# Review

# Protein-*O*-mannosyltransferases in virulence and development

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**Abstract.** Protein-*O*-mannosyltransferases (Pmt proteins) catalyse the addition of mannose to serine or threonine residues of secretory proteins. This modification was described first for yeast and later for other fungi, mammals, insects and recently also for bacteria. *O*-mannosylation depends on specific isoforms of the three Pmt1, 2 and 4 subfamilies. In fungi, *O*-mannosylation determines the structure and integrity of cell walls, as well as cellular differentiation and virulence. *O*-mannosylation of specific secretory proteins of the human fungal pathogen *Candida albicans* and of the bacterial pathogen *Mycobacterium tuberculosis* contributes significantly to virulence. In mammals and insects, Pmt proteins are essential for cellular differentiation and development, while lack of Pmt activity causes Walker-Warburg syndrome (muscular dystrophy) in humans. The susceptibility of human cells to certain viruses may also depend on *O*-mannosyl chains. This review focuses on the various roles of Pmt proteins in cellular differentiation, development and virulence.

**Keywords.** *O*-glycosylation, protein *O*-mannosyltransferase, Pmt proteins, POMT, virulence, development, *Candida*, *Saccharomyces*.

## Introduction

Protein glycosylation was previously considered to be restricted to eukaryotic organisms, while it is evident today that it is widespread among pro- and eukaryotes. Glycosyl residues can be linked to proteins via asparagine (*N*-glycosylation) or via hydroxylated amino acids including serine, threonine and, more rarely, tyrosine, hydroxyproline and hydroxylysine (*O*-glycosylation). A variety of monosaccharides can get *O*-linked to proteins, e.g. *N*-acetylgalactosamine (GalNAc), galactose (Gal) or glucose (Glc), which may or may not become extended further by additional sugars. The first evidence for mannose (Man) attached via *O*-glycosidic bonds to eukaryotic (yeast) proteins was obtained in 1969 [1], while later this modification was discovered also in prokaryotes (reviewed in [2]) and in mammalian cells (reviewed in [3]). The pioneering work by Tanner and co-workers (reviewed in [4]) established basic principles of the mechanisms and genetics of protein *O*-mannosylation. It was recognised that protein-*O*-mannosyltransferases (Pmt proteins) constitute a separate functional class of glycosyltransferases, transferring mannose carried by a polyisoprenoid carrier lipid as the donor substrate to serine or threonine residues of proteins in an α-glycosidic linkage. Pmt proteins are membrane proteins located in the ER membrane (eukaryotes) or the cytoplasmic membrane (prokaryotes) and they function by mannosylating secretory proteins during their membrane translocation, which

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may occur by a co- or posttranslational secretion mechanism.

Concepts regarding the roles of Pmt proteins and *O*-mannosylation have changed considerably over time. Initially Pmt proteins were believed to be specific for fungi, but recently their occurence in prokaryotes and higher eukaryotes, except plantae, was detected. Second, the presence of two and more Pmt isoforms in most species was formerly thought to provide functional redundancy, but later the specificity of Pmt isoforms was recognised. Third, the concept that *O*-mannosylation mainly is important for protein structure and stability was extended later to the essential roles of Pmt proteins for protein enzymatic activity. Furthermore, the relevance of *O*-mannosylated proteins for complex chains of reactions leading to cellular differentiation and development, as well as for virulence traits of microbial pathogens, has been recognised in recent years. The latter findings suggest Pmt proteins as targets for future antimicrobial therapy. This review provides an overview on various complex processes influenced by Pmt-mediated *O*-mannosylation, focusing on recently discovered roles of *O*-mannosylation for the virulence of microbial pathogens.

**Pmt proteins in prokaryotes**

Protein glycosylation among prokaryotes was discovered first in archaea, revealing the presence of *O*- and *N*-chains in S-layer proteins (reviewed in [2]). In bacteria, *O*-glycosylated proteins were found in flagellae (flagellin), pili (pilin) and other secretory proteins. For example, the enteropathogenic bacterium *Campylobacter jejuni* decorates its flagellar subunits by *O*-glycosylation with glycan moieties containing pseudaminic acid residues [5, 6]. Inactivation of many of the corresponding genes was shown to interfere with bacterial virulence by inhibiting motility and autoaggregation, both fundamental features for adherence and invasion of the intestinal epithelium, as well as for biofilm formation [6–8]. *O*-linked flagellar glycosylation was described for many other pathogenic bacteria including *Treponema palladium*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Aeromonas* and *Clostridium* species (reviewed in [9]). Moreover, pili involved in host-bacteria-interactions were found to be *O*-glycosylated in numerous important pathogens, e.g. *Neisseria meningitis, N. gonorrhoea*, *P. aeruginosa* and *Streptococcus parasanguis* (reviewed in [10]).

While the above glycoproteins in S-layers, flagellae or pili are not *O*-mannosylated it is known that a few glycoproteins in bacteria contain mannose in direct glycosidic linkages including the cellulases of *Cellulomonas fimi* and *Streptomyces lividans* [11], the endoglycosidase of *Flavobacterium meningosepticum* [12], the phytotoxin of *Corynebacterium sepedonicum* [13] and the MPB83 and Apa glycoproteins of *Mycobacterium tuberculosis* [14, 15]. Pmt proteins able to carry out *O*-mannosylation have been described only recently in *M. tuberculosis* [16] and in the taxonomically related but non-pathogenic species *Corynebacterium glutamicum* [17]. In *M. tuberculosis* two fully characterised glycoproteins contain threonine residues with linear α-1,2- and α-1,3-oligomannosides; this structure is similar to yeast *O*-chains suggesting that *O*-mannosylation is catalysed by Pmt proteins. Indeed, bioinformatics revealed a single *PMT* gene (*rv1002c*) in the genome of *M. tuberculosis* encoding a Pmt protein sharing 22–24 % identical residues and a similar hydropathy profile compared to Pmt proteins of *Saccharomyces cerevisiae*. Pmt activity was confirmed by overexpression of the *rv1002c* gene in *M. smegmatis* leading to increased Pmt enzymatic activity in membrane preparations, using an assay containing mannosyl-phosphoryl-decaprenol as donor and an artifical peptide as acceptor. Furthermore, proper glycosylation was shown to depend on protein translocation via the *sec* secretory pathway [16]. These results suggested that protein *O*-mannosylation is conserved in *M. tuberculosis* and in eukaryotes. Two of the probable mycobacterial Pmt targets are the immunodominant antigen MPB83 [14] and the cell wall-associated adhesin Apa (alanin- and proline-rich antigen), which was recently shown to bind to lectins of the immune system and for this reason is considered to facilitate colonization and invasion of host cells [18, 19].

Recently, Mahne *et al.* [17] identified a *pmt* gene in *C. glutamicum* encoding a protein with 40 % identical amino acids compared to *M. tuberculosis* Pmt and 15–18 % identity to *Saccharomyces* and *Candida* Pmt proteins. Furthermore, its hydropathy profile suggested a structure similar to *S. cerevisiae* Pmt1, containing 11 predicted transmembrane helices (although only 7 helices were verified experimentally, see below). Interestingly, *C. glutamicum* Pmt lacks a long hydrophilic loop (after the seventh predicted transmembrane helix) considered to be essential for the function of yeast Pmt1, but instead it contains two smaller loops between helices 1 and 2, as well as between helices 7 and 8. Disruption of the *C. glutamicum pmt* gene was shown to completely abolish glycosylation of the four glycoproteins present in culture supernatants of *C. glutamicum* [17]. One of the four known extracellular Pmt target proteins is the resuscitation promoting factor (Rpf2), which is involved in bacterial growth stimulation and intercellular communication

[20, 21], whereas the function of the three other glycoproteins remains to be resolved. By multiple sequence alignments several homologues of the *C. glutamicum* Pmt in other actinomycetales including *M. leprae*, *M. bovis*, *C. diphteriae*, *Streptomyces coelicor* and *Propionibacterium acnes* were identified [17] and open reading frames from a wide range of prokaryotic species have recently been annotated as putative Pmt proteins. The identification and characterization of Pmt proteins in prokaryotes will provide novel insights in the evolution and function of Pmt proteins. Since Pmt-mediated mannosylation appears essential for *M. tuberculosis* and because of the structural differences of bacterial and eukaryotic Pmt proteins, interference with Pmt activity appears to represent an attractive novel strategy to combat bacterial pathogens.

