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The essential endoplasmic reticulum chaperone Rot1 is required for protein *N*- and *O*-glycosylation in yeast

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Abstract

Rot1 is an essential yeast protein originally shown to be implicated in such diverse processes such as β-1,6-glucan synthesis, actin cytoskeleton dynamics, or lysis of autophagic bodies. More recently also a role as a molecular chaperone has been discovered. Here we report that Rot1 interacts in a synthetic manner 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, whereas deletion of *OST6*, a functional homolog of *OST3*, has no effect. Oligosaccharyltransferase activity *in vitro* determined in membranes from *rot1-1ost3*Δ 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. By affinity chromatography a physical interaction between Rot1 and Ost3 was demonstrated. Moreover, Rot1 was found to be involved also in the *O*-mannosylation process, as glycosylation of distinct glycoproteins of this type were affected as well. Altogether the data extend the role of Rot1 as a chaperone required to ensure proper glycosylation.

Keywords: *ROT1*/ *N*-glycosylation/ *O*-glycosylation/ oligosaccharyltransferase/ dolichol/ *Saccharomyces cerevisiae*

Introduction

Protein-glycosylation of eukaryotic secretory and membrane-bound proteins, either in the form of *N*-glycosylation of asparagine residues or *O*-glycosylation of serine/threonine residues, is a fundamental and the most abundant protein modification in lower and higher eukaryotes, serving many intra- and extracellular functions (Helenius and Aebi 2004; Lehle et al. 2006; Ohtsubo and Marth 2006; Spiro 2002).

The pivotal step of the *N*-glycosylation pathway is the *en bloc* transfer of the high mannose core oligosaccharide $Glc_3Man_9GlcNAc_2$ from the lipid carrier dolicholphosphate to selected Asn-X-Ser/Thr sequences of the nascent polypeptide chain in the lumen of the endoplasmic reticulum (ER). The enzyme that catalyzes this process is a multisubunit membrane-complex, called oligosaccharyltransferase (OST) (Dempski and Imperiali 2002; Kelleher and Gilmore 2006; Knauer and Lehle 1999; Schwarz and Aebi 2011; Yan and Lennarz 2005b). Purification of OST from yeast and genetic methods identified nine proteins as constituents of the complex. Five of them, Wbp1, Swp1, Stt3, Ost1 and Ost2, are essential for viability of the cell, whereas Ost3, Ost4, Ost5, and Ost6 are not essential, but are required for maximal OST activity. Apart from Stt3, identified as the catalytic subunit (Hese et al. 2009; Igura et al. 2008; Lizak et al. 2011; Nasab et al. 2008), the specific function of the various other components is largely enigmatic. For the homologous Ost3 and Ost6 proteins it was shown that they are alternatively present in the OST together with the other subunits forming two distinct complexes (Schwarz et al. 2005; Spirig et al. 2005) differing in their substrate-specific activities at the level of individual glycosylation sites (Schulz and Aebi 2009). In addition, Ost3 and Ost6 exhibit oxidoreductase activity and bind specific polypeptides, both non-covalently and via transient disulfide bonds. These subunits are thought to slow down early stages in protein folding, maintaining the nascent polypeptide chain in a glycosylation competent conformation (Schulz et al. 2009). There is also evidence that Ost3/Ost6 may specify the interaction with different translocation complexes (Yan and Lennarz 2005a).

O-glycosylation in yeast is restricted exclusively to *O*-mannosylation of serine/threonine residues forming short linear oligomannose chains consisting of up to four mannose residues. *O*-mannosylation, first identified in yeast (Babczinski and Tanner 1973), was assumed for a long time to occur exclusively in fungi. However, it is now clear that this type of *O*-glycosylation is conserved throughout the animal kingdom, including human (Lehle et al. 2006; Lommel and Strahl 2009). Similarly as for *N*-glycosylation, but differently from other types of *O*-glycosylation, *O*-mannosylation is initiated in the ER, while the nascent

polypeptide is entering the lumen. The initial mannosyltranfer reaction is catalyzed by distinct complexes of the Dol-P-Man:protein *O*-mannosyltransferase (PMT) family, originally discovered in yeast (Gentzsch and Tanner 1996; Strahl-Bolsinger et al. 1993). It has also been shown that the *N*- and *O*-glycosylation machineries may compete for protein substrate (Ecker et al. 2003; Harty et al. 2001).

ROT1, encoding an essential ER-localized membrane protein, was initially reported as a gene required for ß-1,6-glucan synthesis (Bickle et al. 1998; Machi et al. 2004). Mutations of *ROT1* cause pleiotropic defects in cell wall synthesis, cytoskeleton dynamics, or in lysis of autophagic bodies (Takeuchi et al. 2006a; Takeuchi et al. 2006b). More recently it was demonstrated that Rot1 can function as a general chaperone exhibiting anti-aggregation activity *in vitro*, and *in vivo* the *rot1-2* mutation caused an accelerated degradation of several proteins of the secretory pathway via ER-associated degradation (Takeuchi et al. 2008). Furthermore, *ROT1* was shown to interact genetically with *KAR2*, encoding the yeast BiP, suggesting a cooperation of these two protein in the folding of nascent proteins (Takeuchi et al. 2006a).

We previously observed that over-expression of *ROT1* in *sec59-1* cells, impaired in dolichol kinase, suppresses the temperature-sensitive phenotype of this mutant (Orlowski et al. 2007). As dolicholphosphate (Dol-P) is a key component in the formation of lipid-linked oligosaccharide, we asked, whether a direct role of Rot1 in protein glycosylation can be demonstrated. Here we show that *N*-glycosylation is impaired *in vivo* and *in vitro* in *rot1-1ost3*Δ cells and that Rot1 physically interacts with the Ost3 subunit of OST. Quite unexpected, we observe that Rot1 is also required for *O*-mannosylation of proteins.

