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Review

Protein *O*-mannosylation: What we have learned from baker's yeast ☆

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ABSTRACT

Background: Protein *O*-mannosylation is a vital type of glycosylation that is conserved among fungi, animals, and humans. It is initiated in the endoplasmic reticulum (ER) where the synthesis of the mannosyl donor substrate and the mannosyltransfer to proteins take place. *O*-mannosylation defects interfere with cell wall integrity and ER homeostasis in yeast, and define a pathomechanism of severe neuromuscular diseases in humans. Scope of review: On the molecular level, the *O*-mannosylation pathway and the function of *O*-mannosyl glycans have been characterized best in the eukaryotic model yeast *Saccharomyces cerevisiae*. In this review we summarize general features of protein *O*-mannosylation, including biosynthesis of the mannosyl donor, characteristics of acceptor substrates, and the protein *O*-mannosyltransferase machinery in the yeast ER. Further, we discuss the role of *O*-mannosyl glycans and address the question why protein *O*-mannosylation is essential for viability of yeast cells. General significance: Understanding of the molecular mechanisms of protein *O*-mannosylation in yeast could lead to the development of novel antifungal drugs. In addition, transfer of the knowledge from yeast to mammals could help to develop diagnostic and therapeutic approaches in the frame of neuromuscular diseases. This article is part of a Special Issue entitled: Functional and structural diversity of endoplasmic reticulum.

1. Introduction

Glycosylation is the most common and at the same time the most diverse protein modification in eukaryotic cells [1]. Over the years it appeared that a variety of protein-linked glycan structures play crucial roles in numerous cellular processes including cell-cell recognition, signal transduction, and ER protein quality control [2–7]. Especially in the recent past, glycosylation defects in humans have been identified as pathomechanism underlying a diverse set of severe congenital disorders [8–10], highlighting the impact of protein glycosylation on growth and development of eukaryotes.

Fungi and animals share three vital types of protein glycosylation that are initiated in the endoplasmic reticulum (ER): *N*-glycosylation, *O*-mannosylation and glypiation [11,12]. This review will focus on protein *O*-mannosylation.

O-mannosyl glycans were first identified in baker's yeast [13] where they are frequent modifications of secreted and cell wall proteins [14]. These short oligomannose chains [15] are attached *via* a glycosidic bond in alpha anomeric configuration to the hydroxyl group of Ser or Thr residues [16]. Mannosyltransfer of the reducing terminal mannose residue takes place in the ER [17] and is catalyzed by a conserved family of protein *O*-mannosyltransferases (PMTs; see below).

In baker's yeast the initial mannose may be step-wise extended in the cis and medial Golgi apparatus by α1,2-mannosyltransferases of the KTR-family to form α1,2-linked mannodi- and mannotriose [18,19]. Further α1,3-linked mannoses can be added in the medial and trans Golgi by α1,3-mannosyltransferases of the MNN1-family [18,20,21] to form linear oligosaccharides that are in the majority of cases up to five mannosyl residues long. In addition, mannosyl phosphate can be incorporated by the mannosylphosphate transferases Mnn6 and Mnn4, although for the latter transferase activity has not been proven directly [22].

In the following article, we will discuss the main aspects of protein *O*-mannosylation in the ER with emphasis on PMTs of the eukaryotic model organism *Saccharomyces cerevisiae*.

2. The mannosyl donor dolichol phosphate-mannose

The biosynthesis of *O*-mannosyl glycans starts in the ER where dolichol phosphate ß-D-mannose (Dol-P-Man) is the only mannosyl donor in all eukaryotes [23,24]. Dolichol is a long-chain polyprenol derivative that in baker's yeast is composed of 14-17 isoprene moieties

Abbreviations: CWI, cell wall integrity pathway; Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate-mannose; ERAD, ER-associated protein degradation; GPI, glycosylphosphatidylinositol; MIR, mannosyltransferase, inositol triphosphate- and ryanodine receptor; OST, oligosaccharyltransferase; PMT/POMT, protein *O*-mannosyltransferase; SDF2, stromal cell-derived factor 2; SVG, STE vegetative growth; TMD, transmembrane domain; UPR, unfolded protein response

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(C_{70}-C_{85}; prevalent components are C_{75} and C_{80}) [25] terminating in an α-saturated isoprene unit. At the cytosolic leaflet of the ER membrane dolichol is phosphorylated by the dolichol kinase Sec59 [26]. Mannose is then transferred from GDP-α-D-mannose (GDP-Man) to dolichol phosphate (Dol-P) with inversion of the anomeric configuration at the C1 atom of the mannosyl residue [16] (Fig. 1). This mannosyltransfer is catalyzed by GDP-α-D-Man:Dol-P ß-D-mannosyltransferase (EC 2.4.1.83; Dpm1) that belongs to the CAZy glycosyltransferase family 2 (GT2; http://www.cazy.org). Dpm1 was first purified in *S. cerevisiae*, allowing cloning of the corresponding gene [27,28]. Recombinant expression in *Escherichia coli* demonstrated that Dpm1 is the catalytic Dol-P-Man synthase [28]. The knockout of *DPM1* turned out to be lethal in baker's yeast, finally proving that Dol-P-Man is essential for cell viability reflecting its role as mannosyl donor in *N*-glycosylation, *O*-mannosylation and glypiation [29]. Based on homology to the enzyme from baker's yeast, DPM homologues from numerous organisms were identified, which fall into two major subgroups: i) individual membrane proteins, like *S. cerevisiae* Dpm1, that have a single transmembrane helix near the carboxyl-terminus and are fully functional when heterologously expressed in *E. coli* or mammalian cells [28,30]; and ii) proteins, like *Schizosaccharomyces pombe* and human DPM1, that lack the C-terminal hydrophobic region [31] and depend on the membrane proteins DPM2 and DPM3 for full enzymatic activity [32,33]. DPM2 and DPM3 are tethering DPM1 to the ER membrane, thereby stabilizing DPM1 [33], and most likely facilitating interactions with its substrate Dol-P. Recently, a third subgroup has been identified in the human pathogen *Plasmodium falciparum* that lacks DPM2 and DPM3. *Pf*DPM1 bears a single hydrophobic helix near the N-terminus and is functional in *S. pombe*, but not *S. cerevisiae* or human cells [34]. For a detailed discussion of Dol-P-Man synthases other than baker's yeast, we refer to the review by Maeda and Kinoshia [35].