## Fungal Pmt proteins for growth and virulence

Following the early discovery of *O*-glycosylated proteins in fungi [1] it took 20 years before *PMT1*, the first gene linked to this protein modification was identified (reviewed in [2]). The initial step of fungal protein-*O*-glycosylation, the addition of a mannose residue in α1-linkage to the target protein, is conserved in fungi and is catalysed by Pmt proteins. This reaction probably occurs at the lumenal side of the ER-membrane, while the protein is translocating through the Sec61 pore complex, mostly co-translationally but in yeast also by a posttranslational secretion process [22]. In contrast to *N*-glycosylation, a specific *O*-glycosylation sequon could never been defined although serine/threonine-rich regions are preferentially *O*-glycosylated. Further extension of the glycan structures occurs in the Golgi system and is mainly mediated by the Ktr/Mnn-class of mannosyltransferases or other specific sugar transferases (reviewed in [23]), although a large fraction of *O*-chains consists only of a single mannose residue [24]. While initially protein-*O*-glycosylation in fungi was thought to be the linear addition of α-1,2- and/or α-1,3-linked mannose units to serine or threonine residues of respective target proteins it became evident in the last few years that *O*-linked glycosylation in fungi can be more complex and can include the addition of different sugars (glucose, galactose, etc.), branching, use of divergent glycosidic linkages (α-1,6-, β-1,2-, etc.) or the modification of attached sugar residues by phosphate or sulfate groups (reviewed in [25]). Pmt proteins not only have been shown to be required for fungal growth, but also for differentiation processes including cellular polarisation, cell fusion and sensing of external cues. Hence, development and virulence of fungal pathogens are influenced significantly by protein-*O*-mannosylation. Below we summarize the essentials of Pmt function in avirulent yeasts and filamentous fungi and then focus on their roles in pathogenic fungi.

***Avirulent yeasts.*** *S. cerevisiae* encodes a family of seven Pmt proteins, Pmt1–7 (reviewed in [2, 26]). These proteins share 50–60 % overall identity and can be subdivided into three subfamilies, the Pmt1-subfamily (Pmt1, Pmt5, Pmt7), Pmt2-subfamily (Pmt2, Pmt3, Pmt6) and Pmt4 [27] (see Fig. 1). In contrast, the fission yeast *Schizosaccharomyces pombe* only encodes 3 Pmt homologues, one of each Pmt subfamily (designated Oma/Ogm1, 2 and 4 [28, 29]), which is typical of fungi that have not undergone a genomic duplication during their evolution. The topology of ScPmt1 has been analysed and a model of its structure in the ER membrane was suggested containing seven transmembrane domains, an N-terminal cytoplasmic and a C-terminal ER-lumenal end [30]. Two hydrophilic loops (loop 1 and 5) faceing the ER lumen are indispensable for protein function [27]. Identical topologies have been proposed for all other fungal Pmt proteins. ScPmt proteins were found to function as homo- (Pmt4) or heteromeric (Pmt1-Pmt2 subfamily) complexes and corresponding dimers were also reported for *S. pombe* [28, 31, 32]. Specific regions in the N- and C-terminal ends of the Pmt proteins were identified that are important for dimer formation [31, 32]. The Pmt1/Pmt2 heterodimer accounts for most of the *O*-glycosylation activity measured in an enzymatic test using an artificial peptide acceptor substrate. This assay, however, may not reveal activities of all Pmt isoforms. Individual Pmt isoforms are dispensable for growth of *S. cerevisiae*, but some double mutants require osmotic stabilization and *pmt1/2/4* and *pmt2/3/4* triple mutants fail to grow. *S. pombe* Oma2 was reported to be required for growth [27], at variance with another study [29]. These results indicate that Pmt activity, not necessarily of individual isoforms but collectively, is essential for growth of fungi [33]. The reason for this growth requirement may be deduced from the phenotypes of single *pmt* mutations including high sensitivities to cell wall-disturbing agents suggesting striking defects regarding structure and function of the fungal cell wall. Many of the known Pmt target proteins directly or indirectly affect components of the cell wall (Table 1). The Kre9 glycoprotein is required for β-1,6-glucan assembly [34] and it is underglycosylated in both Δ*pmt1* and Δ*pmt2* mutants [35]. *O*-mannosylation appears important for Kre9-function because *kre9* and Δ*pmt1* Δ*pmt2* double mutants share phenotypes including slow growth,

reduced β-1,6-glucan (down to ~20%), an aberrant multi-budded cell morphology and resistance to K1 killer toxin that binds to β-1,6-glucan. Another Pmt-target protein affecting cell wall polysaccharides is the endochitinase Cts1, which allows the separation of mother and daughter cells by cleavage of wall chitin [36]. Cts1 is glycosylated by several Pmt isoforms, mainly by Pmt1 and Pmt2 [35], but also by Pmt4 [37], Pmt6 [38] and Pmt3 in a Δpmt1 Δpmt2 deletion background [32, 33]. Most if not all cell wall proteins covalently bound to glucan (β-1,3- or β-1,6-glucan) are O-mannosylated. Among proteins bound to β-1,3-glucan are Ccw proteins that can be released from the cell wall fraction by mild alkali treatment or laminarinase [39]. Most Ccw proteins were found to be O-glycosylated by Pmt1, Pmt2, Pmt4 and Pmt6 to varying degrees but not by Pmt3 or Pmt5 [39]. Most Ccw proteins belong to a previously identified protein family of "proteins with internal repeats" (PIR; [40]) that are linked to β-1,3-glucan through a novel protein-carbohydrate linkage [41]. Except for mutants lacking Hsp150/Pir2/Ccw7 none of the ccw/pir mutants had a strong phenotype [41, 42]. Simultaneous depletion of multiple members of the Pir-protein family led to slower growth, an increase in cell size and death, and increased sensitivity to cell wall-destabilising agents [40, 42]. Besides being bound to β-1,3-glucan, proteins can get anchored to β-1,6-glucan via a remnant of a glycosylphosphatidylinositol (GPI) anchor, which is cleaved during glucan-attachment. Such proteins include the highly O-glycosylated a-agglutinins Aga1 (Aga2) and α-agglutinin (Sag1), which promote cell agglutination during mating (reviewed in [43]). These agglutinins were found to be O-glycosylated by Pmt1 and/or Pmt2 but not by any other of the remaining Pmt proteins [38, 44, 45] and accordingly pmt1 and pmt2 mutants show cell type-specific unilateral and/or bilateral mating defects [45]. Pmt1 also O-mannosylates other proteins coupled to β-1,6-glucan, such as Srp1/Tir1 and Sed1 [44]. Srp1 belongs to the Srp1/Tip1-family of serine-alanine-rich cell wall mannoproteins that is expressed during anaerobiosis and at low growth temperatures and maintains cell wall integrity under these conditions [46,47], while Sed1 is a major stress-induced GPI glycoprotein that seems to be involved in cell integrity in the stationary growth phase and metal or oxidative stress [48, 49]. Another GPI remnant-linked protein glycosylated by Pmt4 and Pmt6 is gp115/Gas1/Ggp1 [33, 38]. In S. cerevisiae Gas1 belongs to the glycosidase/transglycosidase GH72 family of fungal enzymes involved in cell wall maintenance consisting of five members, Gas1–5 (reviewed in [50]). These enzymes are thought to be cell wall β-1,3-glucanosyltransferases involved in the formation and mainte-

nance of β-1,3-glucan. gas1 mutants show defects typical of cell wall-affected mutants including an abnormal round morphology, an increased sensitivity to cell wall-disturbing agents and a compensatory mechanism to counterbalance the loss of β-1,3-glucan and ensure cell wall integrity.