Results

Synthetic interaction of ROT1 and OST3

OST3 and *OST6* are two closely related, not essential subunits of two distinct OST isoforms, required for efficient *N*-glyosylation (Knauer and Lehle 1999; Schulz and Aebi 2009). As shown in Figure 1, deletion of *OST6* displayed a growth phenotype, when stressed by agents known to interfere with cell wall biogenesis, such as Calcofluor White, caffeine, Congo Red or hygromycin B, whilst the *ost3*Δ strain proved more or less resistant at the concentrations used, similar to wild-type. Deletion of *ROT1* is known to be lethal, but the *rot1-1* mutant is viable and was hardly affected by aforementioned agents. However, growth of a *rot1-1ost3*Δ double mutant was abolished, while the *rot1-1ost6*Δ strain was not affected in a synthetic manner by these drugs and displayed only the phenotype of the *ost6*Δ single mutant.

Similarly, only *rot1-1ost3*Δ cells reveal a temperature-sensitive growth phenotype. Altogether the data suggest a synthetic interaction between Rot1 and Ost3 that does not apply to Rot1 in combination with Ost6.

Rot1 is required for N-glycosylation

The synthetic interaction between *ROT1* and *OST3* pointed to an involvement of *ROT1* in *N*-glycosylation. To investigate this in more detail, we examined the glycosylation status of several well characterized soluble and membrane-bound glycoproteins in single and double mutant strains. As depicted in Figure 2, Western blot analysis showed an underglycosylation of the soluble, vacuolar carboxypeptidase Y (CPY) only in *rot1-1ost3*Δ cells, indicated by the appearance of glycoforms migrating faster than mature CPY, lcking one, two or three *N*-glycan chains. CPY containing four chains is the dominant species in wild-type cells. Similarly, also the vacuolar membrane glycoprotein dipeptidyl aminopeptidase B (DPAP B), the plasma membrane cell wall integrity sensor Mid2, or Gas1, a GPI-anchored β-1,3-glucanosyltransferase involved in cell wall remodeling, displayed an underglycosylation pattern only in *rot1-1ost3*Δ, but not in *rot1-1ost6*Δ and single deletion strains, respectively. The last two proteins carry also *O*-glycans. However, as it will be shown later (see Figure 4), the underglycosylation is due exclusively to the loss of *N*-linked glycans.

*Altered oligosaccharyltransferase and cis-prenyltransferase activity in the rot1-1ost3*Δ *mutant*

The phenotype of underglycosylation of proteins could be caused, amongst others, by a reduced activity of OST, or might be due to a reduced amount of the Dol-P precursor oligosaccharide. We therefore determined OST activity *in vitro* in microsomal membranes isolated from these strains. As shown in Figure 3A, OST activity reflected the underglycosylation observed *in vivo*. Whereas membranes from *ost3*Δ and *ost6*Δ single mutants displayed only a small decrease of activity, in the double mutant *rot1-1ost3*Δ, but not in *rot1-1ost6*Δ, it was severely reduced, indicative for the specific synthetic interaction between Rot1 and Ost3. We also determined the activity of cis-prenyltransferase, the first enzyme committed to the biosynthesis of the dolichol backbone, encoded by *RER2* localized to the ER (Grabinska and Palamarczyk 2002). As can be seen in Figure 3B, also this activity was significantly reduced in a synthetic manner in *rot1-1ost3*Δ cells, indicating that disturbed *Rot1* function in combination with impaired glycosylation due to deletion of *OST3* may affect earlier steps of this pathway as well (for further discussion see below).

Rot1 affects O-mannosylation in vivo and in vitro

The fact that there is a link between *N*- and *O*-glycosylation (Ecker et al. 2003) and both processes in yeast occur while the nascent, unfolded polypeptide is translocated into the ER lumen, prompted us to ask, whether Rot1 may also take an active role in *O*-mannosylation. As mentioned above, the initial mannosyltransfer reaction is catalyzed by heteromeric and homomeric, respectively, complexes of the Pmt1/Pmt2 and the Pmt4 subfamilies. The complexes differ in their target protein substrate specificity (Ecker et al. 2003; Gentzsch and Tanner 1996; Gentzsch and Tanner 1997; Lommel and Strahl 2009). As shown in Figure 4, Western blot analysis of endochitinase Cts1, an exclusively *O*-mannosylated protein, revealed that it migrated faster in *rot1-1ost3*Δ, reflecting a defect in *O*-mannosyltransfer. Such a reduced *O*-mannosyltransferase activity could also be demonstrated *in vitro* in microsomal membranes (Figure 5). Pmt activity in *rot1-1ost3*Δ was decreased to 45% compared to wild-type, *rot1-1* or *ost3*Δ single mutant strains.

Besides Cts1 further *O*-mannosylated proteins were investigated, like Ccw5, Gas1 and Mid2. In the case of Gas1 and Mid2 also *N*-glycosylation of the protein occurs (see above). In order to exclusively monitor *O*-mannosylation of these proteins, in case of Gas1, samples were treated with Endo H to remove *N*-glycan chains, while for Mid2 a mutant variant (Mid2-N35A) was analyzed, in which the single *N*-glycan chain at asparagine residue 35 was deleted by substitution of asparagine to alanine. As can be seen in Figure 4, *O*-mannosylation of these proteins, in contrast to chitinase Cts1, was not altered in *rot1-1ost3*Δ cells. The faster mobility of Gas1 in Figure 4 (compare lanes 1 and 3) was due to a defect in *N*-glycosylation and hence could not be observed upon treatment with Endo H (lanes 4 and 6). Similarly, the mobility shift of Mid2-HA in *rot1-1ost3*Δ (lanes 7 and 8), was not the result of defective *O*-mannosylation, but rather was due to the lack of *N*-glycosylation, as the Mid2-(N35A)-HA mutant variant, lacking the *N*-glycosylation site, had the same mobility in wild-type and *rot1-1ost3*Δ cells (lanes 2 and 6).

