Chapter 11

Impact of Yeast Glycosylation Pathway on Cell Integrity and Morphology

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1. Introduction

The subject of protein N- and O-glycosylation in the yeast *S.cerevisiae* has already been well described in many excellent reviews [1-3]. However, less is known about the occurrence of these processes in the yeast *Candida albicans,* an opportunistic human pathogen.

In the *N*-glycosylation reactions catalysed by the Alg enzymes, dolichyl phosphate (DolP) serves as a lipid acceptor of sugar residues. The Alg enzymes are present both on the cytosolic side of the ER: Alg7, Alg1, Alg2 and Alg11) and on its lumenal face(Alg3, Alg5, Alg6, Alg8-10 and Alg12 [3-6]. Cytosolic Alg enzymes, necessary for viability of yeast and mammalian cells in culture, produce DolPP-$GlcNAc_2Man_5$ from DolP, UDPGlcNAc and GDPMan. There is also circumstantial evidence that DolPP-$GlcNAc_2Man_5$ is delivered into the lumen of the ER by a putative flippase (Rft1p) [7]. Within the ER the DolPP-$GlcNAc_2Man_5$ product is converted to DolPP-$GlcNAc_2Man_9Glc_3$ using DolPMan and DolPGlc as sugar donors [3]. As it was mentioned already DolPMan is also a substrate for protein *O*-glycosylation, where it serves as a donor of the first mannose to be attached to hydroxyl groups of serine and threonine. The second and subsequent mannose residues are transferred directly from GDPMan [1, 2]. DolPMan is also involved in the synthesis of the sugar part of glycosylphosphatidyl inositol anchor in yeast and other eukaryotes [2]. Moreover, a large group of cell wall glycoproteins is attached to the glucan polymers via a GPI-remnant structure.

On the other hand, there is a growing literature describing the involvement of cell wall carbohydrates in fungus-host interactions (for review see [8]), as well as in the maintenance of cell wall integrity [9]. Thus, one can predict a functional link between N-glycosylation and O-mannosylation of cell wall proteins, cell wall integrity and/or fungus–host interactions. Nonetheless, open questions remain concerning the regulatory mechanisms of early events of

protein glycosylation and their impact on the synthesis of outer glycan chain in cell surface glycoproteins of *S. cerevisiae* and *C. albicans*.

A model of the *S. cerevisiae* cell wall has been described. It contains four classes of interacting components: chitin, β1,3 and β1,6 glucan and mannoproteins [10,11]. This structure is similar to that of the *C. albicans* cell wall [11-13].

2. Contribution of the mevalonate pathway to protein glycosylation and cell wall integrity

Mevalonate pathway in yeasts is important not only for ergosterol biosynthesis but also for the production of nonsterol molecules, deriving from farnesyl diphosphate. Formation of cell wall proteins, i.e. the glycosyl- phosphatidylinositol (GPI) anchored (GPI-CWP) and proteins with internal repeats (pir-CWP,) requires, as an initial intermediate, DolP synthesized together with the other isoprenoid lipids in the mevalonate pathway.