Pmt activity also influences the activity of proteins in the cytoplasmic membrane that direct complex cellular functions including sensing, polarisation and cell fusion. Cell wall integrity in S. cerevisiae is regulated through the PKC-MAP-kinase signalling pathway (reviewed in [51]) and this pathway is triggered by the Wsc1, Wsc2 and Mid2 sensors. These proteins are O-glycosylated by Pmt2 and Pmt4 and this modification is required to prevent an aberrant posttranslational processing, which causes lysis without osmotic stabilisation and cell death in the presence of mating-pheromone [52, 53]. These phenotypes can be rescued by overexpression of various components of the PKC pathway corroborating the finding that this pathway is defective in Δpmt2 Δpmt4 mutants [52]. During yeast growth, cells polarise their secretion machinery to allow budding at specific locations of the cell; haploids bud in axial fashion (budding next to sites of previous buddings), while diploids bud in bipolar fashion (also budding at sites opposite of previous buddings). Axial budding requires the Axl2 protein and it was shown that Pmt4-mediated O-mannosylation of Axl2 is absolutely required for its stability; in fact, pmt4 mutants were found in a screening for bipolar budding of haploids [54]. The Axl2 protein could be partially stabilised in pmt4 mutants by blocking N-glycosylation with tunicamycin indicating that O-glycosylation prevents proteolytic cleavage of the protein by blocking the attachment of N-glycosyl chains, which may allow degradation of misfolded ER proteins [55]. Correspondingly, O-glycosylation of the Pir4/Ccw5 protein by Pmt4 appears to prevent abnormal N-glycosylation [56]. Another O-mannosylated membrane protein, Fus1, is predominantly localised to the tips of mating protrusions ("shmoo") through reorganisation processes of lipid rafts [57, 58] and allows fusion of haploids of opposite mating types. Similar to Axl2, Fus1 was found to be hypo-glycosylated in S. cerevisiae pmt4 mutants, which resulted in an aberrant processing of Fus1 and its mislocalisation to Golgi vesicles and endosomes [58]. It was recently shown that membrane integration of Fus1 is a prerequisite for Pmt4-dependent O-glycosylation and localisation, since a deletion of the transmembrane region led to a complete loss of Pmt4-mediated O-glycosylation [59]. The data suggested that modification by Pmt4 (but not by Pmt1) requires a serine/threonine-rich potential attachment site situated adjacent to a transmembrane region or a GPI anchor

**Table 1.** Target proteins of fungal protein-$O$-mannosyltransferases.

| Pmt target proteins | Pmts | Phenotype/function related to $O$-glycosylation defects |
|---|---|---|
| ScKre9 (β-glucan assembly) | ScPmt1/2 | Associated with growth defects; altered cell wall composition/structure reduction of β-1,6-glucan; aberrant multiply budded morphology; mating defects; killer toxin resistant. |
| ScCts1 (cytokinesis) | ScPmt1/2/ (4/6) ScPmt3$^{Δ1/2}$ SpOma1* CaPmt1* | Impaired cell-cell separation; enhanced pseudohyphal growth. |
| ScBar1 (α-factor inactivation) | ScPmt1/2 | Mating defect; supersensitive to α-factor. |
| ScAga1/ScAga2/ScSag1 (sexual cell adhesion) | ScPmt1/2 | Mating defect. |
| ScTir1 (cell wall mannoprotein) | ScPmt1 | Cell wall integrity affected at low temperatures and hypoxia. |
| ScSed1 (GPI glycoprotein) | ScPmt1 | Sensitive to lytic enzymes at stationary phase; sensitive to oxidative and metal stresses. |
| ScPir2 (Hsp150) | ScPmt1/2/ (4) | Sensitive to heat, metal and oxidative stress. |
| ScPir1–4 (cell wall mannoproteins) | ScPmt1/2/ 4/6 | Reduced viability and growth rate; simultaneous mutation of multiple Pir proteins: increased cell size; cell accumulation; reduced mating; sensitive to heat shock, Congo red and calcofluor white. |
| ScKex2 (pro-protein processing) | ScPmt4/ (6) | Cold sensitive growth defect; large, multi-budded cells with attached daughter cells; defective in killer toxin production. |
| ScGas1 (GPI glycoprotein, cell wall assembly) | ScPmt4 | Abnormally round morphology; reduced viability in rich medium; sensitive to cell wall disturbing agents and elevated temperatures; higher chitin content in cell wall; slow growth. |
| ScWsc1/ScWsc2/ScMid2 (cell integrity sensors) | ScPmt1/2/ 4 | Sensitive to various cell wall stresses (temperature, caffeine, etc.) and mating pheromone; reduced β-glucan content; slow growth in low glucose. |
| ScAxl2 (axial budding) | ScPmt4/ (1/2) | Bipolar budding of haploid cells. |
| ScFus1 (mating) | ScPmt4 SpOma4* | Bipolar mating defect. |
| ScRax2 (bipolar budding) | ScPmt4 | Loss of bipolar budding pattern in diploid strains. |
| CaCht3? (ScCts1 homologue) | CaPmt1 | Cell separation defect. |
| CaKre9 (ScKre9 homologue) | CaPmt1 | No hyphal growth but cell accumulation in serum; reduced β-glucan content in cell wall. |
| CaAls1 (agglutinin) | CaPmt1 | Filamentation defect in Lee's medium; defect in biofilm formation? |
| CaSec20 (secretion) | CaPmt1/4 | Essential; at lowered expression levels sensitive to antifungals. |
| CaPir2 (CaHsp150) | CaPmt1 | Expression induced under cell wall weakening conditions. |
| AnWscA (cell integrity sensor) | AnPmtA? | Reduced colony formation, complemented by osmotic stabilisers; sensitive to Congo red. |

*, heterologous expression; Δ1/2, $Scpmt1/pmt2$ deletion background; ?, not proven; (..), minor activity. See text for details.

mediating membrane insertion; this concept could indeed be verified for some predicted membrane proteins. On the other hand, Pmt4-mediated modification of Ccw5, which does not contain a transmembrane region or GPI anchor, suggests that $O$-glycosylation signals may be even more complex.

Several other Pmt target proteins have been defined in yeast, including the proteases Bar1 (Pmt1-, Pmt2-target) and Kex2 (Pmt4-target) [38]. Bar1 is a secreted aspartyl protease produced by MATa cells, which degrades the alpha factor-pheromone, while Kex2 is a subtilisin-like protease within Golgi membranes, able to mature alpha factor- and killer factor-secretion precursors. Mutant alpha factor precursor lacking $N$-glycosylation sites was shown to be $O$-mannosylated by Pmt2 and thereby protected from degradation during posttranslational import into the ER [22, 60]; Pmt2-mediated mannosylation appeared to function by increasing the solubility of misfolded proteins, allowing their secretion, thus preventing overflow of the ERAD machinery [60]. In *S. pombe* a homologue of the *S. cerevisiae* cell integrity sensor Wsc1, SpWsc1, and heterologously expressed ScCts1 chitinase were found to be $O$-glycosylated by Oma1, while localisation of ScFus1 in *S. pombe* cells was Oma4-dependent [28, 29], suggesting that substrate specificities of Pmt protein subfamily members are similar across fungal species.