So far, only Cts1 showed an impairment of *O*-mannosylation. Since chitinase is exclusively mannosylated by the Pmt1-Pmt2 complex in contrast to Gas1, which is a substrate for the Pmt4 complex, and Ccw5 as well as Mid2, which are mannosylated by both Pmt1-Pmt2 and Pmt4, we reasoned that only proteins modified by the Pmt1-Pmt2 complex were affected. Thus, a further target protein of this type, Kre9, was analyzed. However, for unknown reason this protein was not detectable in the *rot1-1ost3*Δ double mutant (Figure 4, lane 5), compared to wild-type or single mutant strains. It is assumed that Kre9 is degraded in

this genetic background, because of defective *O*-mannosylation. A similar observation, i.e. lack of detection, was also made when investigating Kre5 in *rot1-2*, which is another mutant allele of *ROT1* (Takeuchi et al. 2008).

Physical interaction between Rot1 and Ost3

Previous experiments showed that Rot1 and Ost3 interact at the genetic level in a synthetic manner. In order to demonstrate also a physical interaction between these two proteins, a strain was constructed co-expressing *OST3-ZZ* and *ROT1-HA* tagged variants to perform co-immunoprecipitation experiments. A detergent-solubilized extract from microsomal membranes was isolated and applied to either magnetic beads coupled with anti-HA antibody recognizing the HA epitope, or to an IgG-Sepharose affinity matrix recognizing the protein A (ZZ) epitope. After washing to remove non interacting proteins, the eluate fraction was analyzed for Ost3 (upper panel) and Rot1 (lower panel). As shown in Figure 6, both proteins co-fractionated (lanes 5 and 6) with either anti-HA-matrix or the IgG-matrix as bait for pulling down the HA- and ZZ-epitope, respectively. It should be mentioned that Rot1 is prone to aggregation and gives rise to doublet bands (lanes 5 and 6, indicated by asterisks). In control extracts from membranes, expressing only Rot1-HA but no Ost3-ZZ (lane 7), or expressing an unrelated membrane protein, Dpm1-HA (lane 8), no binding to IgG-Sepharose was detectable. In a further control using Sepharose (lane 9) instead of IgG-Sepharose as affinity-matrix, also no interaction was observed. This rules out that Rot1 and Ost3 bind in a non-specific manner.

Discussion

In this study we extend the multifunctional role of Rot1 by demonstrating that it is needed to ensure both proper protein *N*- and *O*-glycosylation. Rot1 is an essential ER protein that originally has been related to cell wall biosynthesis, actin cytoskeleton dynamics (Bickle et al. 1998; Juanes et al. 2007; Machi et al. 2004), and more recently shown to function in protein folding as a molecular chaperone (Takeuchi et al. 2006a; Takeuchi et al. 2008). Rot1 does not have any known functional motif and the details of its molecular function have not been elucidated. Rot1 was shown to be transported to the ER by an SRP-independent posttranslational mechanism involving Sec61/Sec63 and is anchored to the ER membrane by a transmembrane domain at its C-terminal region (Juanes et al. 2008; Juanes et al. 2010).

In the first instance, a phenotypic analysis of the *ROT1* mutant allele *rot1-1* in combination with deletion of *OST3*, encoding a non essential subunit of OST, revealed a specific genetic interaction that causes temperature-dependent synthetic lethality and increased sensitivity of cells when stressed by drugs interfering with cell wall biogenesis (Figure 1). These deficiencies were not observed by deletion of its paralogue *OST6*. An involvement in *N*-glycosylation was then further substantiated by demonstrating that in *rot1-1ost3*Δ cells both soluble and membrane-bound model *N*-glycoproteins were underglycosylated *in vivo*, and in accord with this finding we measured a reduced OST activity *in vitro*. Finally, by co-immunoprecipitation experiments a physical interaction between Rot1 and Ost3 could be verified.

OST is a multifunctional enzyme that acts at a pivotal step of the secretory pathway, at which protein translation and translocation into the ER, biosynthesis of lipid-linked glycosyl donor substrates, as well as protein glycosylation and protein folding converge. In view of the proposed role of Rot1 as a molecular chaperone of the ER, it is not quite unexpected that it is also required during glycosylation of the nascent and yet unfolded protein by assisting or modulating the OST complex. Thus it is conceivable that Rot1 could contribute to the assembly of an optimal complex, or to a better positioning of OST to the polypeptide or translocation site. Alternatively it could help keeping the polypeptide in a glycosylation competent state under certain conditions. It has been reported that the *rot1-2* mutant allele causes activation of the UPR pathway, and *ROT1* genetically interacts with *KAR2 (BIP)* in an allele specific manner suggesting cooperation in protein folding (Takeuchi et al. 2006a; Takeuchi et al. 2008). Kar2/BiP in yeast has been shown to be involved in various steps of protein maturation in the ER. It functions in protein translocation into the ER (Corsi and Schekman 1997), but also at later stages facilitating folding (Simons et al. 1995) and even contributes to ERAD (ER-associated degradation) (Fewell et al. 2001). Worth mentioning in this context is the finding of a genetic interaction between *kar2* and *wbp1* mutations, the letter being an essential component of OST. Interestingly, OST activity in *kar2wbp1* and *wbp1* strains was the same (te Heesen and Aebi 1994) in contrast to the results obtained here with the *rot1-1ost3*Δ mutant.

