File S1

Precursors and Carrier Lipids

This Supporting File contains additional information related to **Precursors and Carrier Lipids**. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Sugar nucleotides

Regulation of glucosamine supply and chitin levels. Glucosamine supply is highly regulated and impacts chitin levels, which increase in response to mating pheromones and cell wall stress. Expression of *GFA1* and *AGM1* is upregulated upon treatment of MAT**a** cells with α-factor (Watzele and Tanner, 1989; Hoffman *et al.* 1994), and is accompanied by an increase in chitin deposition (Schekman and Brawley, 1979; Orlean *et al.* 1985). The cell wall stress-induced increase in chitin synthesis (Popolo *et al.* 1997; Dallies *et al.* 1998; Kapteyn *et al.* 1999; see *Wall Composition and Architecture*) is also accompanied by elevated *GFA1* expression (Terashima *et al.* 2000; Lagorce *et al.* 2002; Bulik *et al.* 2003). Elevation of glucosamine levels by other means also elicits increased chitin synthesis, for chitin levels are correlated with levels of expression of *GFA1* itself (Lagorce *et al.* 2002; Bulik *et al.* 2003), and exogenous glucosamine also leads to increased chitin synthesis (Bulik *et al.* 2003). However, Bulik *et al.* (2003) found that chitin formation was not proportional to UDP-GlcNAc concentration. These observations led to the conclusion that chitin synthesis is proportional to Gfa1 activity but that additional factors, for example a glucosamine metabolite or Gfa1 itself, must modulate chitin levels (Bulik *et al.* 2003). It is also formally possible that additional chitin is in a soluble or intracellular form and not detected in cell wall analyses.

Dolichol and dolichol phosphate sugars

Dolichol phosphate synthesis:

Rer2 and Srt1. Biosynthesis of dolichol starts with the extension of *trans* farnesyl-PP by successive addition of *cis*-isoprene units by the homologous cis-prenyltransferases Rer2 and Srt1 (Sato *et al.* 1999; Schenk *et al.* 2001b). Rer2 is the dominant activity and makes dolichols with 10-14 isoprene units, whereas dolichols made by Srt1 in cells lacking Rer2 contain 19-22 isoprenes, like mammals. *rer2Δ* strains have severe defects in growth and in N- and O-glycosylation, and *SRT1* is a high-copy suppressor of *rer2* mutants (Sato *et al.* 1999). The *rer2Δ srt1Δ* double null is inviable (Sato *et al.* 1999). Rer2 and Srt1 both behave as peripheral membrane proteins (Sato *et al.* 2001; Schenk *et al.* 2001b), but Rer2 is localized to the ER membrane, whereas Srt1 is detected in "lipid particles" (Sato *et al.* 2001).

Dfg10. Dfg10 has a steroid 5α reductase domain, and is responsible for much of the activity that reduces the α-isoprene unit of polyprenol activity. Both *dfg10-100* transposon insertion mutants and *dfg10Δ* strains underglycosylate carboxypeptidase Y to the same extent, and dolichol levels are decreased by 70% in *dfg10-100* cells, with a corresponding increase in unsaturated polyprenol (Cantagrel *et al.* 2010). The biosynthetic origin of the residual dolichol is not known.

Membrane organization of Sec59 dolichol kinase. Sec59 is a multispanning membrane protein whose CTP-binding site is oriented towards the cytoplasm (Shridas and Waechter, 2006).

Dolichol chain length specificity of yeast glycosyltransferases and flippases. The enzymes that act after Rer2 and Srt1 can use shorter chain dolichols. Thus, the growth and glycosylation defects of *rer2Δ* cells can be complemented by expression of the *E. coli cis*-isoprenyltransferase, which generates C_{55} isoprenoids, or of the *Giardia* homologue, which makes C_{55-60} (Rush *et al.* 2010; Grabinska *et al.* 2010). The native glycosyltransferases and flippases must therefore also be able to use shorter chain dolichols as substrates.

Dol-P-Man and Dol-P-Glc synthesis:

Relationship between Dpm1 and Alg5. Alg5 and Dpm1 are most similar in their N-terminal halves, which contain their GT-A superfamily domain, but diverge in their C-terminal halves. Both are likely to catalyze their reactions at the cytoplasmic face of the ER membrane.

Literature Cited

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File S2

N-glycosylation

This Supporting File contains additional information related to ***Biosynthesis of Wall Components Along the Secretory Pathway, N-glycosylation****. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.*

Assembly and transfer of the Dol-PP-linked precursor oligosaccharide:

Steps on the cytoplasmic face of the ER membrane:

Alg7. The Alg7 GlcNAc-1-P transferase, which carries out the first step in the assembly of the Dol-PP-linked precursor is highly conserved among eukaryotes and has homologues in Bacteria, for example MraY, which catalyzes transfers N-acetylmuramic acid-pentapeptide from UDP to undecaprenol phosphate in peptidoglycan biosynthesis (Price and Momany, 2005). GlcNAc-1-P transferases such as Alg7 and MraY have multiple transmembrane domains and amino acid residues important for catalysis by members of this protein family lie in cytoplasmic loops (Dan and Lehrman; Price and Momany, 2005).