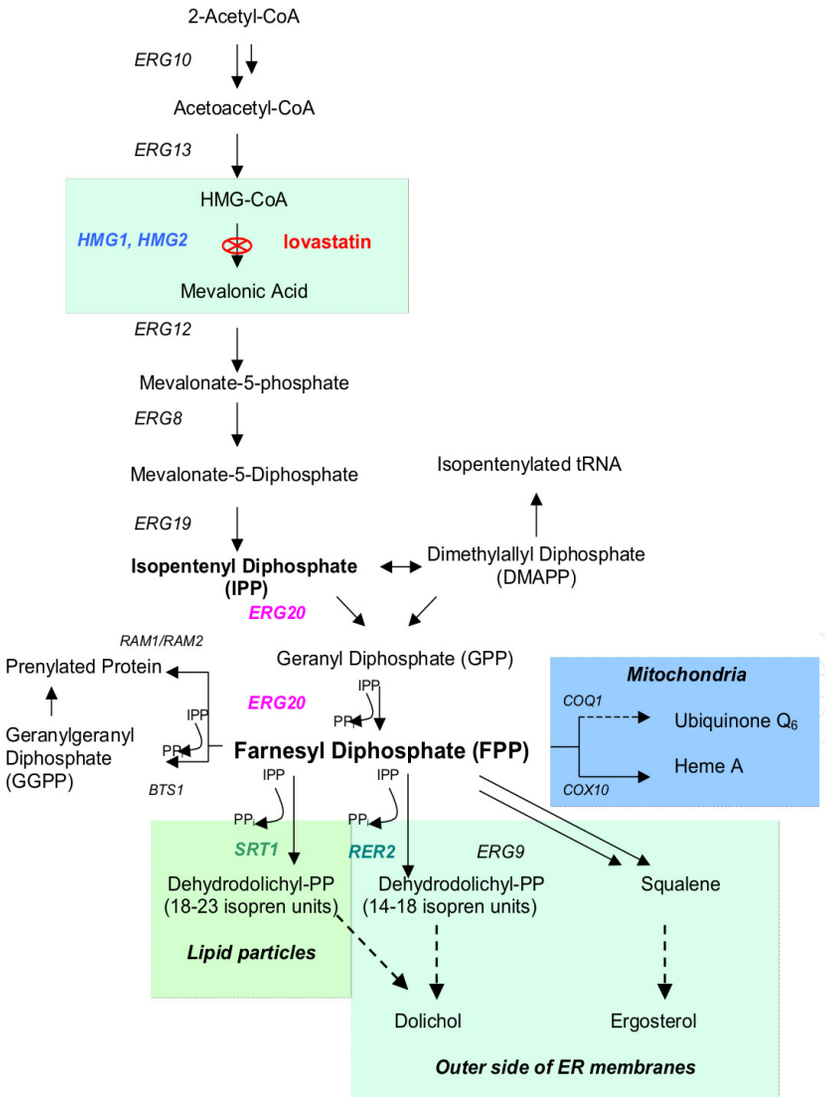


Figure 1. Isoprenoid (mevalonate) pathway in the yeast *Saccharomyces cerevisiae*.

Dolichol biosynthetic pathway has multiple levels of regulation. Thus, its cellular level is amenable to alterations affecting protein glycosylation and, in consequence, the cell wall structure. The pathway is shared with other isoprenoid lipids (Fig.1). The most abundant branch of the pathway, leading to sterol biosynthesis, is one of the main targets for anti-fungal drugs, which exploit the differences in the pathways and the end product - ergosterol in fungal cells and cholesterol in animals. The mevalonate pathway diverges after the synthesis of farnesyl diphosphate by farnesyl diphosphate synthase (FPPS), encoded by the *ERG20* gene.

Products of the subsequent reactions are shown together with the names of the genes, in capital letters, encoding the enzymes catalyzing them. Enzyme affected by lovostatin, an inhibitor of isoprenoid pathway, is indicated (K. Grabinska PhD thesis, IBB 2002)