Why Ost3, but not its homolog Ost6, causes a synthetic interaction with *rot1-1* is not clear at the moment. The identity between both proteins with 21% is rather low, but they display a similar membrane topology containing four transmembrane spanning domains and an ER luminal thioredoxin-like domain at the N-terminal region, for which a crystal structure exists (Schulz et al. 2009). Apart from that no primary structural differences or motifs can be

assigned to Ost3/Ost6 that would allow predicting sites for interaction with Rot1. Ost3 and Ost6 are accessory subunits of two isoforms of the OST complex and contribute to the specificity and efficiency at the level of individual glycosylation sites (Schwarz et al. 2005; Spirig et al. 2005). Ost3/Ost6 function by transiently binding stretches of the nascent substrate to inhibit protein folding and thus increasing glycosylation efficiency of nearby Asn residues (Jamaluddin et al. 2011; Schulz et al. 2009). Polypeptide binding to Ost3/Ost6 is thought to be transient and by mixed disulfide bonds. This is mediated by their ER luminal thioredoxin-like domain causing a structural change in the oxidized form and thus forming a groove complementary to the characteristic of the acceptor peptide stretch. Eukaryotic OST requires flexible domains of unfolded protein substrate, as compared to single protein OST from bacteria, which can also act on folded proteins (Kowarik et al. 2006). From an evolutionary point of view, Ost3/Ost6 are the most recent added subunits of OST (Kelleher and Gilmore 2006) and the physically most peripheral ones (Kelleher et al. 2003). They are also thought to couple OST to the translocation machinery to associate with different translocons (Yan and Lennarz 2005a). But this has been challenged, by recent findings that Ost3 and Ost6 had essentially complementary functions (Schulz and Aebi 2009).

We also observed a decrease of cis-prenyltransferase activity in *rot1-1ost3*Δ cells, a key enzyme in early steps of the glycosylation pathway, encoded by *RER2*. It provides dolichol needed for the formation of glycosyl donor substrates. This deficiency, however, may be different from the effect of Rot1 on OST activity. We hypothesize that this could be the result of an indirect effect of loss of Rot1 function by down-regulating glycosyl donor formation due to defective glycosylation and/or due to poorly folding. Whether the amount of the Dol-P precursor is indeed reduced has not been demonstrated in this study, but it has been reported that over-expression of *RER2* in *sec59-1*, having a defect in dolichol kinase, is able to improve glycosylation. (Orlowski et al. 2007). Hence, activity of Rer2 is indeed able to affect the glycosylation process. Alternatively Rot1 or Rot1/Ost3 could also play a direct, presumably protective function for Rer2. Interestingly, in a genome wide transcriptional profiling in *Candia albicans* the transcript level of *CaROT1* increased by shutting of *CaRER2* (Juchimiuk et al., in preparation). Finally, also an indirect influence by the lack of *O*-mannosylation cannot be ruled out for the decrease of Rer2 activity as Dol-P plays also a role in this process as glycosyl donor substrate.

Our study further revealed for the first time an involvement of Rot1 in *O*-mannosylation at least for some *O*-glycoproteins. Protein *O*-mannosylation in yeast is initiated in the ER by the transfer of mannose from Man-P-Dol to serine and threonine residues during translocation of

the secretory protein into the ER (Ruiz-Herrera and Sentandreu 1975), albeit it is possible in some cases that *O*-mannosylation can occur after the protein has entered the ER (Harty et al. 2001). Considering the role of Rot1 as a general chaperone and the fact that *N*- and *O*-glycosylation can compete for the same protein and therefore must be tightly coordinated, it is not too astonishing that Rot1 is also engaged in *O*-mannosylation, while the target protein is still in its unfolded state. A loss of Rot1 may then cause hypo-mannosylation under specific conditions and situations. Evidence has been presented that *O*-mannosylation might play a general role in the solubilisation of proteins in the ER and defective *O*-mannosylation can result in induction of unfolded protein response (Arroyo et al. 2011; Murakami-Sekimata et al. 2009; Nakatsukasa et al. 2004; Travers et al. 2000) similar to defects in *N*-glycosylation.

Among the several *O*-glycoproteins tested, so far only Cts1 could be identified to be subject to Rot1 deficiency as well as Kre9, which, however, was not detectable in the *rot1-1ost3*Δ background compared to wild-type and single mutants, presumably due to degradation as result of defective *O*-mannosylation. Why the other *O*-mannoproteins analyzed were not affected, can be only a matter of speculation. As mentioned above, *O*-mannosylation is catalyzed by the evolutionarily conserved PMT family classified into PMT1 (comprising Pmt1/5 isoforms), PMT2 (comprising Pmt2/3/6 isoforms) and PMT4 (Pmt4) subfamilies. They form distinct heteromeric (Pmt1-Pmt2) and homomeric (Pmt4-Pmt4) complexes, which in yeast are responsible for the vast majority of mannosyltransferase activity. They exhibit different target site specificities (Hutzler et al. 2007; Lommel and Strahl 2009), the signatures of which are largely elusive and remain to be determined. Merely for Pmt4 protein *O*-mannosyltransferases membrane association was found to be a determinant for substrate recognition (Hutzler et al. 2007). Cts1, a soluble and secreted glycoprotein is known to be substrate exclusively for Pmt1-Pmt2, whereas Gas1 is a target for Pmt4-Pmt4, or in the case of Mid2 and Ccw5 both complexes contribute to glycosylation. Furthermore, it is conceivable that due to a compensatory cooperation other PMT-complexes may contribute to ensure *O*-mannosylation. Thus, it was reported earlier that under certain physiological conditions Pmt1 can also interact with Pmt3, and Pmt5 with Pmt2 (Girrbach and Strahl 2003). Finally, as Gas1, Mid2 and Ccw5 are membrane-bound and anchoring increases residence time in the ER, Pmt4-Pmt4 mediated *O*-mannosylation may have a different kinetics of the mannosyltransfer and is more efficient, perhaps in addition in a posttranslational manner. In this context it is worth mentioning that several mutant secretory proteins that are unable to fold properly are *O*-mannosylated by Pmt1 and/or Pmt2, whereas their wild-type counterparts are not (Coughlan et al. 2004).

