A. (2019) On the Mechanism of Bilayer Separation by Extrusion, or Why Your LUVs Are Not Really Unilamellar, *Biophysical Journal 117*, 1381-1386.

[175] Gouda, A., Sakr, O. S., Nasr, M., and Sammour, O. (2021) Ethanol injection technique for liposomes formulation: An insight into development, influencing factors, challenges and applications, *Journal of Drug Delivery Science and Technology 61*, 102174.

[176] Rigaud, J. L., Pitard, B., and Levy, D. (1995) Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins, *Biochim Biophys Acta 1231*, 223-246.

[177] Amati, A. M., Graf, S., Deutschmann, S., Dolder, N., and von Ballmoos, C. (2020) Current problems and future avenues in proteoliposome research, *Biochemical Society Transactions 48*, 1473-1492.

[178] Dürr, U. H. N., Gildenberg, M., and Ramamoorthy, A. (2012) The Magic of Bicelles Lights Up Membrane Protein Structure, *Chemical Reviews 112*, 6054-6074.

[179] Prosser, R. S., Evanics, F., Kitevski, J. L., and Al-Abdul-Wahid, M. S. (2006) Current Applications of Bicelles in NMR Studies of Membrane-Associated Amphiphiles and Proteins, *Biochemistry 45*, 8453-8465.

[180] Ravula, T., Hardin, N. Z., and Ramamoorthy, A. (2019) Polymer nanodiscs: Advantages and limitations, *Chemistry and physics of lipids 219*, 45-49.

[181] Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002) Self-Assembly of Discoidal Phospholipid Bilayer Nanoparticles with Membrane Scaffold Proteins, *Nano Letters 2*, 853-856.

[182] Brügger, B., Glass, B., Haberkant, P., Leibrecht, I., Wieland, F. T., and Kräusslich, H. G. (2006) The HIV lipidome: a raft with an unusual composition, *Proc Natl Acad Sci U S A 103*, 2641-2646.

[183] Carlson, M. L., Young, J. W., Zhao, Z., Fabre, L., Jun, D., Li, J., Li, J., Dhupar, H. S., Wason, I., Mills, A. T., Beatty, J. T., Klassen, J. S., Rouiller, I., and Duong, F. (2018) The Peptidisc, a simple method for stabilizing membrane proteins in detergent-free solution, *eLife 7*, e34085.

[184] Denisov, I. G., and Sligar, S. G. (2016) Nanodiscs for structural and functional studies of membrane proteins, *Nature Structural & Molecular Biology 23*, 481-486.

[185] Majeed, S., Ahmad, A. B., Sehar, U., and Georgieva, E. R. (2021) Lipid Membrane Mimetics in Functional and Structural Studies of Integral Membrane Proteins, *Membranes (Basel) 11*, 685.

[186] Simon, K. S., Pollock, N. L., and Lee, S. C. (2018) Membrane protein nanoparticles: the shape of things to come, *Biochem Soc Trans 46*, 1495-1504.

[187] Dörr, J. M., Scheidelaar, S., Koorengevel, M. C., Dominguez, J. J., Schäfer, M., van Walree, C. A., and Killian, J. A. (2016) The styrene–maleic acid copolymer: a versatile tool in membrane research, *European Biophysics Journal 45*, 3-21.

[188] Kamilar, E., Bariwal, J., Zheng, W., Ma, H., and Liang, H. (2023) SMALPs Are Not Simply Nanodiscs: The Polymer-to-Lipid Ratios of Fractionated SMALPs Underline Their Heterogeneous Nature, *Biomacromolecules 24*, 1819-1838.