***Filamentous fungi.*** Filamentous fungi comprise medically important fungi including *Aspergillus fumigatus* or *A. flavus*, as well as fungi used for industrial

productions and numerous avirulent species. While *O*-glycan structures are more complex in filamentous fungi than in yeasts (reviewed in [25]) the Pmt proteins catalysing the initial step of *O*-glycosylation are also commonly found in these organisms, as they are in yeasts. *PMT* genes have been recently identified for some filamentous fungi including *A. nidulans*, the black koji mould *A. awamori* and *Trichoderma reesei*, a fungus especially used in hydrolytic enzyme production. In *T. reesei O*-glycosylation is thought to be essential for protein secretion [61] and two genes, *mpg1* and *dpm1*, encoding enzymes that are involved in this process had been identified already some time ago [62, 63]. More recently, a cDNA has been identified that by similarity encoded a potential protein-*O*-mannosyltransferase from *T. reesei* [64]. The corresponding protein PMTI showed highest sequence similarity to members of the Pmt4 subfamily including a seven amino acid-insert in motif A of the enzymatically important loop 5 region that is typical of this Pmt subfamily [27]. Surprisingly, further complementation analyses in the heterologous model *S. cerevisiae* revealed that TrPMTI can probably interact with ScPmt1, suggesting that it may represent a ScPmt2 rather than a ScPmt4 orthologue. However, the role of PMTI in *T. reesei* biology still remains unclear and needs further investigation in the fungus itself.

During an attempt to identify genes involved in the synthesis and localisation of new cell wall material in the filamentous fungus *A. nidulans*, a temperature-sensitive *swoA* mutation was identified, which was later shown to encode a protein-*O*-mannosyltransferase, PmtA, that shows highest similarity to members of the Pmt2 subfamily [65, 66]. Although *pmtA* mutants exhibited greatly reduced *O*-glycosylation activity (to ~6%) *pmtA* mutant strains of *A. nidulans* were viable but showed severe germination and filamental growth defects at restricted temperatures (42 °C) that could be complemented by increasing the osmolarity of the growth medium [65–67]. Furthermore, conidiation of these mutants was reduced to ~20% of wild-type levels [67]. In addition, *pmtA* mutant strains were more sensitive to certain cell wall-destabilising agents indicating that cell wall biogenesis is severely affected. Accordingly, cell wall composition of the mutant was found to be different in comparison to wild-type strains [67]. Similar to *S. cerevisiae* it is possible that the disruption of *pmtA* may lower β-1,6-glucan synthesis due to reduced *O*-glycosylation of a Kre9 orthologue, which in turn increases the chitin content by a compensatory mechanism.

A *pmtA* homologue was isolated from the black koji mould *A. awamori*, which is used for the production of hydrolases and organic acids, as well as in food fermentation. AaPmtA was able to complement the growth defect of an *A. nidulans pmtA* mutant strain and disruption of *pmtA* in *A. awamori* led to similar morphological and growth defects as in the *A. nidulans* mutant [67]. More recently, two additional *pmt* genes have been identified in the *A. nidulans* genome: *pmtB* and *pmtC* (Goto, unpublished results, cited in [25]). *pmtB* mutants show no growth defects except for slightly reduced growth at 42 °C, a hyperbranching phenotype and reduced conidiation. In contrast, *pmtC* mutants show a severe growth defect already at standard growth conditions, conidiation is completely abolished and hyphal cells are unusually swollen. Thus, as in other fungal species, Pmt isoforms in *A. awamori* appear to address specific sets of target proteins. Glucoamylase I (GAI), an enzyme that is secreted by this organism in large amounts, is *O*-glycosylated by PmtA. GAI was also shown to be glycosylated by AnPmtA, when heterologously expressed in *A. nidulans* although with slightly deviating specificity, nevertheless indicating the high degree of homology between AaPmtA and AnPmtA [67]. Other target proteins of PmtA appear to be two potential Wsc cell integrity sensors that have been identified in the *A. nidulans* genome by homology to ScWsc1 and initial analyses revealed that mutations in at least one *wsc* gene resulted in phenotypes similar but not identical to a *pmtA* mutation (Goto, unpublished results, cited in [25]).

***Candida albicans.*** *C. albicans* is the most important human fungal pathogen, causing various forms of superficial and systemic infections in the human host. Five Pmt isoforms were identified in this species (reviewed in [26], Fig. 1) including two Pmt1 subfamily members (Pmt1, Pmt5), two Pmt2 subfamily members (Pmt2, Pmt6) and a sole Pmt4 protein [68–70]. Because *C. albicans* is diploid, homozygous deletion mutants were constructed by inactivating both alleles encoding Pmt isoforms; these were viable in *C. albicans* except for the *pmt2* mutant, similar to the requirement for *PMT2* in some strains of *S. pombe* [28]. This result indicated that *PMT2* is essential for growth and that Pmt6 in this fungus can not complement the loss of Pmt2. Loss of a single *PMT2* allele already sufficed to significantly retarded growth, causing multiple phenotypes discussed below [70]. In addition, while homozygous *pmt1 pmt6* and *pmt4 pmt6* double mutants were viable, double mutants lacking *PMT1* and *PMT4* were not viable [70]. Most homozygous *pmt* mutants grew at normal rates at 30 °C, but the *pmt1* mutant was delayed and failed to grow at 42 °C, as was the case for a *pmt4 pmt6* double mutant and a heterozygous *pmt2/PMT2* strain; the
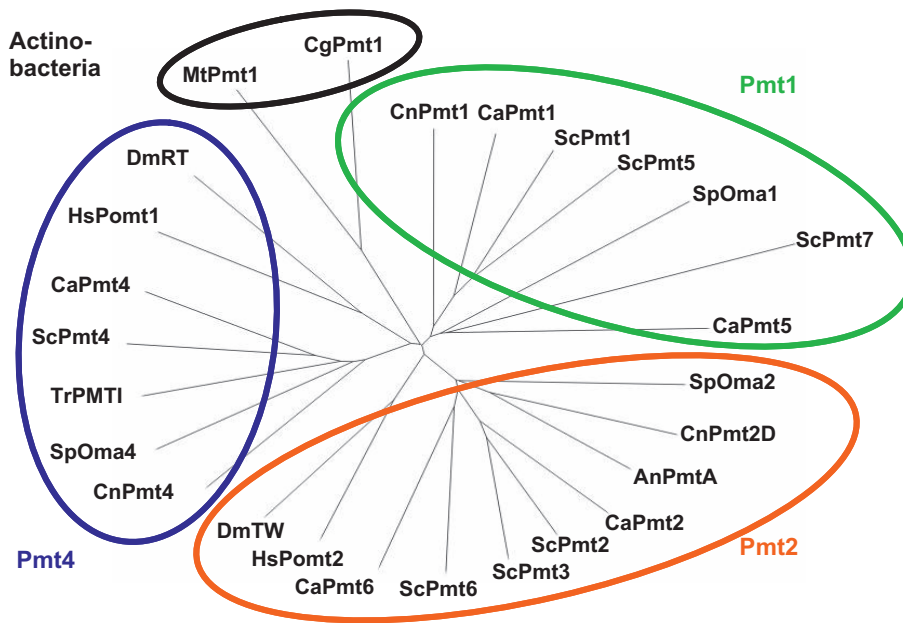
**Figure 1.** Phylogenetic tree of Pmt subfamilies. Primary sequences of published fungal and bacterial protein-O-mannosyltransferases (Pmts) were analysed using the ClustalW algorithm and displayed graphically using TreeView. An, *Aspergillus nidulans*; Ca, *Candida albicans*; Cg, *Corynebacterium glutamicum*; Cn, *Cryptococcus neoformans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mt, *Mycobacterium tuberculosis*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tr, *Trichoderma reesei*.

latter strains were also sensitive to high salt concentrations. Microscopic analyses of the various *pmt* mutants revealed that homozygous *pmt1* or *pmt4* single mutants show a cell separation defect and an aggregation phenotype, as well as decreased hydrophobicity [68, 70], consistent with significant changes in the cell wall composition [70, 71]. Furthermore, homozygous *pmt1* and *pmt4* mutants as well as the *pmt2/PMT2* strains revealed an increased sensitivity towards various antifungal drugs (e.g. hygromycin B, azoles) and cell wall-destabilising agents (calcofluor white, Congo red). Interestingly, while the homozygous *pmt1 pmt6* double and *pmt1* single mutant showed similar antifungal sensitivities, the double mutant was hypersensitive to the iron chelator EDDHA and to caffeine [69]. These results suggest that Pmt1, Pmt2 and Pmt4 account for most of the protein-O-glycosylation activity in *C. albicans*, while Pmt5 and Pmt6 may specifically modulate a much narrower spectrum of target proteins.