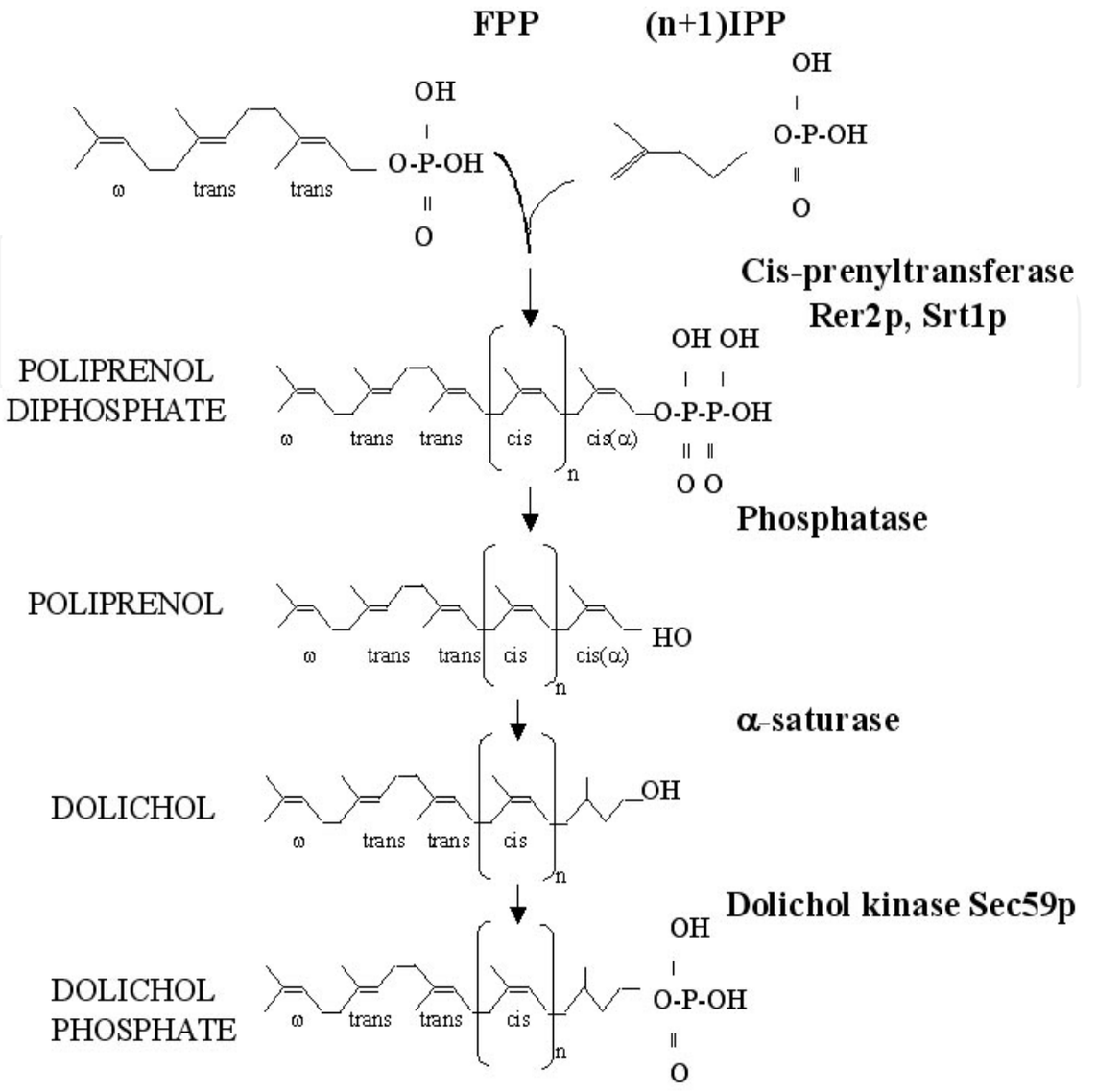
Using a yeast based two hybrid system, we have identified the Yta7 protein interacting with FPPS, and showed that it was membrane-associated and localised both to the nucleus and the endoplasmic reticulum (ER). In order to assess the importance of the mevalonate pathway for cell wall synthesis and its role in cell-wall integrity (Fig 2), we investigated the effects of *YTA7* deletion at the transcriptional level. Our data [14] show that loss of *YTA7* function leads to activation of the genes implicated in cell wall integrity pathway (*CRZ1*, *FKS2*, and *KNR4)* and highlight a possible link between dolichol metabolism and cell wall synthesis.

Moreover, farnesol, which is likely to be derived from dephosphorylation of FPP, inhibits growth of *S. cerevisiae* and *C. albicans.* This inhibition is concomitant with a significant loss of intracellular diacylglycerol (DAG) [15], which is an activator of the PKC1-signaling pathway involved in the maintenance of cell wall integrity (described later, compare Fig.3)

De novo synthesis of dolichol starts with the 1'-4 condensation of farnesyl diphosphate (FPP) with 11-15 isopentenyl pyrophosphate units to form polyprenyl diphosphate (dehydrodolichol diphosphate) (Fig. 2). This reaction is catalysed by *cis*-prenyltransferase (*cis*-Ptase) encoded in *S. cerevisiae* by the *RER2* and *SRT1* genes [16].

Our results indicate that FPP or its derivatives regulate the transcription of *RER2* and *SRT1* genes as well as of *DPM1,* which encodes Dpm1p [17]. To enter the glycosylation pathways the *RER2* and *SRT1* gene products (dehydrodolichol diphosphate) need to be dephosphorylated, reduced and phosphorylated again by CTP-dependent dolichyl kinase, Sec59p [18-20].