[189] Hawkins, O. P., Jahromi, C. P. T., Gulamhussein, A. A., Nestorow, S., Bahra, T., Shelton, C., Owusu-Mensah, Q. K., Mohiddin, N., O'Rourke, H., Ajmal, M., Byrnes, K., Khan, M., Nahar, N. N., Lim, A., Harris, C., Healy, H., Hasan, S. W., Ahmed, A., Evans, L., Vaitsopoulou, A., Akram, A., Williams, C., Binding, J., Thandi, R. K., Joby, A., Guest, A., Tariq, M. Z., Rasool, F., Cavanagh, L., Kang, S., Asparuhov, B., Jestin, A., Dafforn, T. R., Simms, J., Bill, R. M., Goddard, A. D., and Rothnie, A. J. (2021) Membrane protein extraction and purification using partially-esterified SMA polymers, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1863*, 183758.

[190] Jensen, J. W., and Schutzbach, J. S. (1985) Activation of dolichyl-phospho-mannose synthase by phospholipids, *Eur J Biochem 153*, 41-48.

[191] Babczinski, P., Haselbeck, A., and Tanner, W. (1980) Yeast Mannosyl Transferases Requiring Dolichyl Phosphate and Dolichyl Phosphate Mannose as Substrate, *European Journal of Biochemistry 105*, 509-515.

[192] Haselbeck, A., and Tanner, W. (1982) Dolichyl phosphate-mediated mannosyl transfer through liposomal membranes, *Proc Natl Acad Sci U S A 79*, 1520-1524.

[193] Schutzbach, J. S., and Zimmerman, J. W. (1992) Yeast dolichyl-phosphomannose synthase: reconstitution of enzyme activity with phospholipids, *Biochemistry and Cell Biology 70*, 460-465.

[194] Tavares, N. K., VanDrisse, C. M., and Escalante-Semerena, J. C. (2018) Rhodobacterales use a unique L-threonine kinase for the assembly of the nucleotide loop of coenzyme B12, *Molecular Microbiology 110*, 239-261.

[195] Tao, H., Liu, W., Simmons, B. N., Harris, H. K., Cox, T. C., and Massiah, M. A. (2010) Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS, *BioTechniques 48*, 61-64.

[196] Frankel, S., Sohn, R., and Leinwand, L. (1991) The use of sarkosyl in generating soluble protein after bacterial expression, *Proc Natl Acad Sci U S A 88*, 1192-1196.

[197] Orwick-Rydmark, M., Arnold, T., and Linke, D. (2016) The Use of Detergents to Purify Membrane Proteins, *Curr Protoc Protein Sci 84*, 4.8.1-4.8.35.

[198] Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973) Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate, *Journal of bacteriology 115*, 717-722.

[199] Jacob-Dubuisson, F., Buisine, C., Mielcarek, N., Clément, E., Menozzi, F. D., and Locht, C. (1996) Amino-terminal maturation of the Bordetella pertussis filamentous haemagglutinin, *Molecular Microbiology 19*, 65-78.

[200] Burgess, R. R. (1996) [12] Purification of overproduced Escherichia coli RNA polymerase σ factors by solubilizing inclusion bodies and refolding from Sarkosyl, In *Methods in Enzymology*, pp 145-149, Academic Press.

[201] Massiah, M. A., Wright, K. M., and Du, H. (2016) Obtaining Soluble Folded Proteins from Inclusion Bodies Using Sarkosyl, Triton X-100, and CHAPS: Application to LB and M9 Minimal Media, *Curr Protoc Protein Sci 84*, 6.13.11-16.13.24.

[202] Tremblay, G. Y., Daniels, M. J., and Schaechter, M. (1969) Isolation of a cell membrane-DNA-nascent RNA complex from bacteria, *Journal of Molecular Biology 40*, 65-76.

[203] Earhart, C. F. (1970) The association of host and phage DNA with the membrane of Escherichia coli, *Virology 42*, 429-436.

[204] Palanirajan, S. K., Govindasamy, P., and Gummadi, S. N. (2020) Polystyrene adsorbents: rapid and efficient surrogate for dialysis in membrane protein purification, *Scientific Reports 10*, 16334.

[205] Maltoni, G., Scutteri, L., Mensitieri, F., Piaz, F. D., and Hochkoeppler, A. (2022) High-yield production in Escherichia coli and convenient purification of a candidate vaccine against SARS-CoV-2, *Biotechnol Lett 44*, 1313-1322.