Rot1 homologues have been identified so far only in fungi. However, proteins with similar function may also occur in the ER of higher eukaryotes, because of the evolutionary conservation of the *N*- and *O*-glycosylation as well as of the protein folding pathway. A genome-wide screening for Rot1 substrates and further investigation of the role of Rot1 in the mentioned processes will eventually lead to a better understanding of this multifunctional protein.

Material and methods

Strains, plasmids and media

Yeast strains used are listed in Table 1. Strains were grown in YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) or in selective YNBD medium (0.67% YNB, 0.5% casamino acids, 2% glucose) supplemented with amino acids and nucleotide bases, as required.

To construct the expression plasmids pVT100-(TRP1)-Rot1-ZZ and pVT100-(URA3)-Rot1-HA, genomic *ROT1* was amplified from W303-1A by PCR and engineered for cloning purpose with Hind III and BamH I restriction sites at the 5' and 3' ends, respectively, and ligated into the Hind III / BamH I cut vectors pVT100-(TRP1)-ZZ and pVT100-(URA3)-HA, respectively, placing *ROT1* under the control of the constitutive *ADH1* promoter. The constructs were sequenced and the functional expression of Rot1 containing the Protein A (ZZ) epitope or the HA-epitope in frame at the C-terminus were verified by immunoblotting and complementation of the *ts* growth phenotype of the *rot1-1rot2-1* mutant. For PCR amplification of *ROT1* the following primers were used: *ROT1* fw (5'-AGCGGCCGCATGA ATTGGCTGTTTTTGGTCTCGC-3') and *ROT1* rev (5'-AGCGGCCGCTTATTTGAATGGT GCCGATAACCTT AGTAAAGC-3').

Chromosomal deletions of *OST3* and *OST6* were performed using gene specific PCR primers and the *pFA6a-natMX6* cassette as template (Goldstein and McCusker 1999). PCR-products were transformed into *rot1-1*. Homologous integration resulted in the deletion of nucleotides 60–992 from the 1053-nucleotide ORF of *OST3,* and deletion of nucleotides 60-935 from the 999-nucleotide ORF of *OST6*. Successful deletions of genes were confirmed by PCR using 11genomic DNA obtained from nourseothricin resistant transformants. Deletion primers will be provided on request.

For monitoring the glycosylation status the following plasmids expressing C-terminal HA-tagged versions of the respective genes were used: pVT100-Kre9-HA, YEp352-Mid2-HA,

YEp352-Mid2-HA(*N35A*) and YEp352-Ccw5-HA. Transformation into yeast was carried out using standard techniques.

Phenotype analysis of rot1Δ cells

Analysis of drug or temperature sensitivity of different strains was performed on YEPD plates in the presence of the various drugs at 25°C, or for temperature sensitivity at 37°C, respectively, as specified. 3 µL of serial 10-fold dilutions from a liquid culture of mid-logarithmic phase were spotted on agar plates, starting at $3x10^4$ cells and incubated for 72 h.

Biochemical methods

Enzyme assays. Membrane isolation and determination of oligosaccharyltransferase activity was performed as described (Sharma et al. 1981; Zufferey et al. 1995). OST activity was determined according to (Sharma et al. 1981). Determination of PMT activity was carried out according to (Strahl-Bolsinger and Tanner 1991). Cis-prenyltransferase activity was tested as published in (Szkopinska et al. 1997).

Immunoaffinity chromatography. To verify physical interaction between Rot1 and Ost3, yeast membranes were collected by centrifugation, and the membrane pellet was resuspended in 30 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 1 mM DTT, 3% glycerol and adjusted to 100 µL of final volume containing 1% octylglucoside (OG), 0.5 M KCl, 20 mM Tris-Cl, pH 7.4, and 1 mg of membrane protein. After incubation on ice for 25 min, the mixture was centrifuged for 40 min at 120,000 x *g*, and the supernatant was applied for immunoprecipitation. Two different affinity media for were used. (i) IgG-Sepharose 6 (GE Healthcare) was equilibrated with 15 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 150 mM NaCl, and 17.5% glycerol and incubated with solubilized membrane extract for 1 h at 4°C with gentle shaking. Sepharose beads were centrifuged and washed 5 times to remove unbound material with 2 vol. of buffer containing 1.5 mM Tris-HCl, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 0.1% OG, and 17.5% glycerol. Elution was carried out with 100 mM glycine-HCl buffer, pH 3.3, and fractions of 0.4 mL were collected and analyzed by Western blot using antibodies against the ZZ- or HA-epitopes. (ii) As a further affinity material Dynabeads-Protein A (Invitrogen) coupled with anti-HA antibody was applied. Binding and elution was performed following manufacturer's instructions. Dynabeads-Protein A was coupled with 5 µg of anti-HA antibody in 200 µL of antibody binding buffer and incubated under rotation for 10 min at room temperature. After washing four times with washing buffer, beads were incubated with membrane extract for 20 min at room temperature followed again by washing four times with washing buffer.