Biosynthesis of isopentenyldiphosphate (IPP) from mevalonate diphosphate is catalysed by mevalonate pyrophosphate decarboxylase (Fig.1). Subsequently IPP is converted to dimethylallyl diphosphate (DMAPP) and DMAPP (C_5) is condensed with farnesyl diphosphate, composed of 3 isoprene units (C_{15}). This reaction is catalysed by Rer2 and Srt1p. Further elongation of the isoprenol chain occurs, by step wise addition of the 5-carbon isoprene units to reach species-specific chain length. Poliprenol diphosphate is dephosphorylated and reduced by alfa saturase (Dfg10p) to form dolichol. To enter glycosylation pathway dolichol is phosphorylated by CTP-dependent dolichol kinase.



n=10-14 isoprene units for Rer2p and 14-23 for Srt1p products. (Adopted from [21])

Figure 2. Dolichyl phosphate biosynthesis *de novo* in yeast.

Our data indicate that in the rapidly dividing yeast cells "de novo" biosynthesis of dolichyl phosphate described above is a main source of its supply.

However, it is thought that in non dividing cells dolichyl phosphate might be derived mainly from recycling after each cycle of protein glycosylation, either by transfer of the oligosaccharide from DolPPGlcNAc2Man9Glc3 onto acceptor protein or from the transfer across the ER membranes of single glucose (Glc) and mannose (Man) residues from DolPMan or DolPGlc [22].

The length of dolichol molecules is species-specific and in yeast contains 14-18 isoprene units [23]. Although a great deal of progress has been made in the understanding of the enzymatic steps responsible for polyprenyl chain length termination and conversion of dehydrodolichol to dolichol, some open questions still remain. *In vitro*, cell extracts and

membrane fractions from *S. cerevisiae* catalyse the biosynthesis of dolichol backbone i.e. dehydrodolichol [24] whereas in *vivo* yeast synthesise dolichols. On the base of the results obtained so far it was assumed that in *S. cerevisiae* polyprenyl diphosphates synthesised *in vitro* undergo immediate dephosphorylation and reduction of its alpha residue [24, 25]. A similar conclusion was reached for the rat liver system [26] and in general for metazoan eukaryotic cells [22].

3. Cell wall alteration resulting from the defect in dolichol and dolichyl phosphate formation

It has been shown that depletion of GDPMan pyrophosphorylase activity in *C.albicans* [27] leads to cell lysis, inefficient cell separation, impaired bud growth, clumping and flocculation, as well as increased sensitivity to a wide range of antifungal drugs. GDPMan pyrophosphorylase (*Ca SRB*1 encoded) catalyses the transfer of Man-1P to GDP and is responsible for the biosynthesis of GDPMan, the major sugar donor in yeast and filamentous fungi. On the other hand, overexpression of GDPMan pyrophosphorylase encoding gene (*mpg1*) in the filamentous fungus *Trichoderma reesei* resulted in a two-fold increase in GDPMan level, overglycosylation of secreted proteins, increased transcription of *dpm1*, DolPMan-synthase encoding gene, and increased enzyme activity [28]. Thus, it was assumed that the cellular level of GDPMan is one of the factors playing a regulatory role in protein glycosylation.

In this work we have concentrated on the assembly of mono- and oligo-saccharide lipid carrier (DolP), which is another substrate in protein glycosylation, and on it's effect on cell wall integrity and cell morphology.

In *C.albicans,* which unlike *S.cerevisiae* is an obligatory diploid, we initially cloned the genes involved in dolichol biosynthesis (*CaRER2 CaSRT1*) and phosphorylation (*CaSEC59*). To construct mutants defective in Rer2p or Sec59p activities, we used "URA-blaster" cassette [29] to delete one chromosomal copy of the gene. Since the genes under investigation were either essential or generated mutants with strong phenotypes, we adopted a conditional mutant approach and the second copy of the gene was placed under the control of a regulatable promoter.

By growing the strains in repressive conditions we were able to demonstrate that the defect in dolichol backbone synthesis or its phosphorylation, resulted in the aberrant cell wall structure and increased sensitivity to some antifungal drugs. Moreover, the normal morphogenesis of the fungus, e.g. hyphae formation, was prevented (Juchimiuk et al. 2011, in preparation).