[206] Li, M., Zhu, P., Huang, Z., Huang, Y., Lv, X., Zheng, Q., Zhu, Z., Fan, Z., Yang, Y., and Shi, P. (2022) Aspirin damages the cell wall of Saccharomyces cerevisiae by inhibiting the expression and activity of dolichol-phosphate mannose synthase 1, *FEBS Letters 596*, 369-380.

[207] Tomita, S., Inoue, N., Maeda, Y., Ohishi, K., Takeda, J., and Kinoshita, T. (1998) A Homologue of Saccharomyces cerevisiae Dpm1p Is Not Sufficient for Synthesis of Dolichol-Phosphate-Mannose in Mammalian Cells, *Journal of Biological Chemistry 273*, 9249-9254.

[208] Schutzbach, J. S., Zimmerman, J. W., and Forsee, W. T. (1993) The purification and characterization of recombinant yeast dolichyl-phosphate-mannose synthase. Site-directed mutagenesis of the putative dolichol recognition sequence, *Journal of Biological Chemistry 268*, 24190-24196.

[209] Wilson, I. B. H., Taylor, J. P., Webberley, M. C., Turner, N. J., and Flitsch, S. L. (1993) A NOVEL MONO-BRANCHED LIPID PHOSPHATE ACTS AS A SUBSTRATE FOR DOLICHYL-PHOSPHATE MANNOSE SYNTHETASE, *Biochemical Journal 295*, 195-201.

[210] Ballweg, S., Sezgin, E., Doktorova, M., Covino, R., Reinhard, J., Wunnicke, D., Hänelt, I., Levental, I., Hummer, G., and Ernst, R. (2020) Regulation of lipid saturation without sensing membrane fluidity, *Nature Communications 11*, 756.

[211] Browman, D. T., Resek, M. E., Zajchowski, L. D., and Robbins, S. M. (2006) Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER, *J Cell Sci 119*, 3149-3160.

[212] Zhang, Y., Li, X., Becker, K. A., and Gulbins, E. (2009) Ceramide-enriched membrane domains—Structure and function, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1788*, 178-183.

[213] Bock, T., Chen, W.-H., Ori, A., Malik, N., Silva-Martin, N., Huerta-Cepas, J., Powell, S. T., Kastritis, P. L., Smyshlyaev, G., Vonkova, I., Kirkpatrick, J., Doerks, T., Nesme, L., Baßler, J., Kos, M., Hurt, E., Carlomagno, T., Gavin, A.-C., Barabas, O., Müller, C. W., van Noort, V., Beck, M., and Bork, P. (2014) An integrated approach for genome annotation of the eukaryotic thermophile Chaetomium thermophilum, *Nucleic Acids Research 42*, 13525-13533.

[214] Bossuyt, X., and Blanckaert, N. (1993) Topology of nucleotide-sugar:dolichyl phosphate glycosyltransferases involved in the dolichol pathway for protein glycosylation in native rat liver microsomes, *Biochem J 296 (Pt 3)*, 627-632.

[215] Schutzbach, J. S. (1997) The role of the lipid matrix in the biosynthesis of dolichyl-linked oligosaccharides, *Glycoconjugate journal 14*, 175-182.

[216] Maeda, Y., Tomita, S., Watanabe, R., Ohishi, K., and Kinoshita, T. (1998) DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular

localization and stabilization of DPM1, and binding of dolichol phosphate, *The EMBO Journal 17*, 4920-4929.

[217] Orlean, P. (1992) Enzymes that recognize dolichols participate in three glycosylation pathways and are required for protein secretion, *Biochemistry and Cell Biology 70*, 438-447.

[218] Zembek, P., Perlińska-Lenart, U., Rawa, K., Górka-Nieć, W., Palamarczyk, G., and Kruszewska, J. S. (2011) Cloning and functional analysis of the dpm2 and dpm3 genes from Trichoderma reesei expressed in a Saccharomyces cerevisiae dpm1Δ mutant strain, *392*, 517-527.