Alg13/Alg14. These proteins function as a heterodimer to transfer the second, β1,4-GlcNAc-linked GlcNAc to Dol-PP-GlcNAc (Bickel *et al.* 2005; Chantret *et al.* 2005; Gao *et al.* 2005). Soluble Alg13, assigned to GT Family 1, is the catalytic subunit and associates with membrane-spanning Alg14 at the cytosolic face of the ER membranes (Averbeck *et al.* 2007; Gao *et al.* 2008). Alg13 and 14 are homologous to C and N-terminal domains, respectively, of the bacterial MurG polypeptide, which adds N-acetylmuramic acid to undecaprenol-PP-GlcNAc in peptidoglycan synthesis (Chantret *et al.* 2005).

Alg1. This β1,4-Man-T, assigned to GT Family 33, transfers the first mannose from GDP-Man to Dol-PP-$GlcNAc_2$ (Couto *et al.* 1984).

Alg2. This protein is a member of GT Family 4. Remarkably, Alg2 has both GDP-Man: Dol-PP-$GlcNAc_2Man$ α1,3-Man-T and GDP-Man: Dol-PP-$GlcNAc_2Man_2$ α1,6-Man-T activity and successively adds an α1,3-Man and an α1,6 Man to the Dol-PP-linked precursor (O'Reilly *et al.* 2006; Kämpf *et al.* 2009).

Alg11. Alg11, also a member of GT Family 4, adds the next two α1,2-linked mannoses (Cipollo *et al.* 2001; O'Reilly *et al.* 2006; Absmanner *et al.* 2010). *alg11D* mutants are viable though growth-defective, and accumulate Dol-PP-$GlcNAc_2Man_3$, as well as some Dol-PP-$GlcNAc_2Man_{6-7}$ (Cipollo *et al.* 2001; Helenius *et al.* 2002). The latter are aberrant glycan structures formed when Dol-PP-$GlcNAc_2Man_3$ is translocated to the lumen and acted on by lumenal Man-T.

Heterologous expression and membrane topology of Alg1, Alg2, and Alg11. Alg1, Alg2, and Alg11 are catalytically active when expressed in *E. coli* (Couto *et al.* 1984; O'Reilly *et al.* 2006). The catalytic region of Alg1 is predicted to be cytoplasmic, and experimentally derived models for the membrane topology of Alg2 and Alg11 also place catalytic domains at the cytoplasmic side of the ER membrane (Kämpf *et al.* 2006; Absmanner *et al.* 2009), although not all predicted hydrophobic helices in Alg2 and Alg11 span the ER membrane, rather, they lie in its cytoplasmic face.

Complex formation by early-acting Alg proteins. There is evidence from analyses by coimmunoprecipitation and size exclusion chromatographic analyses for higher order organization of the proteins involved in the cytoplasmic steps of the yeast dolichol pathway. Alg7, 13, and 14 associate in a hexamer (Noffz *et al.* 2009). Alg1 forms separate complexes containing either Alg2 and Alg11, although the latter two do not interact with one another (Gao *et al.* 2004). Formation of these multienzyme complexes may in turn facilitate channeling of Dol-PP-linked intermediates to successive membrane-associated transferases.

Transmembrane translocation of Dol-PP-oligosaccharides:

After Dol-PP-$GlcNAc_2Man_5$ is generated on the cytoplasmic face of the ER membrane, it is somehow translocated to the lumenal side of the membrane where subsequent sugars are transferred from Dol-P-sugars (Burda and Aebi, 1999; Helenius & Aebi, 2002). The presumed Dol-PP-oligosaccharide flippase likely prefers the heptasaccharide as substrate, but the presence of shorter oligosaccharides on proteins in both the *alg2*-Ts and *alg11Δ* mutants (Jackson *et al.* 1989; Cippolo *et al.* 2001) indicates that truncated oligosaccharides can be translocated as well.

The Rft1 protein is a candidate for the protein Dol-PP-$GlcNAc_2Man_5$ flippase (Helenius *et al.* 2002). Strains deficient in Rft1 accumulate Dol-PP-$GlcNAc_2Man_5$, but are unaffected in O-mannosylation or in GPI anchor assembly, ruling out a deficiency in Dol-P-Man supply to the ER lumen. Because the few N-glycans chains that were still transferred to the reporter protein carboxypeptidase Y in Rft1-depleted cells were endoglycosidase H sensitive, the activity of Alg3, which adds the α1,3-Man required for substrate recognition by endoglycosidase H, was unaffected. Moreover, high level expression of *RFT1* partially suppresses the growth defect of *alg11Δ* and leads to increased levels of lumenal Dol-PP-$GlcNAc_2Man_{6\text{-}7}$ and an increase in carboxypeptidase Y glycosylation, consistent with the notion of enhanced flipping of the suboptimal flippase substrate Dol-PP-$GlcNAc_2Man_3$ (Helenius *et al.* 2002).