Recently, we have cloned an ortholog of *S.cerevisiae DFG10* from *C.albicans* (orf 19.7841). *S.cerevisiae DFG10* encodes a protein with 3-oxo-5-alpha-steroid 4-dehydrogenase activity, lately postulated to be involved in dehydrodolichol reduction [30]. The latter was based on the finding that the mutant, named *dfg10-100*, isolated in a genetic screen for the strains defective in filamentous growth [31], was shown to be defective in N-linked glycosylation.

This defect was conferred by orf encoding the human SRD5A3 protein, involved in the reduction of dehydrodolichol diphosphate into dolicholdiphosphate. Thus, it was assumed that SRD5A3 is the human ortholog of the yeast *DFG10* gene encoding dehydrodolichyl diphosphate reductase. The product of *DFG10* shows 25% amino acid identity and 43% similarity with the human SRD5A3 protein, forming with others SRD proteins the steroid 5a-reductase family [30].

Double deletion of the *C.albicans* orf19.7841 rendered viable *Cadfg10* mutant strain. Its biochemical analysis revealed that the strain produces 70% of dehydro- and 30% of dolichols, whereas in the wild type, parental strain only dolichols are synthesized. The mutant strain was also oversensitive to tunicamycin, an inhibitor of N-glycosylation. Indeed, a defect in protein glycosylation was confirmed by assessing the degree of glycosylation of the marker glycoprotein hexosaminidase. Moreover, the *Cadfg10* mutant exhibits abnormal hyphal growth and increased resistance to some antifungal agents, thus indicating alterations of the *C.albicans* cell wall (Janik et al., manuscript in preparation).

However, based on our results, only 30% of dehydrodolihol is reduced to dolichol in *Cadfg10* null mutant. Thus, it is possible that *Ca*Dfg10p is not the only protein involved in dehydrodolichol reduction. Another likely candidate is an ortholog of *S.cerevisiae TSC 13*, encoding steroid reductase, i.e. putative enoyl reductase, encoded in *C.albicans* by orf 19.3293. However, the possible role of orf 19.3293 in dolichol synthesis is now under investigation.

We have also studied alterations in cell wall composition and integrity in *S. cerevisiae dpm1-1* mutant impaired in DolPMan formation and in the *sec59* mutant impaired in dolichol kinase activity. In our earlier work we were able to demonstrate that overexpression of the *S.cerevisiae DPM1* gene, encoding DolPMan synthase in *T.reesei* and *Aspergillus nidulans* led to increased secretion of fully glycosylated proteins, concomitant with alteration of the cell wall ultrastructure (*T.reesei*) or with accumulation of overproduced glycoproteins in the periplasmic space (*A.nidulans*) [32, 33].

For the *S. cerevisiae dpm1-1* as well as for the *sec59-1* mutants we observed an increased sensitivity to Calcofluor White (CFW) and an upregulated chitin level. Both mutated strains were also oversensitive to a variety of the external agents including antifungal drugs [34].

We have shown that the *sec59* and *dpm1* mutants are affected in cell wall composition [35]. A search for multicopy suppressors of the mutant phenotype, resulted in the isolation of the *RER2* gene , which as described above, is involved in the synthesis of the dolichol backbone and enhances protein glycosylation. In addition, the *sec 59-1* phenotype could be rescued by overexpression of the *ROT1* gene, encoding the endoplasmic reticulum Rot1 protein. The latter is implicated in cell wall biogenesis and acts as a chaperone for misfolded proteins [36, 37]. Recently, we have shown also that Rot1 interacts with Ost3, one of the nine subunits of the oligosaccharyltransferase complex, the key enzyme of *N*-glycosylation. Deletion of *OST3* in the *rot1-1* mutant causes a temperature sensitive phenotype as well as sensitivity towards compounds interfering with cell wall biogenesis, such as Calcofluor White, caffeine, Congo Red and hygromycin B. Oligosaccharyltransferase activity determined *in vitro* in membranes

from *rot1-1ost3Δ* cells was found to be decreased to 45% compared to wild-type membranes, and model glycoproteins of *N*-glycosylation, like carboxypeptidase CPY, Gas1 or DPAP B, displayed an underglycosylation pattern. A physical interaction between Rot1 and Ost3 was demonstrated by affinity chromatography. Moreover, Rot1 was also found to be involved in the *O*-mannosylation process, as glycosylation of distinct glycoproteins of this type was affected as well. Altogether, it can be assumed that Rot1 acts also as a chaperone required to ensure proper glycosylation [38].