[219] Haselbeck, A. (1989) Purification of GDP mannose: dolichyl-phosphate O-β-D-mannosyltransferase from Saccharomyces cerevisiae, *European Journal of Biochemistry 181*, 663-668.

[220] Orlean, P. (1990) Dolichol phosphate mannose synthase is required in vivo for glycosyl phosphatidylinositol membrane anchoring, O mannosylation, and N glycosylation of protein in Saccharomyces cerevisiae, *Molecular and cellular biology 10*, 5796-5805.

[221] Xing, J., Forsee, W. T., Lamani, E., Maltsev, S. D., Danilov, L. L., Shibaev, V. N., Schutzbach, J. S., Cheung, H. C., and Jedrzejas, M. J. (2000) Investigations of the Active Site of Saccharomyces cerevisiae Dolichyl-Phosphate-Mannose Synthase Using Fluorescent Labeled Dolichyl-Phosphate Derivatives, *Biochemistry 39*, 7886-7894.

[222] Zimmerman, J. W., and Robbins, P. W. (1993) The hydrophobic domain of dolichyl-phosphate-mannose synthase is not essential for enzyme activity or growth in Saccharomyces cerevisiae, *The Journal of biological chemistry 268*, 16746-16753.

[223] Linke, D. (2009) Detergents: an overview, *Methods Enzymol 463*, 603-617.

[224] Krishnarjuna, B., and Ramamoorthy, A. (2022) Detergent-Free Isolation of Membrane Proteins and Strategies to Study Them in a Near-Native Membrane Environment, *Biomolecules 12*.

[225] Doskocz, J., Dałek, P., Przybyło, M., Trzebicka, B., Foryś, A., Kobyliukh, A., Iglič, A., and Langner, M. (2021) The Elucidation of the Molecular Mechanism of the Extrusion Process, *Materials (Basel) 14*.

[226] Vitrac, H., Bogdanov, M., and Dowhan, W. (2013) In vitro reconstitution of lipid-dependent dual topology and postassembly topological switching of a membrane protein, *Proceedings of the National Academy of Sciences 110*, 9338-9343.

[227] Albright, C. F., Orlean, P., and Robbins, P. W. (1989) A 13-amino acid peptide in three yeast glycosyltransferases may be involved in dolichol recognition, *Proc Natl Acad Sci U S A 86*, 7366-7369.

[228] Schutzbach, J. S. (1994) Is there a "dolichol recognition sequence" in enzymes that interact with dolichols and other polyisoprenoid substrates?, *Acta biochimica Polonica 41*, 269-274.

[229] Shibaev, V. N., Veselovsky, V. V., Lozanova, A. V., Maltsev, S. D., Danilov, L. L., Forsee, W. T., Xing, J., Cheung, H. C., and Jedrzejas, M. J. (2000) Synthesis of dolichyl phosphate derivatives with fluorescent label at the omega-end of the chain, new tools to study protein glycosylation, *Bioorganic & medicinal chemistry letters 10*, 189-192.

[230] Sprung, I., Ziegler, A., and Flitsch, S. L. (2002) Enzymatic synthesis of beta-mannosyl phosphates on solid support, *Chemical communications (Cambridge, England)*, 2676-2677.

[231] Gharwalová, L., Palyzová, A., Marešová, H., Kolouchová, I., Kyselová, L., and Řezanka, T. (2021) Identification of Homologous Polyprenols from Thermophilic Bacteria, *Microorganisms 9*.

[232] Bruszewski, T. E., Fergus, C. L., and Mumma, R. O. (1972) Thermophilic fungi: IV. The lipid composition of six species, *Lipids 7*, 695-698.

[233] Renne, M. F., and de Kroon, A. I. P. M. (2018) The role of phospholipid molecular species in determining the physical properties of yeast membranes, *FEBS Letters 592*, 1330-1345.