Dissociation of the complex was carried out by adding 20 µL of elution buffer and analyzed by Western blotting using antibodies against the ZZ- or HA-epitopes.

Western blot analysis. Following SDS-PAGE, proteins were blotted to nitrocellulose, blocked with 5% non-fat milk and decorated with specific antibodies. CPY was analyzed from a total cell lysate obtained by breakage with glass beads and removal of membranes by centrifugation at 48,000 x *g*. The membrane fraction was used for immunodetection of Mid2-HA, Mid2-(N32A)-HA and diaminopeptidase B (DPAP B). Chitinase isolated from cell walls was detected according to (Immervoll et al. 1995; Kuranda and Robbins 1991). Analysis of Ccw5, released from cell walls by 30 mM NaOH overnight at 4°C, was carried out according to (Mrsa et al. 1997).

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Abbreviations

ER, endoplasmic reticulum; CPY, carboxypeptidase Y; DPAP B;, diaminopeptidase B; OST, oligosaccharyltransferase; protein A, ZZ; hemagglutinin, HA

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Figure legends

Fig. 1. Synthetic genetic interaction of *ROT1* and *OST3*

Analysis of cell growth in dependency of drugs and temperature. 3 µL of a serial 1:10 dilution of the indicated strains grown in liquid medium were plated on YEPD plates containing no additions, 5 µg/mL Calcofluor White, 5 mM caffeine, and 15 µg/mL Congo Red or 20 µg/mL hygromycine B starting with $3x10^4$ cells (left). Plates were incubated for 72 h at 25°C in the case of drug treatment, or at 37°C for the analysis of temperature sensitivity.

Fig. 2. Rot1 is required for *N*-glycosylation *in vivo*

Analysis of glycosylation status of CPY, DPAP B, Mid2-HA and Gas1 was analyzed by Western blot. CPY was probed with monoclonal anti-CPY antiserum. The positions of the mature form of CPY (mCPY) and of the underglycosylated forms, lacking one to three oligosaccharide chains, are indicated. Samples of 100 µg protein were loaded for DPAP B and Gas1 and were detected with anti-Gas1 or anti-DPAP B antisera. 20 µg were applied for Mid2-HA glycosylation analysis; membranes were probed with anti-HA antibody.
150 µg of protein was analyzed for CPY with anti-CPY antiserum.

Fig. 3. Determination of OST and cis-prenyltransferase activity *in vitro*

(A) OST activity was determined by measuring the transfer of radiolabeled [^{14}C]chitobiose from Dol-PP-[^{14}C]GlcNAc$_2$ as glycosyldonor to the synthetic hexapeptide YNLTSV. (B) *Cis*-prenyltransferase activity was measured in presence of radiolabeled [^{14}C]FPP. The reaction products were analyzed by RP18 HPTLC and quantified by liquid scintillation counting. Microsomal membranes served as enzyme source. The data shown are the average of three independent experiments.

Fig. 4. Rot1 affects *O*-mannosylation *in vivo*

Analysis of *O*-mannosylation of Cts1, Ccw5-HA, Kre9-HA, Gas1 and Mid2-HA. HA-epitope-tagged Ccw5 was extracted and analyzed from cell wall fraction. Endo H treatment was used to distinguish between *N*- and *O*-mannosylation of Gas1. In presence of Endo H the *O*-mannosylation status can be diagnosed. The Mid2-(N32A) mutant lacks the *N*-glycosylation site allowing to exclusively analyze *O*-mannosylation of the protein. Blots were probed in the case of Cts1 and Gas1 with protein specific antibodies, or otherwise with anti-HA specific antibody.

Fig. 5. Determination of Pmt activity *in vitro*

Pmt activity of microsomal membranes from different strains, as specified, was measured as indicated in *Material and methods*.

Fig. 6. Physical interaction of Rot1 and Ost3

A detergent solubilized membrane extract from cells expressing *OST3*-*ZZ* and *ROT1*-HA was applied to magnetic beads coupled with antiHA (lanes 1-5) or IgG-Sepharose (lanes 6-8), and, as a control, on Sepharose matrix without coupled IgG (lane 9). Membrane fraction (lane 1), solubilized membrane extract (lane 2), washing fractions (lanes 3 and 4), and eluate fractions (lanes 5-9) were analyzed by Western blot with anti-IgG- (for Ost3-ZZ) and anti-HA- (for Rot1-HA) antibodies, respectively. As further controls, to rule out that Rot1-HA binds non-specifically to IgG-Sepharose, extracts expressing only Rot1-HA (lane 7) or Dpm1-HA(lane 8), but no Ost3-ZZ, were analyzed. Rot1 is subject to aggregation and prone to form doublet bands, as indicated by asterisks.

Table I. Strains used in this study

Strain	Genotype	Source
SS330	*MATa ade2-101 ura3-52 his3Δ200 tyr1*	Vijayraghavan et al. 1998
W303-1A	*MATa leu2-3 leu2-112 his3-11 his3-15 ura3-1 ade2-1 trp1-1*	Euroscarf
MH272-1da	*MATa leu2-3,112 ura3-52 trp1 his3::HIS4*	Bickle et al. 1998
MB134-1a	MH272-1da *rot1-1*	Bickle et al. 1998
rot1-1ost3	MH134-1a *ost3::natMX6*	This work
rot1-1ost6	MH134-1a *ost6::natMX6*	This work
ost3Δ	MH272-1da *ost3::natMX6*	This work
ost6Δ	MH272-1da *ost6::natMX6*	This work
OST3^{ZZ} *ROT1*^{HA}	MH272-1da pVT100-(*TRP1*)-*OST3*^{ZZ} ;pVT100-(*URA3*)-*ROT1*^{HA}	This work