As already mentioned, a defect in protein O-mannosylation in fungi results in impaired cell wall integrity [9]. This process is initiated at the luminal side of the ER. The key enzyme of O-mannosylation is protein -O-mannosyltransferase (Pmtp) catalysing direct transfer of Man from DolPMan into the serine/threonine OH group in acceptor protein. This is followed by the addition of a short linear glycan, composed of mannosyl residues, directly from GDPMan. Whereas O-mannosylation is initiated in the ER, further modifications of the glycan chain occur in the Golgi apparatus. In *S. cerevisiae* the Pmt proteins are encoded by seven *PMT* genes [39]. A family of *PMT* genes was also identified in *C. albicans* [40] and in another fungal pathogen *Cryptococcus neoformans* [41]. Phylogenetic analysis of Pmt proteins revealed that they can be grouped in three subfamilies, Pmt1p, Pmt2p and Pmt4p [42]. The functions of Pmt proteins were studied, and it was demonstrated that in *S.cerevisiae* O-mannosylation affects protein stability, localisation and transport from the ER [43]. In *C.albicans* O-mannosylation is important for morphogenesis, adherence to host cells and virulence [44]. Characterization of the *PMT* gene family in *C. neoformans* revealed that Pmt proteins play a crucial role in maintaining cell morphology and cell wall integrity [41]. Similarly, in the filamentous fungus *T.reesei* diminished overall activity of Pmt proteins resulted in decreased O- as well as N-glycosylation and aberrant cell wall composition [45].

Studies of the effect of tunicamycin revealed the effect of the dolichol dependent protein N-glycosylation on *C. albicans* biofilm development. In its normal niche *C.albicans* forms biofilms that are attached to cell surfaces. In addition to the mucosal surfaces, such biofilms are often formed on implanted medical devices [46, 47]. Fully mature *C.albicans* biofilms consist of a complex of yeast, hyphae and pseudohyphae and exhibit increased resistance to antifungal drugs [48, 49]. Tunicamycin is a nucleoside antibiotic, produced by *Streptomyces lysosuperficus* that blocks the transfer of GlcNAc-1-P from UDPGlcNAc to DolP. This reaction, catalysed by the Alg7 protein, initiates the formation of DolPP-oligosaccharide and hence the whole N-glycosylation process. It has been demonstrated that physiological concentrations of tunicamycin display a significant inhibitory effect on biofilm development and maintenance, without affecting overall cell growth or morphology. Based on the above, conclusion was reached underlying the role of N-glycosylation in the developmental stages of biofilm formation [50]

4. Cell morphology in Golgi glycosylation mutants

A number of data indicates that a defect in glycosylation process occurring in the Golgi stack might affect cell morphology and virulence.

Initial steps of protein N- and O-glycosylation described so far occur in the ER. "Dolichol-dependent" glycosylation ends with the formation of DolPP-oligosaccharide (DolPPGlcNAc2Man9Glc3) and subsequent transfer of the oligosaccharide to the beta amido group of asparagine within the N-glycosylation site (Asn/X/Ser). Such a glycosylated peptide undergoes partial trimming, which is species-specific and in yeast involves removal of the three Glc and one Man residues. Partially processed glycopeptide is transported to the Golgi stack where the saccharide part (GlcNAc2Man8) undergoes further processing and maturation. Whereas glycosylation reactions occurring in the ER are well conserved in the eukaryotic cells, N-glycan processing in the Golgi is greatly diverse. In yeast, the core structure of glycoproteins is hypermannosylated (up to 200 Man residues), forming a backbone made up of alpha 1,6 linked residues, branched by alpha 1,2- and alpha 1,6 mannosyl residues. In addition, another mature core structure occurs in the Golgi, i.e. GlcNAc2Man8-13 [51]. A number of Golgi mannosyltransferases is involved in the synthesis of the sugar backbone and branching. It offers a vast majority of modifications of the sugar structure. The *OCH1* gene encodes an alpha 1,6 mannosyltransferase initiating addition of the outer sugar chain (www.yeastgenome.org). Deletion or depletion of *OCH1* causes lethality or slow-growth. The *och1* mutants cannot form high mannose oligosaccharides (~50+ Man residues) so they have decreased levels of cell wall mannoproteins, causing weakness and defects in bud formation and hypersensitivity to agents that attack the cell wall (calcofluor white, hygromycin B and SDS). Weakening of cell walls in hypotonic solution can be partially suppressed by the addition of osmotic stabilizers such as salt or sorbitol.