*C. albicans pmt1* mutants were shown to be defective in *in vitro* O-mannosylation of an artificial peptide used to measure ScPmt1 activity and *CaPMT1* restored O-mannosylation of the Pmt1 substrate chitinase in *S. cerevisiae*, confirming the functional relatedness of Pmt1 in both species [68]. Few specific target proteins for Pmt isoforms have been described in *C. albicans*. Examination of *pmt* mutants for various potential O-mannosylation targets revealed that Pmt1 accounts for the O-glycosylation of the cell wall proteins Kre9, Pir2 and Als1 [68, 70]. On the other hand, similar to *S. cerevisiae* the *C. albicans* Axl2 protein responsible for bud site selection was also found to be O-glycosylated by Pmt4, partially protect-

ing it from proteolytic cleavage [70]. Sec20, which is an essential ER membrane protein in yeast that functions as a tSNARE component in retrograde vesicle traffic, was described as another target of Pmt1 and Pmt4. Lack of Pmt O-mannosylation and removal of O-mannosylation sites led to rapid degradation of Sec20 [72]. Thus, Sec20 is the first example of an essential component of the eukaryotic secretion machinery, whose stability and thereby function depends on Pmt activity. Interestingly, reduced expression of *SEC20* led to supersensitivity phenotypes known of *pmt1* and *pmt4* mutants, suggesting that these *pmt* phenotypes arise at least in part because of defective Sec20.

Recently, a transcriptomal analysis of *C. albicans pmt* mutants grown under various conditions has been performed confirming that *pmt* mutants respond to defects in O-glycosylation by manifold compensatory mechanisms [73]. It was shown that cellular metabolic flow is altered in *C. albicans pmt* mutants resulting in downregulation of glycolysis and glycerol production but leading to an increase in the biosynthesis of activated sugars, which are predominantly used in protein glycosylation and cell wall biogenesis. These events were thought to occur in *pmt* mutants to avoid osmotic pressure (by the osmolyte glycerol) against the weakened cell wall and to upregulate compensating glycosylation reactions [73]. In addition, there seems to be a compensatory mechanism within protein-O-glycosylation itself since it was found that expression of individual *PMT* genes is to some degree dependent on the activity of the other Pmt proteins. Finally, through Pmt1 inhibitory experiments it could be shown that the cellular adaptation of *C. albicans* cells to loss of protein-O-glycosylation can be divided

into an immediate response that is dependent on several general stress response signalling pathways including PKA and PKC signalling or the calcium-dependent phosphatase calcineurin (Cna1), while long-term adaptation on the other hand seems to be mostly dependent on calcineurin [73]. The known downstream targets of calcineurin, the Crz1 and Crz2 transcription factors, appear not to be involved in this adaptation, suggesting the existence of yet unknown downstream regulatory components. Phosphorylation of the Cek1 MAP kinase was shown to become significantly upregulated in *pmt1* but not other *pmt* mutants, suggesting that a "SVG"-like pathway that has been described in *S. cerevisiae* as a response to defective *N*-glycosylation and includes the Cek1-homologue Kss1 [74], is activated by defective Pmt1-mediated *O*-glycosylation in *C. albicans*. In contrast, increased phosphorylation of the MAP kinase of the PKC-pathway, Mkc1, was not observed in any of the *pmt* mutants, unlike in *och1* mutants defective in *N*-glycosylation [75], indicating different responses to defective *O*- and *N*-glycosylation. Further experiments are needed for a better understanding of the complex cellular adaptation processes, by measuring the kinetics of transcriptomal, signalling and physiological events following defects in *O*-mannosylation. In this direction, it has already been shown that transcriptomal patterns following short-term inhibition by the relatively Pmt1-specific rhodanine-type inhibitors [73] are different from transcriptomal patterns of *pmt1* mutants (see below).

*C. albicans* virulence has been linked to several factors, most strikingly to its ability to switch between different morphological forms, particularly a budding yeast form and a true hyphal form (dimorphism) [76]. All homozygous *pmt* mutants except the *pmt5* mutant showed a defect in hypha formation on some solid inducing media and the heterozygous *pmt1/PMT1*, *pmt2/PMT2* and *pmt6/PMT6* were also defective in filamentation [68, 70]. Interestingly, while deletion of a single *PMT1* allele led to an intermediate filamentation defect, deletion of one *PMT6* allele sufficed to obtain the full phenotype of the homozygous mutant; deletion of a single *PMT4* allele had no effect. These results indicate that *PMT* alleles contribute differently to the overall phenotype. Contact to a solid surface appears important to reveal the contribution of Pmt proteins in dimorphism, because *pmt* mutants were still able to form hypha during induction in liquid media, especially in the presence of serum. Possibly, *O*-mannosylated proteins on the fungal cell surface sense surface contact and trigger morphogenesis. In agreement with the *pmt* mutant phenotypes at least two of the Pmt target proteins were shown to be required for filamentation, since homozygous *kre9*

and *als1* mutants exhibited a strong delay in filamentation in normoxia [77, 78]. Recently, we discovered that the combination of surface growth and hypoxia as well as embedded growth (presumably leading to oxygen deprivation) trigger hyphal morphogenesis by a different signalling pathway than during normoxia [79]. Surprisingly, while the homozygous *pmt1* and *pmt1 pmt6* mutants showed a filamentation defect under embedded and hypoxic conditions similar to standard inducing conditions, all other mutants filamented similar to wild-type cells. In contrast, the heterozygous *pmt2/PMT2*, the homozygous *pmt4*, and especially the homozygous *pmt4 pmt6* mutant strain actually were hyper-filamentous under both embedded and hypoxic conditions [70]. These findings indicate that a deficiency in Pmt function does not result in a general filamentation defect (e.g. by an impaired component required for filament structure), but it may interfere with the signaling pathways transmitting the hyphae-inducing environmental signals. In support of this notion it was found that the morphogenetic defects of the *pmt6* single mutants in normoxia, yet of no other *pmt* mutant, could be suppressed by overexpression of genes encoding for components of the signalling pathways triggering filament induction [69]. These results suggest that a sensor protein *O*-mannosylated by Pmt6 functions upstream of the known PKA/Efg1/MAPK signaling pathways. Consistent with a rather unique function of this specific Pmt protein in *S. cerevisiae*, Pmt6 was found not to interact with any other Pmt protein in *S. cerevisiae*, still forming a protein complex of unknown composition [32].