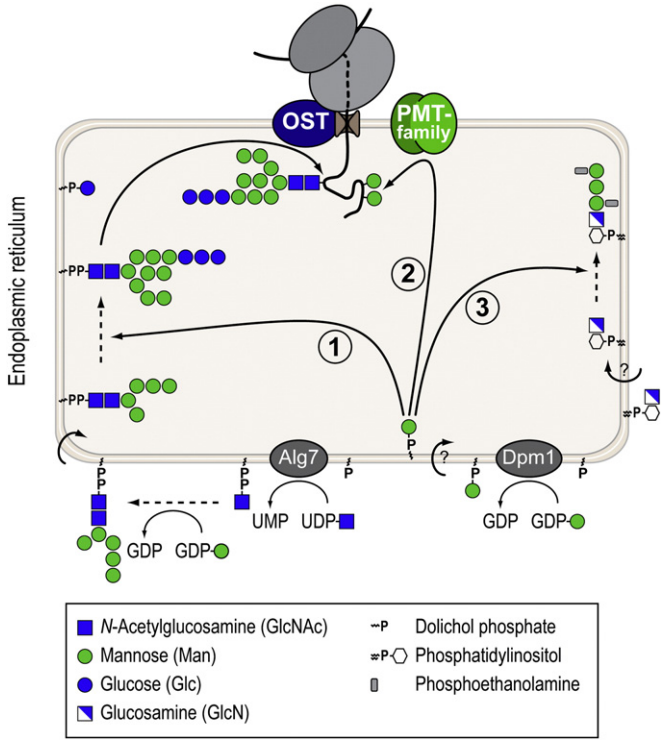

Fig. 1. Protein glycosylation in the ER. Dol-P-Man serves as mannosyl donor for the initial steps of *N*-glycosylation, *O*-mannosylation and glypiation. It is synthesized on the cytosolic face of the ER membrane by Dpm1 catalyzing the mannosyltransfer from GDP-Man to Dol-P. Dol-P-Man is flipped across the ER membrane and used as mannosyl donor for the assembly of the core oligosaccharide (1), transfer on Ser/Thr residues by PMTs (2), and biosynthesis of GPI anchors (3).

S. cerevisiae Dpm1 is a membrane protein that is facing the cytosolic side of the ER membrane [28]. Thereby Dpm1 can interact with both, the water-soluble mannosyl donor substrate GDP-Man in the cytosol and the highly hydrophobic mannosyl acceptor Dol-P within the ER membrane. Up to date, the three-dimensional (3D) crystal structure of yeast Dpm1 has not been resolved, however, usage of Dol-P and synthetic Dol-P analogs containing a fluorophore group provided a 3D structural model of the enzyme with bound GDP-Man, Dol-P and Mg^{2+} ions to trace substrate binding and catalytic sites [36,37]. A two-domain structure was predicted with an N-terminal domain, which is made up of parallel ß-strands, and a C-terminal domain consisting of antiparallel ß-strands, each flanked by α-helices on both sides. The model infers that the active site is a long shallow and wide cleft spanning the two domains of the protein with the GDP-binding site located in the N-terminal region whereas mannose and Dol-P binding is restricted to the C-terminal domain. Interactions of Dpm1 with Dol-P are restricted to a few isoprene units and the phosphate group at the α-terminus. Further, the model indicates that the amino acid residues Tyr12, Asp44, Asp97, and Arg212, which are conserved among DPMs from various organisms, form the active site [37].

The resulting Dol-P-Man is flipped from the cytosolic to the luminal leaflet of the ER membrane (Fig. 1), a process that most likely depends on a transport facilitator also referred to as flippase [38–40]. Dpm1 itself was proposed to act as flippase [41], but was subsequently ruled out as playing a direct role in Dol-P-Man flip-flopping [42]. Although numerous approaches over the last 30 years have addressed the translocation of Dol-P-Man [39], and despite the recent success in reconstituting Dol-P-Man transport into proteoliposomes containing rat liver ER membrane proteins [43], Dol-P-Man flippases have not yet been identified in eukaryotes. Interestingly, based on NMR and molecular modeling studies on the membrane orientation of dolichols and their interaction with dolichol recognition sequence peptides (also present in Dpm1), Zhou and Troy [44] constructed the hypothesis that dolichol-induced changes of the membrane structure may have the potential of forming a membrane channel that could facilitate the movement of hydrophilic sugar chains across hydrophobic membranes. However it is done, Dol-P-Man flipping into the ER lumen remains a mystery, both in mammals and yeast.

3. The PMT family of protein *O*-mannosyltransferases

In the mid-70s, ribosomal profiles of regenerating yeast protoplasts labeled with radioactive Dol-P-Man revealed that in the presence of cycloheximide protein *O*-mannosylation was mainly detected at the polysomal level [45]. Since then it is generally accepted that *O*-mannosylation is initiated while proteins are translocated into the ER lumen. More recently, it became obvious that various unfolded proteins receive *O*-mannosyl glycans while they are retained in the ER, showing that *O*-mannosylation can also occur in the ER after protein translocation (see below).