Mannan branching involves Mnn1p, one of five *S. cerevisiae* proteins of the MNN1 family. It is alpha-1,3-mannosyltransferase, an integral membrane glycoprotein of the Golgi complex, required for addition of alpha1,3-mannose linkages to N- and O-linked oligosaccharides,. Mnn1 mutant exhibits decreased sensitivity to hygromycin B [52]. It has been demonstrated that a double deletion of the *OCH1* and *MNN1* genes renders a strain which is unable to grow at non-permissive temperature and is defective in cell cytokinesis. On the other hand, no clear effect on the cell integrity was observed [53]. However, the role of Mnn5 protein (an iron regulated alpyha-1,2 mannosyltransferase) from *C.albicans* in cell wall integrity maintenance was demonstrated [54]. A *mnn5* delta mutant contains a reduced amount of cell wall mannan and hypersensitivity to the cell wall damaging agents. Disruption of the *C. albicans OCH1* homologue resulted in a temperature-sensitive growth defect and cellular aggregation. Outer chain elongation of *N*-glycans was absent in the null mutant, demonstrated by the lack of the alpha 1,6-linked polymannose backbone and the underglycosylation of *N*-acetylglucosaminidase. Moreover, the null mutant was hypersensitive to a range of cell wall perturbing agents. These mutants had near normal growth rates *in vitro* but exhibited decreased virulence in a murine model of systemic infection. Based on this the importance of *N*-glycan outer chain epitopes to the host-fungal interaction and virulence was assumed [55, 56].

5. Evidence for the role of glycan in cell defence mechanisms

The fungal cell wall is a highly dynamic structure, essential for the shape and stability of the cell. Thus in yeast and fungi cell wall integrity is tightly controlled by the activation of the protein kinase C –dependent MAP-kinase pathway [57] (Fig. 3).