Biofilms are an important concerted life form of microorganisms on solid surfaces, which significantly influences the virulence of pathogens by surface-anchoring and resistance to antimicrobial compounds. *C. albicans* was shown to form biofilms on various solid supports, which are highly resistant to most antifungal drugs (reviewed in [80]). Biofilm formation was defective in mutants unable to form hyphae and microscopic inspection of biofilms showed a defined structure containing a basal layer consisting predominantly of yeast-form cells and an upper layer consisting mainly of hyphae. The above-discussed role of Pmt proteins in the hydrophobicity, structure and function of the *C. albicans* cell surface, as well as in its morphogenesis on solid surfaces suggested an important role of *O*-mannosylation in biofilm biogenesis. Indeed it was found that homozygous *pmt1* and heterozygous *pmt2* mutants were severely reduced in biofilm formation in static polystyrene wells, while homozygous *pmt4* and *pmt6* mutants showed moderately reduced biofilm formation and a *pmt5* mutant was not defective [81]. Defects in biofilm formation

were not due to defects in morphological changes of *C. albicans* since microscopic analyses showed that the biofilms and/or microcolonies obtained even for the *pmt1* mutant consisted of yeast and filamentous cells. The importance of Pmt1-dependent *O*-glycosylation for biofilm formation was also demonstrated in a continuous-flow microfermenter model [81]. In this model system adhesion of *pmt1* mutant cells to Thermanox plastic (polyolefin polyester) was greatly reduced while *pmt4*, *pmt5* and *pmt6* mutants adhered at wild-type levels. However, in contrast to *pmt5* and *pmt6* mutant cells that produced biofilm at wild-type levels, biofilm dry mass of *pmt4* mutant cells was two times lower, indicating that Pmt4 may be important for later stages of biofilm production under these conditions. Interestingly, among other agglutinins Als1, a Pmt1 target protein, was found to be upregulated during biofilm formation in *C. albicans* [82] and may therefore be important for biofilm formation, as it has been shown for the Als3 agglutinin [83]. In addition, the two transcription factors Bcr1 and Ace2, which both are involved in *ALS1* regulation, contribute to biofilm formation [83]. Therefore, protein-*O*-glycosylation may have a huge impact on biofilm formation by either interfering with the regulation or direct modification of specific cell wall proteins important for the different stages of biofilm formation. Thus, the reduced virulence of individual *C. albicans pmt* mutants (see below) may also be due to reduced biofilm-dependent host-pathogen interactions that are thought to be important for pathogenicity of *C. albicans* [84].

The contribution of different Pmt isoforms to *C. albicans* virulence was tested in different models of infection (Table 2). *pmt* mutants were examined in a model of hematogenously disseminated candidiasis (HDC) using tail vein injections of either CD2F1 or BALB/c mice. The homozygous *pmt1* mutant was found to be avirulent and the *pmt6* mutant was slightly attenuated in both infection models, but interesting differences were obtained for the other *pmt* mutants. While the *pmt4* mutant was avirulent in the BALB/c model it was attenuated in CD2F1 mice. Surprisingly, the *pmt5* mutant was found to be reduced for virulence in the CD2F1 model, although it had not shown any other defective phenotypes during *in vitro* tests [68 – 70, 85, 86]. The reduced virulence in the HDC-assay was not correlated with defective hypha formation described above, since all *pmt* mutants formed hyphae *in vivo*, although no fungal filaments could be found in kidneys of mice infected with *pmt1* mutant cells [85, 86]. This finding still allows the assumption that a blockage of filamentation may be a decisive factor in special niches of the human host. Therefore, it was of interest to determine the behaviour of *pmt* mutants in

localised yet complex models of infection, including reconstituted human epithelium (RHE) or engineered human oral mucosa (EHOM). As in the HDC model, interesting differences regarding the susceptibility of both localised infection models to specific *pmt* mutants was observed. Interestingly, the importance of Pmt5 in virulence was again detected in the EHOM model system, which closely mimicks normal skin. The homozygous *pmt1* and *pmt5* mutants did not cause an obvious damage to cells of the EHOM model, in contrast to the *pmt4* and *pmt6* mutants [85]. Furthermore, *pmt1* and *pmt5* mutant cells showed extremely reduced filament formation in the EHOM model system indicating that in this model system morphological changes seem to be related to their ability of damaging epithelial cell layers. In contrast to the EHOM model, the *pmt5* mutant was not attenuated for virulence in the RHE model, while all other homozygous *pmt* mutants and the heterozygous *pmt2* strain showed significantly reduced virulence, measured by the release of the marker enzyme LDH [85]. Microscopic analyses revealed that all mutants tested were able to form hyphae in this model system suggesting that filamentation defects seen *in vitro* are most likely not responsible for their reduced ability to cause damage to the RHE. Sanchez *et al.* [87] confirmed the reduced virulence of *pmt1* and *pmt6* in yet another infection model, measuring damage to human umbilical endothelial cells. In this system the authors noted that both *pmt* mutants formed shorter hyphae compared to control cells.

The results obtained for the different virulence model systems demonstrate that pathogenicity of *C. albicans* is often but not always related to cellular morphology and is a complex interaction of various virulence factors. For example, an important factor for infection is the ability to adhere to host cells and it was shown that *pmt1* and *pmt6* mutants were defective in adhesion to endothelial or epithelial cells [68, 69]. In addition, the ability of *C. albicans* cells to damage cell layers in RHE or EHOM models may in part be related to defective protease secretion and this was indeed the case for the *pmt1* mutant and to a lesser extent for the *pmt2*, *pmt4* and *pmt5* mutants [85, 86]. Further virulence factors that may also be affected by defects in protein-*O*-glycosylation by specific Pmt proteins may include phospholipase activity that was found to be reduced in *pmt2/PMT2* mutant cells, or a reduced sensitivity to killing by cells of the host immune system as it was found for the resistance of homozygous *pmt5* mutant to neutrophils [86]. Thus, the course of infection may be determined significantly by the way the host immune system responds to specific *O*-mannosylation defects. Interestingly, IL-10 production by macrophages was stimulated especially

**Table 2.** Biofilm formation and virulence[1] of *C. albicans pmt* mutants.

| Strain tested | Biofilm | RHE | EHOM | HDC | |
|---|---|---|---|---|---|
| | | | | CD2F1 | BALB/c |
| *PMT/PMT* | + | + | + | + | + |
| *pmt1/pmt1* | – | –/+ | –/+ | – | – |
| *pmt2/PMT2* | –/+ | –/+ | n.d. | – | n.d. |
| *pmt4/pmt4* | +/– | –/+ | + | –/+ | – |
| *pmt5/pmt5* | + | +/– | –/+ | –/+ | + |
| *pmt6/pmt6* | +/– | –/+ | + | –/+ | –/+ |

[1]  Virulence of *C. albicans* strains was tested using reconstituted human epithelium (RHE), engineered human oral mucosa (EHOM) and in a mouse model of hematogenously disseminated candidiasis (HDC) using CD2F1 mice [85] or BALB/c mice [70]. +, full virulence; –, no virulence; –/+, +/–, weakened virulence; n.d., not determined.

by the *pmt5* mutant, in contrast to the *pmt2/PMT2* heterozygous strain that led to increased IL-12 [86]. In addition, a block of *O*-mannosyl extension beyond the one-mannose stage decreased lymphokine production by human mononuclear cells; this study also revealed that (extended) *O*-chains are bound by the TLR4 receptor [88].

***Cryptococcus neoformans.*** *Cryptococcus neoformans* is an opportunistic human fungal pathogen mainly causing a severe meningoencephalitis in immunocompromised patients and several extracellular factors have been identified that are important for virulence (reviewed in [89]). Similar to *S. pombe* the basidiomycetous yeast *C. neoformans* was found to contain only three protein-*O*-mannosyltransferases CnPmt1, CnPmt2 and CnPmt4 [90]. While in analogy to *S. pombe* and *C. albicans* the *PMT2* gene seems also to be essential in *C. neoformans*, *pmt1* and *pmt4* disruption strains were viable, but a *pmt1 pmt4* double mutation was synthetic lethal ([90] and Lengeler, unpublished results). Similar to *pmt* mutant strains in other yeasts *pmt1*- and *pmt4*-deficient cryptococcal cells show unusual cellular morphologies including increased cell size, aberrant cell shape and a cell separation defect. In addition, the biogenesis of vacuolar structures seems also to be defective ([90] and Lengeler, in preparation). Furthermore, *pmt1* as well as *pmt4* mutants showed growth defects at elevated growth temperatures (39 °C) and low SDS concentrations, were more sensitive to osmotic stress, and *pmt4* mutants were also more sensitive to the antifungal drug amphotericin B ([90] and Lengeler, in preparation). Similar phenotypes have previously been linked to defects in the PKC cell integrity pathway in *C. neoformans* [91], again corroborating the fact that cell wall integrity is severely affected by mutations in protein-*O*-glycosylation. Interestingly, expression of the *CnFKS1* gene, a target of the Mpk1-dependent cell integrity pathway [92] is induced by

amphotericin B in wild-type but not in *pmt4* mutant cells [90]. *FKS1* encodes the cryptococcal catalytic subunit of β-1,3-glucan synthase [93] and reduced levels of Fks1 may result in decreased levels of cell wall compensatory functions promoted by the cell integrity pathway.