In the ER lumen, dolichol phosphate ß-D-mannose: protein *O*-mannosyltransferases (PMTs; EC 2.4.1.109) are the key enzymes that specifically initiate protein *O*-mannosylation. These enzymes catalyze the transfer of a mannosyl residue from Dol-P-ß-D-Man to Ser and Thr residues of secretory and membrane proteins in α-D-mannosidic linkage [16] (Fig. 1). As in the case of Dpm1 the first PMT (Pmt1) was purified from baker's yeast leading to the cloning of the corresponding *PMT1* gene [11,46,47]. Based on homology to Pmt1, a family of protein *O*-mannosyltransferases comprising at least six members (Pmt1-Pmt6) was found in *S. cerevisiae* [48–51]. In the following years it turned out, that PMTs are conserved throughout the fungal and animal kingdoms, and are even present in some

bacteria and archaea [52,53]. PMTs are classified as CAZy family GT39 (http://www.cazy.org). They represent generic members of the GT-C glycosyltransferase superfamily. Their predicted architecture is that of large polytopic membrane proteins located in the ER or the plasma membrane which display variations of a DXD motif in the first luminal/extracellular loop region close to the membrane surface [54,55]. Like PMTs, most GT-Cs are inverting glycosyltransferases that utilize lipid phosphate-activated sugar donors. Very recently, the first three-dimensional structure of a GT-C family member has been solved [56]. The X-ray structure of oligosaccharyltransferase (OST) from *Campylobacter lari* revealed that PglB features an N-terminal transmembrane region consisting of 13 TMDs, and a C-terminal periplasmic region. Stt3, the eukaryotic homologue of bacterial OST, has been suggested to be distantly related to PMTs (http://pfam.sanger.ac.uk/family/PMT; A. Bateman personal observation), indicating that similarities in either the architecture of the enzyme or the catalytic mechanism might be present.

In eukaryotes, the PMT-family can be subdivided into three major clades referred to as PMT1, PMT2 and PMT4 [57,58]. In *S. cerevisiae*, two members of the PMT1 clade, three members of the PMT2 clade and a single member of the PMT4 clade are present [51]. Redundancy of PMT1 and PMT2 members is also observed in yeasts of the class Saccharomycetes and in the opportunistic fungus *Candida albicans* and might have evolved by genome/gene duplication as discussed earlier [52]. In contrast, in other yeasts and in filamentous fungi only a single member of each, PMT1, PMT2 and PMT4 is present [11,59–64]. Interestingly, PMT1s are restricted to fungi, since in animals, only one member of each, the PMT2 and the PMT4 clade is found [65,66]. For more information on PMTs and protein *O*-mannosylation of fungi other than *S. cerevisiae*, of animals and of bacteria we refer to previous reviews [52,53,58,59,67–69].

3.1. Structure-function relationship of PMT complexes

PMTs are ER membrane glycoproteins comprising seven transmembrane spanning domains (TMDs) [70]. The carboxyl-terminus and the hydrophilic regions loop1 (between TMD1 and TMD2) and loop5 (between TMD5 and TMD6) are located on the luminal side of the ER membrane, whereas the amino-terminus, a hydrophobic region between TMD4 and TMD5, and the hydrophilic loop6 (between TMD6 and TMD7) are facing the cytosol (Fig. 2A). PMTs form distinct complexes among each other and complex formation is mandatory to acquire full mannosyltransferase activity in yeasts and in mammals [71–73]. In baker's yeast, PMT1s (Pmt1, Pmt5) form heterodimeric complexes with PMT2s (Pmt2, Pmt3). Pmt1-Pmt2 complexes represent the predominant PMT1/PMT2 *O*-mannosyltransferase activity *in vitro* and *in vivo*, although Pmt5-Pmt3 complexes are present [72]. In the absence of the corresponding complex partner also Pmt1-Pmt3 and Pmt5-Pmt2 interactions occur, suggesting a compensatory cooperation to maintain *O*-mannosyltransfer in the ER. In contrast to PMT1/PMT2 members, Pmt4 forms homodimeric complexes. The same protein regions, especially an invariant arginine residue in TMD2, are important for both hetero- and homomeric interactions [72].

Yeast Pmt1-Pmt2 complexes have been characterized using a photo-reactive mannosyl acceptor peptide [74]. The photo-probe could be specifically cross-linked to the Pmt1-loop1 domain (Fig. 2A). Mutations of a DE motif, which is highly conserved in the loop1 region of PMTs from pro- and eukaryotes, hampered binding of the photo-probe suggesting that this signature constitutes or is part of the acceptor binding and/or the catalytic site. PMTs are inverting glycosyltransferases that most likely employ a direct displacement S_N2-like mechanism involving an active-site aspartate or glutamate side chain as an enzymatic base catalyst [55]. Very often, at least three acidic amino acids are involved in this reaction mechanism. However, since in Pmt1-Pmt2 complexes exchange of a single