[234] Boumann, H. A., Gubbens, J., Koorengevel, M. C., Oh, C. S., Martin, C. E., Heck, A. J., Patton-Vogt, J., Henry, S. A., de Kruijff, B., and de Kroon, A. I. (2006) Depletion of phosphatidylcholine in yeast induces shortening and increased saturation of the lipid acyl chains: evidence for regulation of intrinsic membrane curvature in a eukaryote, *Mol Biol Cell 17*, 1006-1017.

[235] Casares, D., Escribá, P. V., and Rosselló, C. A. (2019) Membrane Lipid Composition: Effect on Membrane and Organelle Structure, Function and Compartmentalization and Therapeutic Avenues, *Int J Mol Sci 20*.

[236] Janssen, M. J., Koorengevel, M. C., de Kruijff, B., and de Kroon, A. I. (2000) The phosphatidylcholine to phosphatidylethanolamine ratio of Saccharomyces cerevisiae varies with the growth phase, *Yeast 16*, 641-650.

[237] Jensen, J. W., and Schutzbach, J. S. (1988) Modulation of dolichyl-phosphomannose synthase activity by changes in the lipid environment of the enzyme, *Biochemistry 27*, 6315-6320.

[238] Del Bel, L. M., and Brill, J. A. (2018) Sac1, a lipid phosphatase at the interface of vesicular and nonvesicular transport, *Traffic 19*, 301-318.

[239] Han, G., Gupta, S. D., Gable, K., Bacikova, D., Sengupta, N., Somashekarappa, N., Proia, R. L., Harmon, J. M., and Dunn, T. M. (2019) The ORMs interact with transmembrane domain 1 of Lcb1 and regulate serine palmitoyltransferase oligomerization, activity and localization, *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1864*, 245-259.

[240] Breslow, D. K., Collins, S. R., Bodenmiller, B., Aebersold, R., Simons, K., Shevchenko, A., Ejsing, C. S., and Weissman, J. S. (2010) Orm family proteins mediate sphingolipid homeostasis, *Nature 463*, 1048-1053.

[241] Hung, W.-C., Lee, M.-T., Chung, H., Sun, Y.-T., Chen, H., Charron, Nicholas E., and Huang, Huey W. (2016) Comparative Study of the Condensing Effects of Ergosterol and Cholesterol, *Biophysical Journal 110*, 2026-2033.

[242] van den Brink-van der Laan, E., Antoinette Killian, J., and de Kruijff, B. (2004) Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1666*, 275-288.

[243] Stone, M. B., Shelby, S. A., and Veatch, S. L. (2017) Super-Resolution Microscopy: Shedding Light on the Cellular Plasma Membrane, *Chem Rev 117*, 7457-7477.

[244] von Filseck, J. M., Vanni, S., Mesmin, B., Antonny, B., and Drin, G. (2015) A phosphatidylinositol-4-phosphate powered exchange mechanism to create a lipid gradient between membranes, *Nature Communications 6*, 6671.

[245] Janik, A., Sosnowska, M., Kruszewska, J., Krotkiewski, H., Lehle, L., and Palamarczyk, G. (2003) Overexpression of GDP-mannose pyrophosphorylase in Saccharomyces cerevisiae corrects defects in dolichol-linked saccharide formation and protein glycosylation, *Biochim Biophys Acta 1621*, 22-30.

[246] Lehrman, M. A. (1991) Biosynthesis of N-acetylglucosamine-P-P-dolichol, the committed step of asparagine-linked oligosaccharide assembly, *Glycobiology 1*, 553-562.

[247] Radenkovic, S., Fitzpatrick-Schmidt, T., Byeon, S. K., Madugundu, A. K., Saraswat, M., Lichty, A., Wong, S. Y. W., McGee, S., Kubiak, K., Ligezka, A., Ranatunga, W., Zhang, Y., Wood, T., Friez, M. J., Clarkson, K., Pandey, A., Jones, J. R., and Morava, E. (2021) Expanding the clinical and metabolic phenotype of DPM2 deficient congenital disorders of glycosylation, *Mol Genet Metab 132*, 27-37.