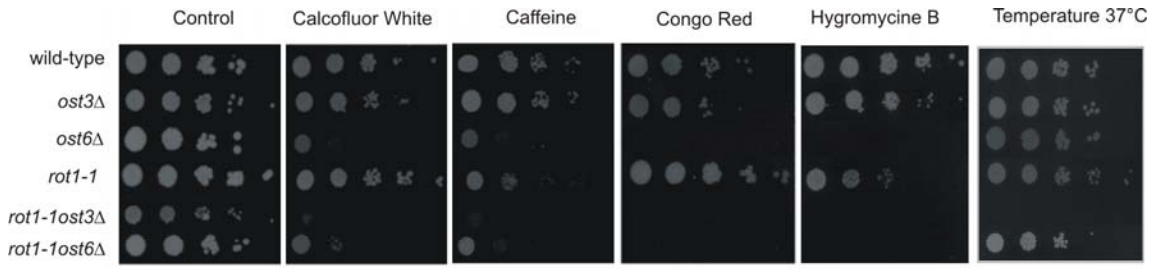

Fig. 1

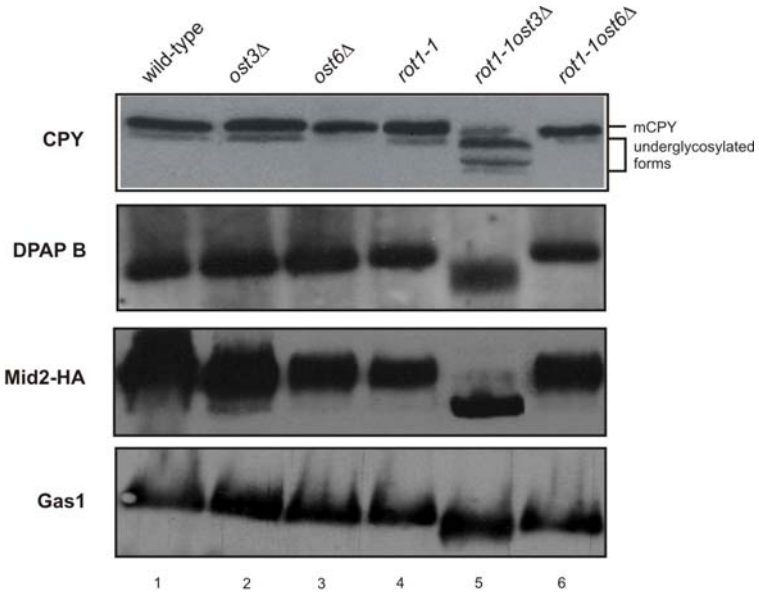

Fig. 2

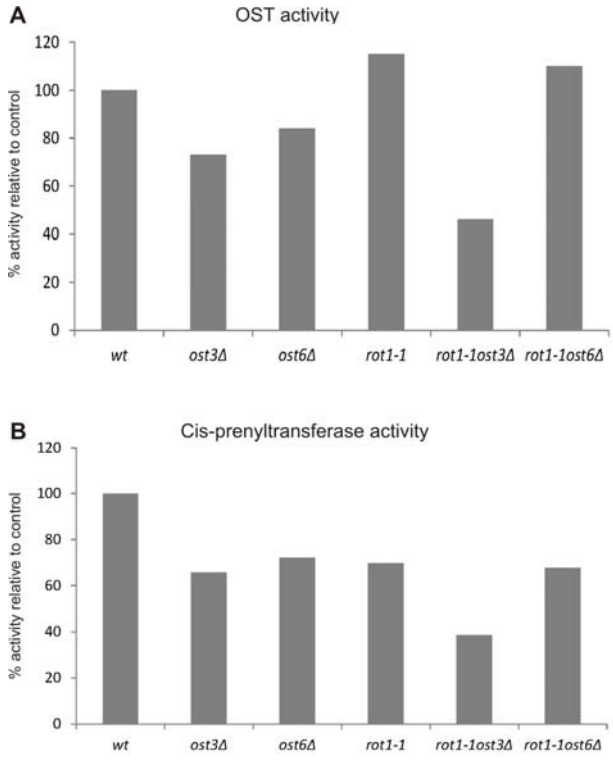

Fig. 3