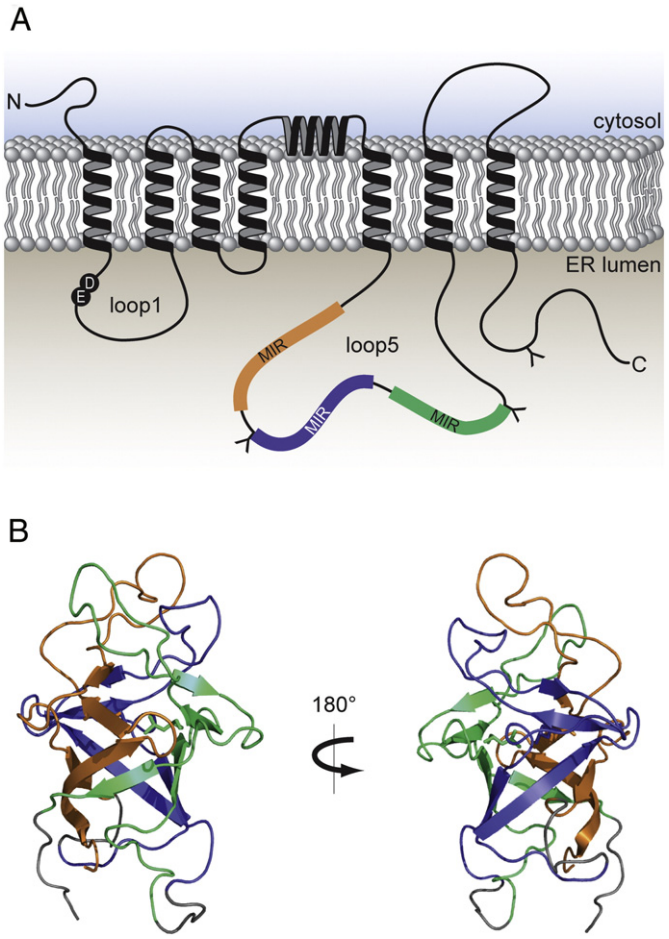

Fig. 2. Molecular structure of PMT proteins. (A) Topology model of Pmt1 according to [71]. The conserved Asp-Glu (DE) motif in loop1 and *N*-glycosylation sites (Y) are indicated. In loop5, the MIR motifs are colored in orange, blue, and green. (B) Ribbon representation of a Pmt4 loop5-MIR domain model, modeled by the SWISS-MODEL server [135] based on the *At*SDF2 structure. A ß-trefoil fold is made up of the three MIR motifs (color coded as in (A)) giving rise to a pseudo 3-fold symmetry. The view on the right is rotated by 180° along a vertical axis with respect to the view on the left.

acidic residue of the DE motif does not abolish enzymatic activity [74] it is unlikely that one of those plays a role as direct catalyst. More likely, they might be involved in the coordination of divalent cations that position the catalytic base to interact with the substrate and/or electrochemically stabilize the lipid phosphate leaving group [55]. In line with this, it was shown that divalent Mg^{2+} cations stimulate yeast PMT activity, even though chelators did not inhibit enzyme activity *in vitro* [75,76]. When the DE motif was replaced with alanine residues mannosyltransferase activity of both, Pmt1-Pmt2 and Pmt4 complexes was completely lost. In contrast, single replacement of either the aspartate or the glutamate residue abolished transferase activity of homodimeric Pmt4 complexes, although the same mutations caused only a moderate reduction of enzymatic activity of heterodimeric Pmt1-Pmt2 complexes [74]. These data suggest that DE motifs of dimeric complexes could contribute to the catalytic site (D77E78 in Pmt1 and D92E93 in Pmt2; D80E81 in Pmt4), thereby offering a possible explanation why physical interactions between two PMT molecules are obligate for mannosyltransfer activity in baker's yeast. However, in *S. pombe*, deletion of PMT2, but not of PMT1 results in cell death, although they form a heteromeric complex and no other PMT1/PMT2 members are conserved which could complement for PMT2 activity [77]. There are several ways to explain these discrepancies however, they are largely notional. Thus, to ultimately understand

the molecular mechanisms underlying the mannosyltransfer reaction, structural analysis of PMT complexes is mandatory for the future.

All eukaryotic PMTs contain three so called MIR motifs (mannosyl transferase, inositol triphosphate- and ryanodine receptor) [78] within the ER-luminal loop5 domain (Fig. 2A). In baker's yeast, this MIR domain is essential for enzymatic activity *in vitro* and *in vivo* [57], although its deletion did not affect binding of a photo-reactive peptide substrate [74]. Because bacterial and archaeal PMTs have a truncated loop5 lacking the MIR domain [52], it is highly unlikely that this region is directly involved in catalysis but rather plays a yet unknown ER-specific role. Interestingly, SDF2 (stromal cell-derived factor 2)-type proteins show significant homology to the MIR domain of the PMT-loop5 region. SDF2-type proteins are small hydrophilic ER proteins that are conserved throughout the animal and plant kingdoms, but are absent in fungi [79,80]. They are ER stress-induced components that interact with the molecular HSP40 co-chaperone ERdj3 and in turn with the HSP70 chaperone BiP, and thus might play a role in protein folding [80–82]. We recently solved the 3D-crystal structure of *At*SDF2 [80,83]. It revealed a typical ß-trefoil fold, which is found in a number of proteins that share little sequence similarities, but among those chaperone interactors are frequently found [84]. Based on the *At*SDF2 structure, the loop5-MIR domain of yeast PMTs has been modeled (A. Schott and S.S., unpublished data) (Fig. 2B). Most interesting, alanine replacement of conserved leucine residues that resulted in improper folding and aggregation of *At*SDF2 [80], also affected *Sc*Pmt1 activity [57] and folding of Pmt1-loop5 (V. Girrbach and S.S., unpublished data), supporting the significance of the model. In analogy to SDF2-type proteins, the PMT loop5-MIR domain might interact with ER chaperones in order to retain an unfolded mannosylation competent state of substrate proteins while they are entering the ER and/or to trigger *O*-mannosylation of un-/misfolded proteins (see below). However, both options have to be proven in the future. In contrast to the Pmt1 domains in the ER lumen, little is known about the regions that are oriented towards the cytosol, except that they are crucial for mannosyltransferase activity [57].