Virulence of *C. neoformans* is linked to several well-defined extracellular virulence factors including melanin production, capsule formation or lytic enzyme secretion. An analysis of *pmt1* and *pmt4* mutant strains revealed no obvious differences regarding the most important virulence factors in comparison to wild-type cells except for a severe defect in melanin production that was found for the *pmt4* mutant in our hands (Lengeler, in preparation) but not by Olson and colleagues [90]. Whether this conflicting result is due to subtle differences in *pmt4* mutant strain construction still remains unclear. Virulence of the *pmt1* and *pmt4* mutants was subsequently tested in a macrophage phagocytose/killing assay and different mouse virulence models ([90] and Lengeler, in preparation). It has recently been described that cryptococcal strains showing a flocculation phenotype ("clump[+]") were more amenable to complement-activated phagocytosis by macrophages in comparison to regular yeast cells and therefore are less virulent in a mouse model [94]. Since *pmt4* mutant cells show an aberrant cell morphology and pronounced cell aggregation, and also display a severe melanin defect, it was no surprise that this mutant showed an extremely reduced rate of survival in the macrophage killing assay and was significantly attenuated for virulence in the mouse model systems. More surprisingly, the *pmt1* mutant strain that had no obvious defect in any virulence factor tested and displayed less severe morphological abnormalities also showed reduced survival rates in the macrophage killing assay and was even more attenuated for virulence in the mouse model systems compared to the *pmt4* mutant ([90] and Lengeler, in preparation). *pmt4*-deficient strains were

found to show enormous differences in the overall pattern of mannosylated proteins, when SDS-extracted cell wall proteins were analysed by 1D and 2D gel electrophoresis followed by staining of glycoproteins [90]. Furthermore, it has previously been shown by various laboratories that extracellular mannoproteins are important for many immunological aspects of *C. neoformans*-host interactions including T cell activation [95–97] and therefore, defects in *O*-glycosylation may dramatically impair the virulence of *C. neoformans* by altering immunological aspects of pathogenesis. It will therefore be of interest to analyse whether *pmt1* mutant strains display similar differences in cell wall mannoprotein composition and which proteins are specifically affected by the *pmt1*, but also *pmt4* mutation. The identification of immediate targets for individual cryptococcal Pmt proteins may provide further explanations for the defects in pathogenicity found for the *pmt1* and *pmt4* mutants of *C. neoformans*.

## Pmts in higher eukaryotes: roles in development and disease

While there is no evidence for Pmt proteins in algae, plants and protozoa, they are known to occur in insect and mammalian cells. The first discoveries of *PMT*-type genes in multicellular eukaryotic organisms were the *rt* gene in *Drosophila melanogaster* [98] and its homolog in human cells, designated *POMT1* [99].
In *D. melanogaster* the *rotated abdomen* (*rt*) and *twisted* (*tw*) genes encode homologues of the fungal Pmt4 and Pmt2 proteins, respectively (also designated dPOMT1/DmPOMT1 and dPOMT2/DmPOMT2, respectively) [98, 100, 101]. Recessive mutations in the *rt* gene led to poorly viable flies with defects in embryonic muscle development and abdomens twisted clockwise by 60 to 90° [98]. This phenotype was also detected following RNA interference (RNAi) knockdown of *dPOMT1* [100]. Likewise, recessive mutant alleles of the *tw* gene, as well as RNAi knockdown flies, also presented the twisted abdomen phenotype [100, 101]. Genetic interaction of mutant *rt* and *tw* alleles suggested that both genes affect the same molecular processes. Partial reduction of both *rt* and *tw* gene products in a heterozygous *rt/+* strain carrying a *tw* knockdown mutation under conditions not revealing *tw* deficiency led to a clear synergistic, twisted abdomen phenotype. Furthermore, simultaneous RNAi-knockdown of both *rt* and *tw* genes resulted in lethality [100]. Surprisingly, the *tw^1* mutant allele was also reported to suppress the phenotype of several *rt* mutations in a dominant manner [101]. However, it appears that this particular genetic interaction was due to a special feature of the *tw^1* allele rather than a genuine antagonism on the *rt O*-mannosylation pathway. Overproduction of dPOMT1 and/or dPOMT2 in SF21 insect cells revealed their *O*-mannosylation activity on human α-dystroglycan, but only if both proteins were co-expressed [100], supporting similar experiments on human POMT proteins [102]. Taken together, the results indicate that both *rt* and *tw* are involved in the same developmental pathway, carrying out non-redundant functions. Different molecular mechanisms may explain collaboration of dPOMT1 and dPOMT2 proteins, including dimer formation in *O*-mannosylation of specific sites, a requirement for target *O*-mannosylation by the other isoform and their enzymatic modification of each other. During development the *rt* and *tw* genes are largely co-expressed, although during early stages of the embryonic development *rt* expression is dominant, which suggests that dPOMT1 functions on its own during this stage [100]. The *Drosophila* α-dystroglycan gene, *Dg*, encodes the possible molecular target of dPOMT proteins [103], although there is only a partial overlap between *Dg* expression and *rt/tw*-expression in tissues. Because a gene encoding a homologue of hPOMGnT1 is missing in the *Drosophila* genome its *O*-mannosyl chains appear not to become elongated as in human cells [100]. *In situ* hybridization revealed high levels of *rt* and *tw* transcripts in the invaginating gut, corresponding to the region of epidermal segment border cells [101]. This result strongly suggests that *O*-mannosylation activity is required for the development of muscle attachment sites in *D. melanogaster*. Pmt-type proteins appear widely distributed among insects, since an EST of a *tw* gene homolog was also reported in mosquito (*Anopheles gambiae*) cells.
During the last 10 years it has become clear that several mammalian secretory proteins are *O*-mannosylated and typically contain the glycosyl chain Siaα2–3Galβ1–4GlcNAcβ1–2Manα1 attached to serine or threonine residues (reviewed in [104]). α-dystroglycan, a cell adhesion glycoprotein required for integrity of muscle cells and for neuronal migration during development, is considered as one of the main targets for *O*-mannosylation [105]. The discovery of *D. melanogaster rt* as the gene encoding a yeast Pmt4 homolog sparked the discovery of the human *POMT1* gene [99] and subsequently led to the assignment of *POMT1* mutations to a subset of patients suffering from Walker-Warburg syndrome [106]. A second gene encoding a yeast Pmt2 homologue was also discovered, designated *POMT2* [107] and later shown to be mutated in other cases of this syndrome [108]. *POMT* genes are located on different chromosomes (*POMT1:* 9q34.1; *POMT2:* 14q24.3) [99, 108].