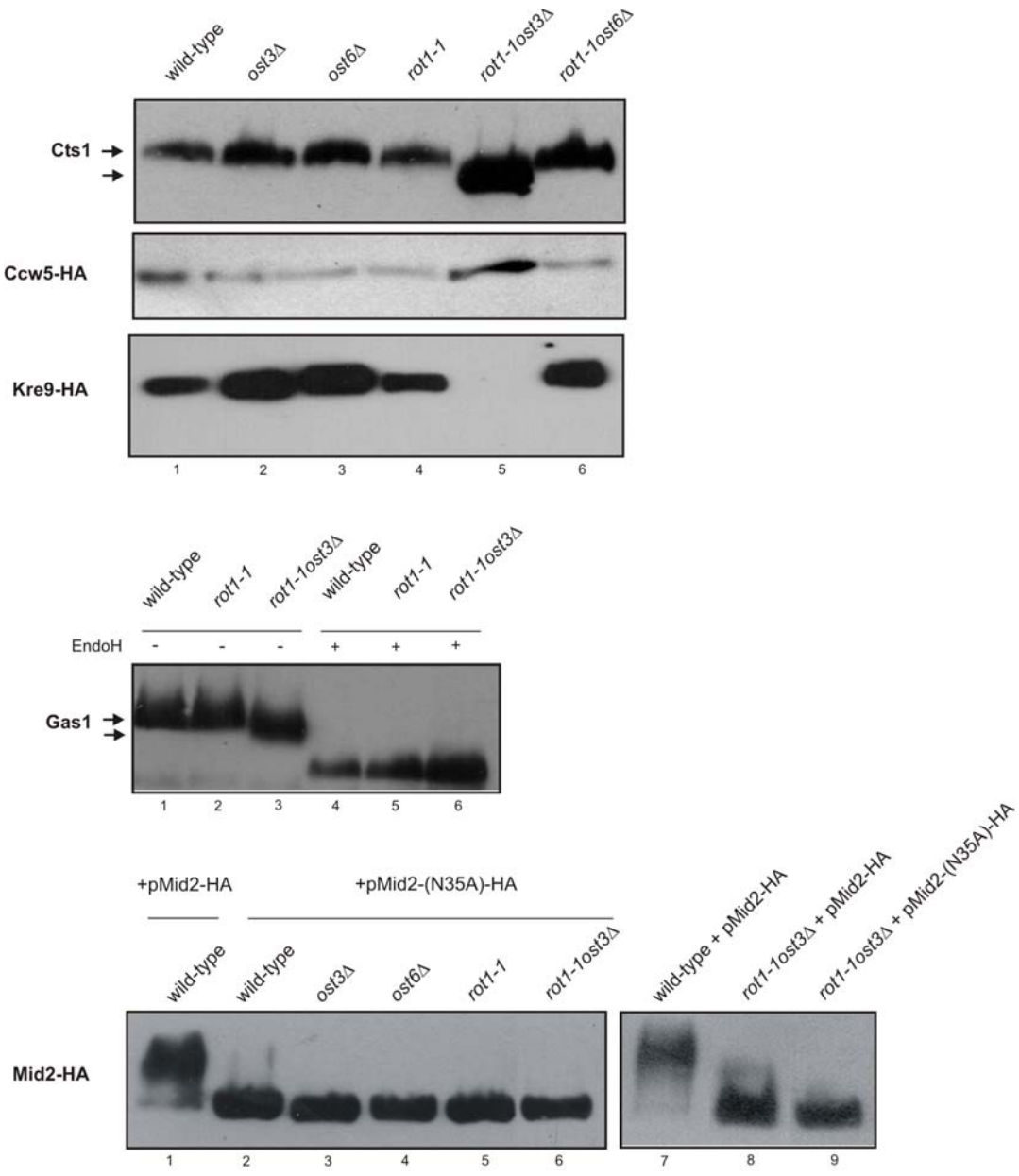

Fig. 4

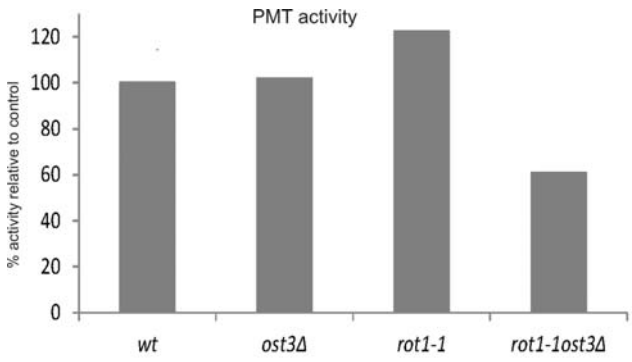

Fig. 5

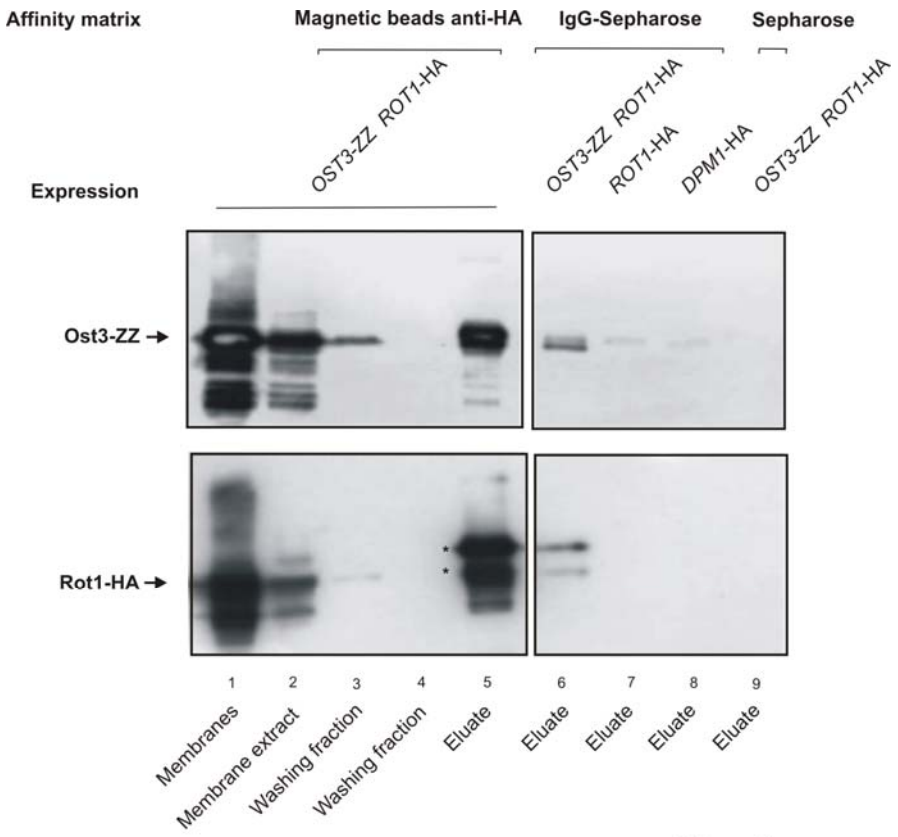

Fig. 6