3.2. PMT protein substrates and substrate specificity

In the course of the last 20 years, many *O*-mannosylated proteins have been identified including secretory proteins, proteins anchored to the plasma membrane and the cell wall, or proteins located in organelles of the secretory pathway [14,85–88] (Fig. 3). In most of the cases, these proteins are substantially *O*-mannosylated and the mannosyl chains are clustered in distinct Ser/Thr-rich regions (Fig. 4A). An extreme example is the extensively *O*-mannosylated cell surface flocculin Flo11/Muc1 with 685 out of 1367 amino acids being Ser/Thr residues [89]. A recent computational analysis rated that in baker's yeast, about 45% of 594 signal peptide-containing proteins (according to the SignalP server) have at least one region of 20 or more amino acids with a minimum Ser/Thr content of 40% [90]. Around 18% of the proteins with signal peptide were predicted to contain 1-2 highly *O*-mannosylated Ser/Thr-rich regions with a mean length of ~67 amino acids and a minimum of 25% *O*-glycosylated residues.

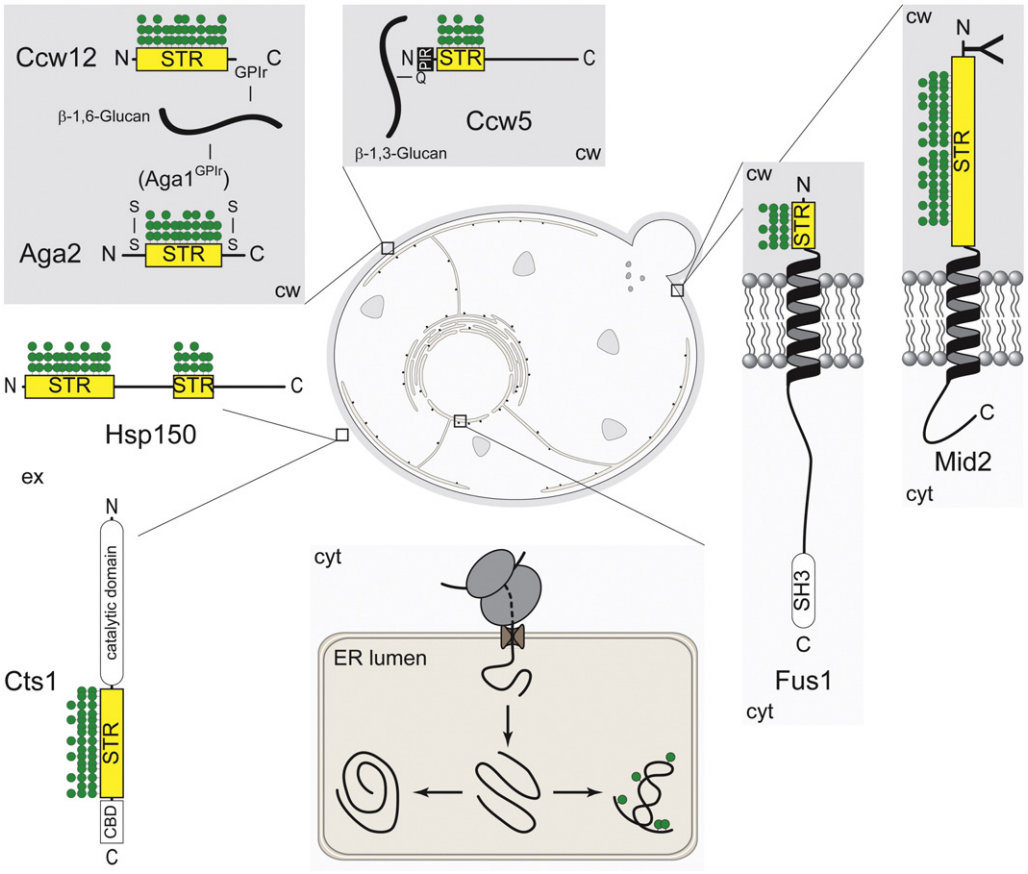

Fig. 3. Occurrence of *O*-mannosylated proteins in *S. cerevisiae*. Mannoproteins have been identified including proteins anchored to the plasma membrane such as the sensor protein Mid2, and Fus1 that is involved in cell fusion [93,94]. In addition, many secretory proteins such as chitinase (Cts1) and proteins anchored to the cell wall (*e.g.*, Aga2, Ccw5, Ccw12) are highly *O*-mannosylated [136–138]. Typically, *O*-mannosyl glycans are clustered in Ser/Thr-rich regions, and affect stability or targeting of some of these proteins. In addition, specific proteins are *O*-mannosylated only in cases where they are not properly folded (for details see 4.2). Mannose residues are depicted in green and *N*-glycans (Y) are indicated; STR, Ser/Thr-rich region; GPIr, GPI remnant; cw, cell wall; cyt, cytosol; ex, extracellular space.