[248] Tian, J., Tian, L., Chen, M., Chen, Y., and Wei, A. (2022) Low Temperature Affects Fatty Acids Profiling and Key Synthesis Genes Expression Patterns in Zanthoxylum bungeanum Maxim, *Int J Mol Sci 23*.

[249] Takahashi, D., Li, B., Nakayama, T., Kawamura, Y., and Uemura, M. (2013) Plant plasma membrane proteomics for improving cold tolerance, *Front Plant Sci 4*, 90.

[250] Suutari, M., Liukkonen, K., and Laakso, S. (1990) Temperature adaptation in yeasts: the role of fatty acids, *Microbiology 136*, 1469-1474.

[251] Suutari, M., Liukkonen, K., and Laakso, S. (1996) Temperature gradient incubator for the study of alterations occurring with fatty acids of Saccharomyces cerevisiae when approaching extreme growth temperatures, *Journal of Microbiological Methods 25*, 207-214.

[252] Buczkowska, A., Swiezewska, E., and Lefeber, D. J. (2015) Genetic defects in dolichol metabolism, *Journal of Inherited Metabolic Disease 38*, 157-169.

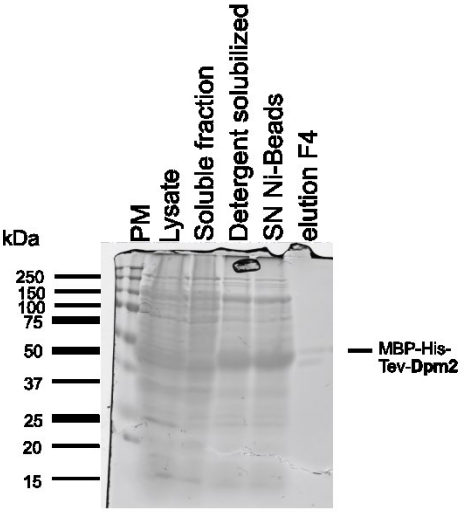
[253] Zdrazilova, L., Kuchar, L., Ondruskova, N., Honzik, T., and Hansikova, H. (2020) A new role for dolichol isoform profile in the diagnostics of CDG disorders, *Clinica Chimica Acta 507*, 88-93.

[254] Haeuptle, M. A., Welti, M., Troxler, H., Hülsmeier, A. J., Imbach, T., and Hennet, T. (2011) Improvement of Dolichol-linked Oligosaccharide Biosynthesis by the Squalene Synthase Inhibitor Zaragozic Acid*, *Journal of Biological Chemistry 286*, 6085-6091.

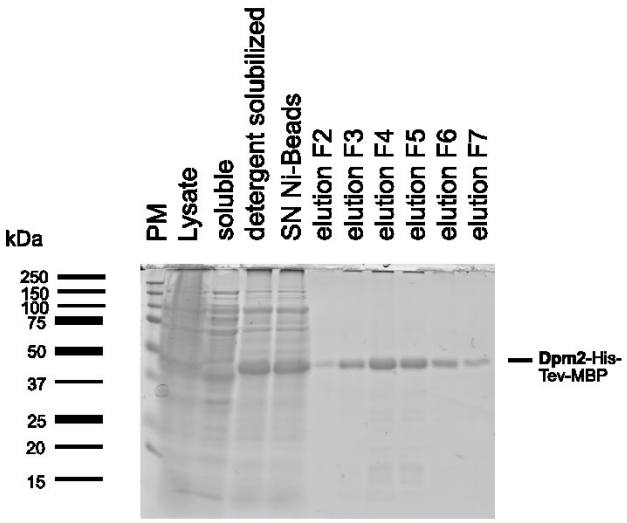
6 Supplement

Coomassie stained Gels of protein purifications of different Dpm2 constructs.