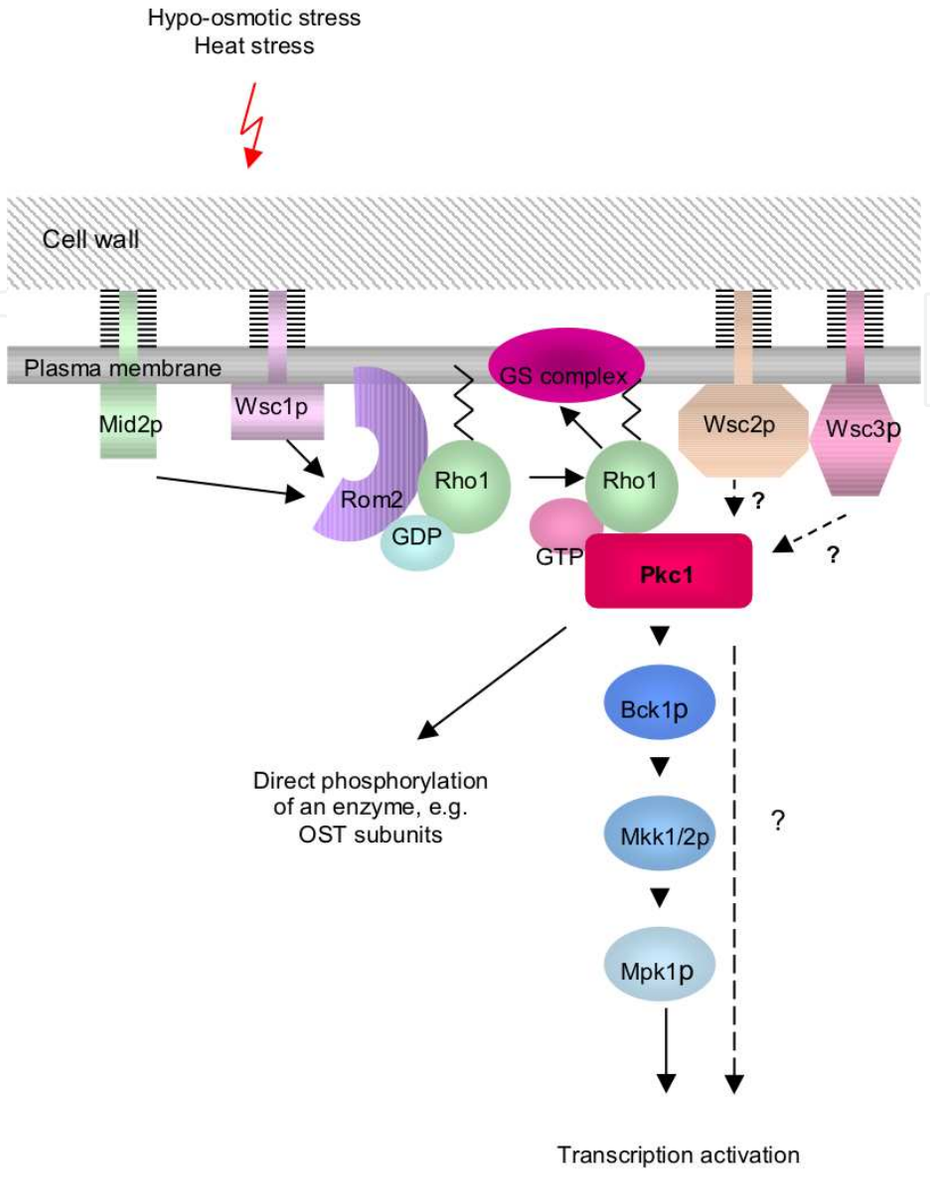


Figure 3. Cell wall integrity pathway (CWIP) in *Saccharomyces cerevisiae*

The sensors of the cell wall damage located in the plasma membrane (Mid2p, Wsc1-3p) pass the signal thorough the Rom1p to Rho1p, which in turn activates the Pkc1p- dependent cascade of MAP kinases (Bck1p, Mkk1/2p) Mpk1 kinase activates its main targets Swi4/6p and Rlm1p, transcription factors that activate expression of the cell wall genes (adopted from [57]).

Efficiency of this process depends, among others, on the glycosylation status of the receptor proteins located in the plasma membrane [58]. It has been demonstrated that the plasma membrane protein Mid2, a putative mechanosensor, responds to cell wall stresses and changes in the cell morphology induced by pheromone treatment. The response is related to the glycosylation status of the Mid2 protein, which is highly O-glycosylated and contains two potential glycosylation sites, one of them at Asn-35, carrying N-linked sugars. It was demonstrated that O-glycosylation is responsible for the stability of the protein, whereas the

presence of the N-linked sugar chain is a prerequisite for its function as a sensor of external stimuli.

Our data [35] indicate that impairement of dolichol kinase (Sec59p) activity is concomitant with a defect in plasma membrane/cell wall Gas1p glycosylation and the activation of the CWIP. These results were further confirmed by the analysis of the cell wall composition of the *sec59-1* mutant as compared to the parental wild type. The cell wall of the mutant strain contains a significantly increased amount of chitin and beta-1,6 glucan (2.7 and 1.7 fold, respectively). Simultaneously we observed an activation of the CWIP in *sec59-1*, as compared to the wild type cells. All these differences were abolished by overexpression of the suppressor *RER2* gene, leading to increased synthesis of the oligosaccharide carrier (DolP) and protein glycosylation.

An activation of the cell defence mechanisms was also observed in *C.albicans OCH1* mutants, affected in outer sugar chain elongation (compare above).

Together, based on the knowledge acquired so far, it can be assumed that the glycosylation pathway in yeast and fungi offers many levels of regulation which might influence the final quality and quantity of cell wall glycoproteins and consequently cell surface immunogeneity and the fungal-host interaction. This includes also the processes occurring in the ER i.e. dolichol biosynthesis and glycosylation steps involving dolichol.

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