*POMT1* is expressed mainly in fetal brain, skeletal muscle and testis, whereas *POMT2* has its highest expression in testis and occurs in the acrosome of spermatids, a cap-like structure derived from the Golgi [107]. The latter POMT2-form is encoded by an elongated transcript due to differential transcriptional initiation [107]. Walker-Warburg syndrome is characterised by congenital muscular dystrophy, structural brain defects leading to a "cobblestone"-appearance and to eye malformations. The underlying molecular reason for most cases of this disease is a malfunctioning of the dystroglycan complex due to underglycosylation of its α-dystroglycan subunit. α-dystroglycan is normally heavily *O*-glycosylated, increasing the size of the unmodified protein from 72 kDa to 150–200 kDa. Correct glycosylation of α-dystroglycan is required for binding to the laminin, agrin and neurexin components of the basement membrane, the specialised sheet of extracellular matrix that surrounds muscle and other cells. It is known that α-dystroglycan is situated outside of muscle cells in a non-covalent linkage to β-dystroglycan in the cell membrane (sarcolemma), which in turn is linked to F-actin via dystrophin. Linkage of the extracellular matrix to the actin cytoskeleton is necessary to stabilize the plasma membrane and to ensure correct assembly of the basement membrane; in its absence, degeneration of muscle cells occurs. During normal brain development, the "*glia limitans*" basement membrane prevents neurons from migrating out of the brain into the subarachnoid space; gaps in the *glia limitans* that are caused by a failure to firmly link α-dystroglycan cause escape of neurons from the cortex, leading to a cobblestone morphology and other brain defects (reviewed in [109]).

The function of hPOMT1 and hPOMT2 as Pmt proteins has been verified by an enzymatic assay, using bacterially-synthesized α-dystroglycan as the substrate [110]. Co-expression of both genes was necessary to obtain increased Pmt activity in microsomal extracts of transfected cells; the use of octyl-thioglucoside as the solubilising detergent was crucial to obtain activity. As expected, mutated versions of *POMT1* derived from Walker-Warburg patients did not function in the enzymatic assay [110]. These results suggested that interaction of both POMT proteins is required for enzymatic activity and co-localisation and co-immunprecipitation experiments have supported this notion [110]. As discussed above for the *D. melanogaster* POMT proteins, the molecular mechanisms leading to Pmt activity by the co-expression of hPOMT proteins remain to be established. Interestingly, the formation of heteromeric complexes among Pmt isoforms appears to have changed during evolution, since no interaction of

Pmt4 and Pmt2 (the homologues of POMT1 and POMT2, respectively) was detected in yeast [32]. *O*-chains initiated by hPOMT proteins are subsequently extended to yield the structure Siaα2–3Galβ1–4GlcNAcβ1–2Manα1-Ser/Thr and defects in chain elongation have been associated with diseases related to the Walker-Warburg syndrome (reviewed in [109]). The attachment of GlcNAc to the mannose residue is mediated by POMGnT1 (protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase) and defects of this enzyme occur in muscle-eye-brain disease [111]. POMGnT1 appears to functionally interact with fukutin, a protein defective in Fukuyama-type congential muscular dystrophy [112, 113]. Both proteins co-localize in the Golgi and co-immunoprecipitation and two-hybrid analyses demonstrated their direct interaction. Furthermore, POMGnT1 enzymatic activity is reduced in a transgenic mouse carrying an insertion mutation in the fukutin gene [114]. Similar to fukutin, fukutin-related protein is required for α-dystroglycan glycosylation in the Golgi and its defect leads to congenital muscular dystrophy 1C [115]. The *LARGE* gene encodes yet another component involved in α-dystroglycan glycosylation and its mutation causes congenital muscular dystrophy [116]. The LARGE protein interacts with the globular N-terminal domain of α-dystroglycan to increase its degree of glycosylation of the central mucin-like domain; the N-terminal domain is cleaved off during later stages of α-dystroglycan secretion to the cell surface [117]. Remarkably, overexpression of *LARGE* was shown to restore α-dystroglycan glycosylation in cells of patients suffering from distinct types of congenital muscular dystrophy, suggesting a novel therapeutic route for treatment [118]. The LARGE protein encodes a putative glycosyltransferase partially homologous to bacterial α-glycosyltransferase and mammalian β-1,3-*N*-acetylglucosaminyltransferase; however, since these types of transferases are not known to modify α-dystroglycan, it is assumed that overproduction of LARGE recruits and/or activates glycosyltransferases that are not used in cells with normal levels of LARGE. The powerful tools of mouse genetics have been used to confirm results obtained for human cells, because POMT1 and 2 proteins also occur in rodents [119, 120]. The defects of the spontaneous mouse model Large^Myd containing mutation of LARGE closely resemble the human disease [121] and could be complemented by the human *LARGE* gene [118]. Targeted disruption of the *POMT1* homologue in mouse showed defects in basement membrane formation, leading to early embryonic lethality [119] and "knock-in"-mice carrying a mutated fukutin gene showed reduced POMGnT1 activity [114].

Another aspect of *O*-mannosyl chains in mammals relates to their function as receptors for viruses. α-dystroglycan has been recognised as the cellular receptor for arenaviruses including the Lassa fever virus, which represents a major threat for human health by causing hemorrhagic fever [122]. Virus binding occurs by the mucin-type domain of α-dystroglycan and depends on the activity of the LARGE protein [123]. Competition between arena viruses and the natural ligand laminin for α-dystroglycan provide a molecular explanation for tissue tropism and for pathomechanisms triggered by such viral infections. It has been speculated that the prevalence of α-dystroglycan defects in the human population may be due to a positive selection for heterozygotes with reduced mortality to arenavirus infections [123].

## Pmt inhibitors

Given their many functions in virulence and development it seems promising to use Pmt proteins as targets for chemotherapeutic intervention. One obvious possibility is to block Pmt functions in pathogenic bacteria or fungi. Orchard *et al.* [124] described derivatives of rhodanine-3-acetic acid that were active against Pmt1 of *C. albicans*. This class of inhibitors appears quite specific for the Pmt1 isoform, because it blocked growth of a *pmt4* mutant, consistent with defective growth of *pmt1 pmt4* double mutants, but not of *pmt1* mutant lacking the target of the inhibitor [70, 73]. Furthermore, treatment with the inhibitor generated phenotypes characteristic of *pmt1* mutants, including defective hyphal morphogenesis and increased sensitvities to aminoglycosides [124]. In addition, the inhibitor was used in transcriptomal analyses and revealed upregulation of a number of genes that were also detected in a *pmt1* mutant [73] and in its presence biofilm-formation was blocked [81]. The observed specificity of this inhibitor is an encouraging fact considering the occurence of Pmt-homologues also in mammalian cells. Although use of the rhodanine-type inhibitors in the clinic has not yet been described, it appears that Pmt isoform-specific inhibitors can be developed. This is a very positive perspective considering the described essential roles of Pmt proteins in the wide-spread tuberculosis- and candidiasis-causing microorganisms. On the other hand, agents to overcome Pmt isoform-deficiency may be of great interest, given the lack of Pmt function in severe human diseases including muscular dystrophy. In this sense, the study of adaptation mechanisms to defective Pmt activity will be of great interest

and may offer again novel targets for medical intervention.

## Concluding remarks

Numerous questions regarding the molecular function of Pmt proteins and their targets, especially with regard to their roles in virulence and development, remain to be solved. One pertinent problem concerns the specificity of target recognition by each Pmt isoform. No consensus sequence for *O*-mannosylation is known, suggesting relatively specific Pmt isoform-target interactions that need to be defined. Such experiments may also help to define novel specific targets, e.g. for cellular differentiation, virulence and development. Such proteins then need to be characterised, in particular for their functional dependence on *O*-mannosylation. Another important topic of research will be to further characterise interactions of Pmt proteins, not only with regard to dimer formation but also in their functional context, e.g. in association with the secretory pore. Furthermore, adaptation mechanisms to Pmt deficiency and chemotherapeutic modulation of Pmt activity are largely unexplored. The rate at which knowledge on Pmt proteins has expanded during recent years promises exciting further developments in this field.

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