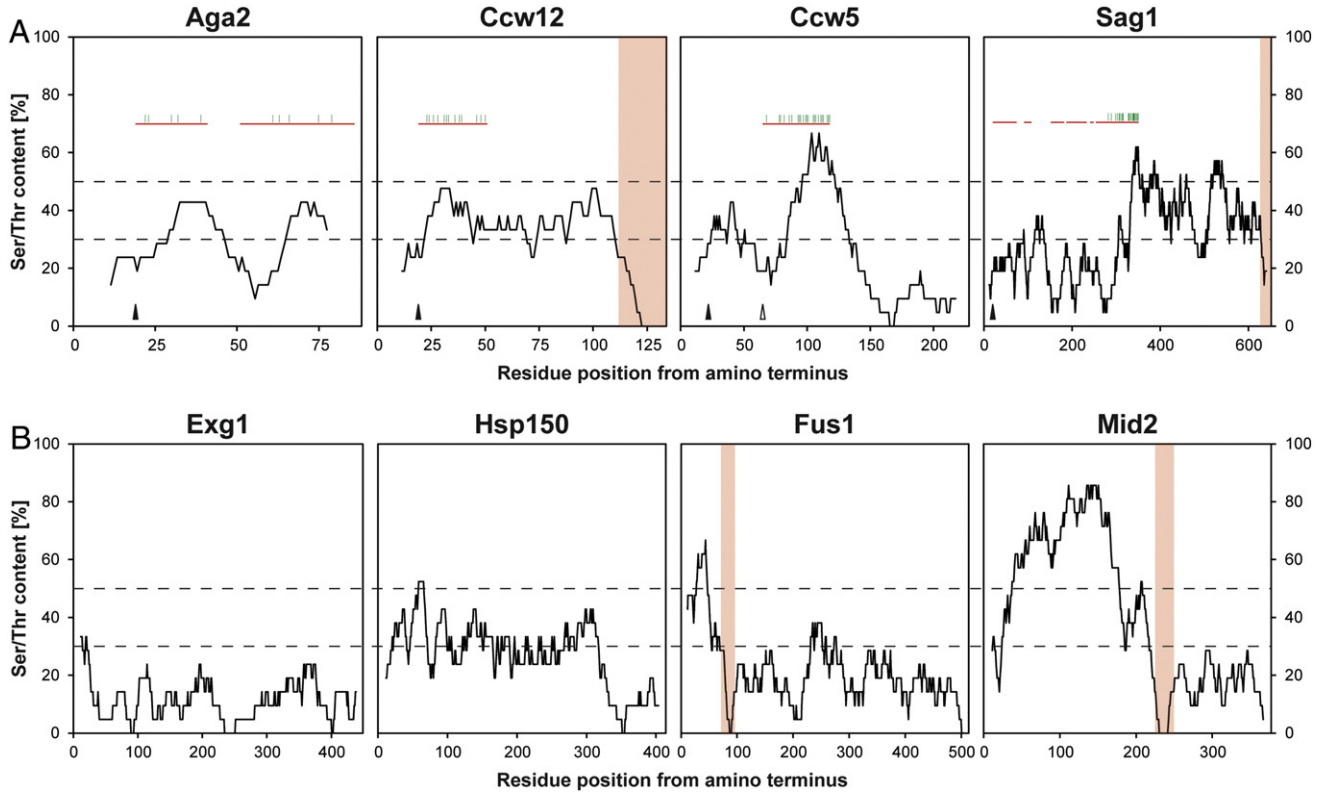

Fig. 4. PMT substrates are especially rich in Ser/Thr residues. The Ser/Thr content of proteins was calculated with a window size of 21 amino acids (A, B). (A) Clustering of *O*-mannosyl glycans within Ser/Thr-rich regions of the cell wall proteins Aga2, Ccw12, Ccw5, and Sag1. Typically, the Ser/Thr content of regions bearing *O*-mannosyl glycans is above 30%. Sequenced peptides (indicated as red lines) and experimentally verified *O*-mannosylation sites (marked in green) are shown according to [96,131,136,137]. Signal peptide and Kex2 cleavage sites are denoted by filled and open arrows, respectively. Hydrophobic protein regions replaced by GPI anchors are illustrated as red boxes. (B) Comparison of Ser/Thr content between non-substrate Exg1 [139], Pmt1-Pmt2 substrate Hsp150 [91], Pmt4 substrate Fus1 [93], and Pmt1-Pmt2, Pmt4 substrate Mid2 [94]. TMDs are depicted as red boxes.

Pmt1-Pmt2 and Pmt4 complexes exhibit specificity towards their numerous protein substrates (Fig. 4B) of which some have dozens of *O*-mannosylation sites. Various *S. cerevisiae* proteins have been experimentally proven to be predominantly mannosylated by Pmt1-Pmt2 (*e.g.*, Kre9, Bar1, Pir2/Hsp150, and Aga2), yet others by Pmt4 (*e.g.*, Kex2, Gas1, Axl2 and Fus1) [91–93]. Besides, there are examples where Pmt1-Pmt2 and Pmt4 complexes act on the same protein but mannosylate distinct regions, such as Cts1, Mid2, Mtl1, the WSC-family members and Ccw5 [91,94–96]. Notably, Pmt1-Pmt2 complexes act on both, soluble and membrane proteins, whereas Pmt4 mannosylates membrane-bound proteins, whereby the nature of the membrane anchor is not relevant for recognition [97]. Moreover, various proteins with moderate Ser/Thr content have been described to be *O*-mannosylated by Pmt1-Pmt2 only when they are not properly/efficiently folded (see 4.2.), whereas Pmt4-derived mannosylation of misfolded proteins has not been observed yet. Even if mannosyltransferase activity is measured *in vitro*, different specificities towards mannosyl acceptors are obvious. *In vitro*, Pmt1-Pmt2 complexes transfer mannose from Dol-P-Man to Ser and Thr residues of synthetic peptides ranging from tri- to oligomers [46,75]. Derivatives of the pentapeptide YATAV turned out to be potent *in vitro* acceptors with K_m values in the micromolar range [49,98]. However, mannosyltransfer of Pmt4 to synthetic peptides could not be detected [50]. We recently showed that Pmt4, but not Pmt1-Pmt2 activity can be measured *in vitro* using an assay that has been established to detect PMT activity in mammals [74]. In this assay, a recombinant GST-fusion protein of a mammalian PMT substrate is serving as mannosyl acceptor [24], demonstrating that yeast Pmt4 and mammalian PMT complexes share substrate specificities, at the very least *in vitro*. It is noteworthy, that *O*-mannosyl glycans in mammals so far have only been found on membrane associated proteins [99–101], suggesting that mammalian PMTs and yeast Pmt4 recognize similar features of their protein substrates also *in vivo*. The basis of the different substrate specificities of PMT complexes is not known yet, since in contrast to other types of glycosylation, signals for *O*-mannosylation of Ser and Thr residues appear to be not just linear sequences of a protein's primary structure both in fungal and in animal cells. Although various signature sequences had been suggested, none proved itself true [46,102,103].