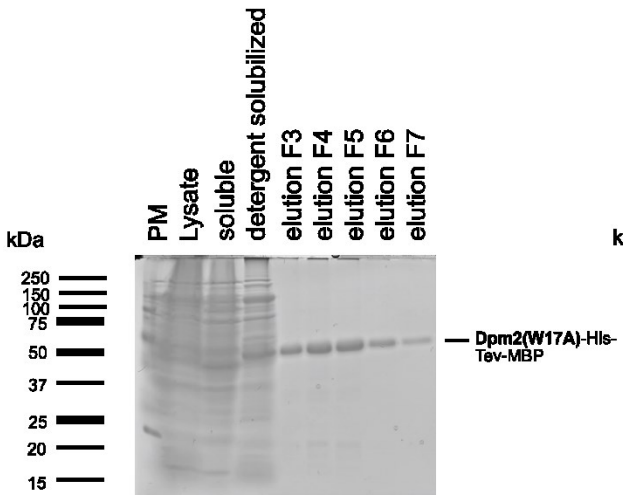
MBP-Tev-His-Dpm2



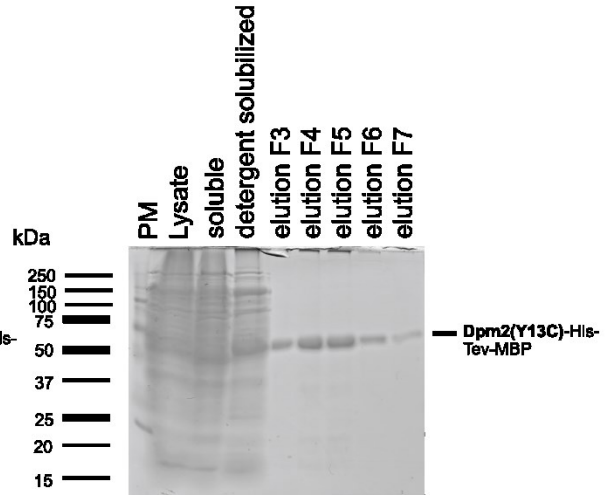
Dpm2-His-Tev-MBP



Dpm2(W17A)-His-Tev-MBP

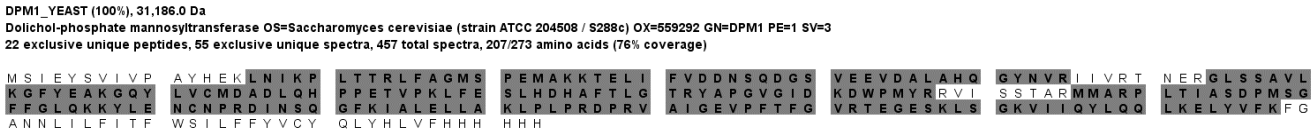


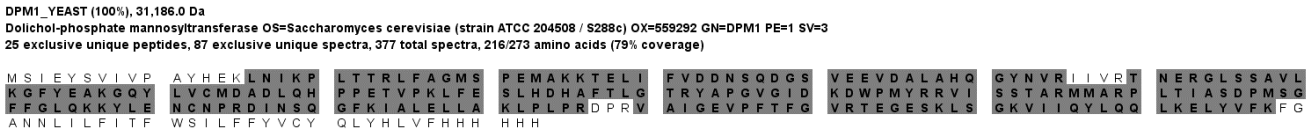
Dpm2(Y13C)-His-Tev-MBP



Mass spectrometric analysis of His-Dpm1

Mass spectrometric analysis was performed by Core Facility for Mass Spectrometry & Proteomics (CFMP) at the ZMBH. Proteins were digested with trypsin and analyzed by LC-MS. Protein was identified using Proteome Discoverer 2.3.0.523. The peptide coverage of two independent runs is shown in grey.