4. The role of protein *O*-mannosylation in baker's yeast

Protein *O*-mannosylation is an essential protein modification in fungi, animals, and humans [50,51,64,77,104,105]. In *S. cerevisiae*, the analysis of viable *pmt*Δ mutants revealed that *O*-mannosyl glycans are important for the stability, localization and/or function of various secretory and membrane proteins and hence affect diverse cellular processes such as cell polarity, cell wall integrity, mating and filamentation [52,95,106]. The simultaneous knockout of particular combinations of PMTs (*e.g.*, *pmt1pmt2pmt4*Δ) turned out to be lethal [50,51].

4.1. Protein O-mannosylation in an essential protein modification

Why is *O*-mannosylation crucial for the survival of yeast cells? The transcriptional response upon inhibition of *O*-mannosylation, threw light on this question. In response to inhibition of all PMT

family members by a rhodanine-3-acetic acid derivative three major stress signaling pathways turned out to be activated: i) the unfolded protein response (UPR); ii) the cell wall integrity (CWI) pathway, which provides a means to repair damages to the cell wall; and iii) the STE vegetative growth (SVG) pathway, which promotes cell wall integrity in parallel with CWI. Evidently, ER homeostasis and cell wall integrity are primarily affected when *O*-mannosylation is blocked [107].

Nascent secretory and membrane proteins synthesized at the rough ER are translocated, potentially glycosylated and soon begin to fold. Conditions that interfere with protein folding induce the accumulation of misfolded proteins in the ER and thereby trigger a protective response termed the UPR and eventually, ER-associated protein degradation (ERAD) [108,109]. The transcriptional program of the UPR consists of genes involved in a wide range of cellular processes comprising protein folding and glycosylation (including *PMT1*, *PMT2*, *PMT3*, and *PMT5*), protein degradation, lipid biosynthesis and cell wall construction [110]. In baker's yeast, under normal growth conditions proteins receive *O*-mannosyl glycans while they are entering the ER [45] (M. L. and S.S., unpublished data). Transcriptional profiling showed that the UPR is activated when *O*-mannosylation is blocked [107], most likely due to the accumulation of abnormal proteins since *O*-mannosyl glycans affect solubility, maturation, and/or targeting of various proteins [52]. Even in viable pmtΔ mutants the UPR is constitutively induced under normal growth conditions [111,112] (U. Schermer and S.S., unpublished data), and synthetic lethal interactions between *HAC1*, the key transcription factor of the UPR, and individual *PMT*s proved that the UPR is crucial for survival of yeast cells when *O*-mannosylation is diminished [107]. Since many proteins involved in cell wall biosynthesis and structure are highly *O*-mannosylated (see 3.2.), decreased glycosylation most likely results in aberrant cell wall mannoproteins and, consequently, the CWI and the SVG pathways are activated to buffer accruing cell wall defects.

The yeast cell wall is of vital importance. It is composed of ß-glucan (~50%), chitin (~2%) and mannoproteins (~48%) that are highly *N*-glycosylated, *O*-mannosylated or both [86,113]. Mannoproteins are structural and functional important cell wall components, and their carbohydrate moieties regulate cell wall permeability and determine characteristics of individual proteins that are crucial for cell wall biogenesis and structure [14,86]. Thus, glycosylation mutants feature cell wall-related phenotypes such as aberrant cell wall morphologies and susceptibility towards cell wall disturbing agents [47,51,114,115]. During vegetative growth and periods of environmental stress the CWI MAP kinase pathway compensates for diverse cell wall defects *inter alia* by enhancing the biosynthesis of certain mannoproteins, chitin- and glucan-synthases [116]. In parallel, the SVG pathway promotes cell wall integrity under vegetative growth conditions especially when biosynthesis of mannoproteins is hampered [117,118]. Inhibition of PMTs triggers the CWI pathway, and in some pmtΔ mutants the CWI pathway is constitutively activated [94,107] which goes along with an increased cell wall chitin content [51]. The CWI pathway also results in the induction of the UPR [119]. However, in response to inhibition of *O*-mannosylation initiation of the UPR occurs at least in part in a CWI pathway-independent manner, since expression of many of the induced UPR target genes is activated neither by a constitutive active CWI pathway nor in cell wall mutants [107]. In addition to the CWI pathway, the SVG signaling cascade is induced upon inhibition of PMTs, and its central transcription factor Ste12 is crucial for vegetative cell growth when *O*-mannosylation is low [107]. Ste12 is also a key regulator of the MAPK pathways controlling mating of haploid yeast cells, and filamentous growth [120]. Interestingly, inhibition of *O*-mannosylation resulted in an at least partially Ste-dependent repression of mating and filamentation genes, which appeared in strikingly reduced mating and invasive growth efficiencies of haploid yeast cells. ChIP analysis suggested that PMT inhibition is triggering the displacement of the transcription factor Ste12 from mating and filamentation genes towards targets of the SVG pathway [107]. In this way, processes which involve extensive cell wall remodeling are prevented, to protect cells from lysis when mannoprotein biosynthesis is impaired.