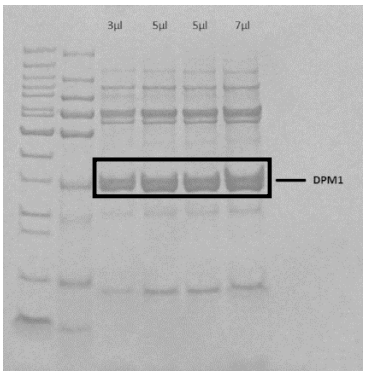




Dpm1 sequence

10	20	30	40	50
MSIEYSVIVP	AYHEKLNIKP	LTTRLFAGMS	PEMAKKTELI	FVDDNSQDGS
60	70	80	90	100
VEEVDALAHQ	GYNVRIIVRT	NERGLSSAVL	KGFYEAKGQY	LVCMDADLQH
110	120	130	140	150
PPETVPKLFE	SLHDHAFTLG	TRYAPGVGID	KDWPMYRRVI	SSTARMMARP
160	170	180	190	200
LTIASDPMSG	FFGLQKKYLE	NCNPRDINSQ	GFKIALELLA	KLPLPRDPRV
210	220	230	240	250
AIGEVPFTFG	VRTEGESKLS	GKVIIQYLQQ	LKELYVFKFG	ANNLILFITF
260				
WSILFFYVCY	QLYHLVF			

Coomassie of purified Dpm1 protein and analyzed band



Biotinylated α-Dystroglycan peptide (pαDG), used for testing Pmt4 activity

The sequence was taken from Bausewein et al. 2016 [21]

pαDG [401-412]

IRPTMTIPGYVEPTAVATPP-K(Biot)

Danksagung

Die meiste Zeit während des Schreibprozesses war diese letzte Seite gefüllt mit Zweifeln und meinen Mühen, die richtigen Worte und Bedeutung zu finden. Ich bin extrem glücklich, dass ich das alles schlussendlich löschen konnte um nun diese Seite mit meiner Dankbarkeit zu füllen. Diese Arbeit wäre nicht möglich ohne die Unterstützung folgender Personen:

Britta Brügger, die es mir ermöglicht hat meine Dissertation im Rahmen dieses Projektes zu verfassen. Danke für all die Ratschläge, Idee und die ganze Unterstützung. Danke, dass du an mich und meine Arbeit glaubst.

Rainer Beck, der besonders zu Beginn meiner Arbeit mein Mentor war. Danke für all die unzähligen Brainstormingsessions und inspirierenden Diskussionen.

Ich möchte mich auch bei meinen TAC Mitgliedern **Sabine Strahl** und **Christian Thiel** bedanken. Danke für all die Vorschläge, Anmerkungen und Unterstützung.

Im Brügger-Lab möchte ich vor allem jenen danken, die praktisch zu dieser Arbeit beigetragen haben. **Alexia, Thomas, Dipali** und den Lipidomicsleuten **Iris, Timo, Christian, Jochen und Jürgen.** Danke für eure Unterstützung.

Außerdem möchte ich allen aktuellen und ehemaligen PhD Studenten danken, besonders **Diego, Nethi** und **Helena**. Und natürlich auch **Alina**. Danke fürs Zuhören, Aufmuntern und all die wunderbare Zeit innerhalb und außerhalb der Universität.

Ich möchte auch den Studenten danken, die mich in der Arbeitsgruppe unterstützt haben. Besonders **Sonja, Franzi und Chrissi**, auch wenn ich nicht eure Betreuerin war, habe ich die Zeit mit euch sehr genossen.

Danke auch an **Abaya, Elena**, **Ellen** für die schöne gemeinsame Zeit.

Ein Großer Dank geht auch an **Marion** und **Bruni**, meine Heidelberg- und Coronafamilie. Vielen Dank, dass ihr immer für mich da seid, mich unterstützt und natürlich für all die schönen Badmintonspiele.

Ein riesen Dank geht auch an meine Eltern, **Daggi** und **Wolle**. Danke für all eure Liebe und Unterstützung. Und an meine Geschwister **Leonie** und **Till**. Ich hoffe ihre seid stolz auf eure große Schwester.

Und natürlich meinem Verlobten, **Johannes**. Danke für deine grenzenlose Liebe.