Both, the CWI and the SVG pathways boost *inter alia* the biosynthesis of cell wall mannoproteins to counteract cell wall defects [121,122]. In addition, the UPR is activated to deal with the high protein load of the ER. Like most ER chaperones and members of the ERAD machinery, *PMT1*, *PMT2*, *PMT3* and *PMT5* are transcriptionally induced during the UPR, further emphasizing their role in ER homeostasis [110]. Absence of *O*-mannosylation however, could provoke an infinite loop, finally causing cell death due to proteotoxic effects of accumulating aberrant mannoproteins in the secretory pathway.

4.2. Protein O-mannosylation and ER homeostasis

In addition to adding *O*-linked mannose to proteins entering the ER, PMTs might have more specific functions during protein folding in the ER. In recent years it turned out that various proteins are *O*-mannosylated after translocation inside the ER by Pmt1-Pmt2 complexes when they are misfolded [123,124]. Especially when ER-stress is triggered by tunicamycin (block of *N*-glycosylation), *O*-mannosylation is significantly increased and proteins that are normally not *O*-mannosylated receive *O*-mannosyl glycans [123]. Based on these findings, it was proposed that prolonged ER residence allows modification of exposed *O*-mannosyl glycan acceptor sites within misfolded proteins. To date, the fate of only a handful of artificial aberrant proteins has been analyzed that receive *O*-mannosyl glycans whereas their wild-type counterparts do not [111,123–126]. Some of them are more protected against degradation [123,125], while others are degraded by the ERAD pathway [124,126]. Very recently, evidence was presented that Pmt1-Pmt2 complexes have the capacity to interact with ER chaperones involved in oxidative protein folding (Pdi1 and Ero1); the Hrd1 complex, which is functioning in ERAD; and the p24 protein complex, which participates in ER export of GPI-anchored proteins [127]. A model has been suggested where Pmt1-Pmt2 complexes directly assist protein folding of the GPI-anchored protein Gas1. In cases where ER export competent folding cannot be achieved, interaction with Pmt1-Pmt2 might be extended. Consequently, *O*-mannosylation increases and targets Gas1 for ERAD or post-ER degradation [127]. For other examples of heterologously expressed or misfolded proteins it has been reported that *O*-mannosylation by PMTs increases their solubility and renders them less dependent on the ER chaperone BiP/Kar2 [111,126,128]. As a consequence, aberrant proteins bearing *O*-mannosyl glycans exit the ER for normal secretory transport and are either degraded in the vacuole or secreted from the cell [111]. When deletion of *PMT2* was combined with mutants of ERAD components, yeast cells exhibited enhanced UPR. Thus, it was proposed that *O*-mannosylation might function as a fail-safe mechanism by solubilizing aberrant proteins that deluge from the ERAD pathway thereby reducing the load for ER chaperones [111]. While analyzing the extracellular domain of the plasma membrane protein Fus1 which is modified by Pmt4 [93], we observed that in the absence of the membrane anchor Fus1 is entering the secretory pathway. The majority of the protein however, is not mannosylated and accumulates in the ER and a post-ER compartment [97]. Interestingly, a fraction of the mutant protein is *O*-mannosylated by Pmt1-Pmt2 and secreted into the medium, thereby supporting the model that *O*-mannosyl glycans enhance the solubility of misfolded proteins and facilitate their exit from the ER. Due to its complexity, to date the physiological function of *O*-mannosylation for ER homeostasis still needs to be defined precisely.

5. Concluding remarks

In recent years, an interdependence between protein *O*-mannosylation and *N*-glycosylation became more and more obvious in baker's yeast. Both types of glycosylation are initiated in the ER, *O*-mannosylation by distinct PMT complexes and *N*-glycosylation by the membrane protein complex OST [129]. Activity of both enzyme complexes depends on the essential ER chaperone Rot1 [130], and they can act on the same acceptor protein (*e.g.*, [94–96,131]). It was shown that PMTs and OST compete for acceptor protein substrates, and that *O*-mannosylation can precede *N*-glycosylation, suggesting that PMTs have the potential to alter the number of *N*-linked glycans [96] (M.L. and S.S., unpublished data). The transcriptional profiles of cells in which *O*-mannosylation [107] or *N*-glycosylation [110] was selectively blocked, revealed that both defects result in activation of the UPR, the CWI and the SVG pathways suggesting an overlapping impact of *O*- and *N*-glycosylation on ER homeostasis and the yeast cell wall. The idea that *O*- and *N*-linked glycans of cell wall mannoproteins can at least partially compensate for each other in order to maintain cell wall integrity, is further supported by the finding that transcription of the Golgi mannosyltransferases *OCH1*, *KTR2* and *MNT4* that are involved in the biosynthesis of the outer chain of *N*-linked high mannose carbohydrates [21,115,132,133] is enhanced when *O*-mannosylation is decreased [107]. As a *quid pro quo*, inhibition of *N*-glycosylation increases transcription of *PMT*s and of the α1,2-mannosyltransferase *KTR1*, which is involved in the extension of *O*-linked glycan chains in the Golgi [133]. Further, epistatic miniarray profiles revealed strong genetic interactions between *N*- and *O*-glycosylation machineries in the early secretory pathway [134]. To tackle these interconnections remains a challenging task for the future.

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