However, although the above findings are consistent with Rft1 being the flippase itself, this role could not be demonstrated in biochemical assays for flippase activity, for sealed microsomal vesicles or proteoliposomes depleted of Rft1 retained flippase activity, and in fractionation experiments, flippase activity could be separated from Rft1 (Franck *et al.* 2008; Rush *et al.* 2009).

Lumenal steps in Dol-PP-oligosaccharide assembly:

Alg3. This α1,3-Man-T is a member of GT Family 58, and transfers the precursor's sixth, α1,3-Man from Dol-P-Man, making the glycan sensitive to endoglycosidase H (Aebi *et al.* 1996; Sharma *et al.* 2001). Alg3's Dol-P-Man:Dol-PP-$GlcNAc_2Man_5$ Man-T activity can be selectively immunoprecipitated from detergent extracts of membranes (Sharma *et al.* 2001), providing strong evidence that Alg3 and its yeast homologues in the dolichol and GPI assembly pathways are indeed glycosyltransferases.

Alg9 and Alg12. Alg9, a member of GT Family 22, transfers the seventh, α1,2-linked Man to the α1,3-Man added by Alg3 (Burda *et al.* 1999; Cipollo and Trimble, 2000). Alg12, also a GT22 Family member, next adds the eighth, α1,6-Man to the α1,2-linked Man just added by Alg9 (Burda *et al.* 1999), whereupon Alg9 acts again to add the ninth Man, in α1,2 linkage, to the α1,6-Man added by Alg12 (Frank and Aebi 2005). The second activity of Alg9 was uncovered in *in vitro* assays in which *alg9Δ* and *alg12Δ* membranes were tested for their ability to elongate acceptor Dol-PP-$GlcNAc_2Man_7$ isolated from *alg12Δ* cells. These experiments established that Alg12 requires prior addition of the seventh Man by Alg9, even though Alg12 does not transfer its Man to that residue, and that the Alg12 reaction precedes Alg9's second α1,2 mannosyltransfer (Frank and Aebi 2005).

Alg6, Alg8, and Alg10. Alg6 and Alg8, members of GT Family 57, act successively to transfer two α1,3-linked glucoses to extend the second α1,2-Man added by Alg11, and lastly, Alg10, assigned to GT Family 59, completes the 14-sugar Dol-PP-linked oligosaccharide by adding a third, α1,2-Glc (Reiss *et al.*, 1996; Stagljar *et al.*, 1994; Burda and Aebi, 1998).

Shared transmembrane topology of Dol-P-sugar-utilizing transferases. The six Dol-P-sugar-utilizing transferases are members of a larger protein family that includes the Dol-P-Man-utilizing Man-T involved in GPI anchor biosynthesis (Oriol *et al.* 2002). The results of *in silico* analyses of the sequences of these proteins suggested they have a common membrane topology and 12 transmembrane segments, and a membrane organization recalling that of membrane transporters, which is consistent with the idea that each protein translocates its own Dol-P-linked sugar substrate (Burda and Aebi, 1999; Helenius and Aebi, 2002). It also plausible that these transferases operate in multienzyme complexes to facilitate substrate channeling.

Oligosaccharide transfer to protein:

Truncated oligosaccharides can be transferred to protein. The results of analyses of the N-linked glycans present on protein in mutants defective in the assembly of the Dol-PP-linked precursor oligosaccharide indicate that a range of structures smaller than $GlcNAc_2Man_9Glc_3$ can be transferred *in vivo*. However, full-size Dol-PP-$GlcNAc_2Man_9Glc_3$ is the preferred OST substrate *in vitro*, and the observation that mutants that make smaller precursor oligosaccharides have a synthetic phenotype

with OST mutants indicates the preference exists *in vivo* as well (Knauer and Lehle, 1999; Zufferey *et al.* 1995; Reiss *et al.* 1997; Karaoglu *et al.* 2001). This preference does not reflect differences between the binding affinities of Dol-PP-$GlcNAc_2Man_9Glc_3$ and smaller oligosaccharides at the OST active site, rather, it has been proposed that OST has an allosteric site that binds $GlcNAc_2Man_9Glc_3$ as well as smaller oligosaccharides, in turn activating the catalytic site for $GlcNAc_2Man_9Glc_3$ and acceptor peptide binding. Binding of a truncated oligosaccharide at the allosteric site, however, enhances $GlcNAc_2Man_9Glc_3$ binding more strongly, and so ensures preferential utilization of the full-size precursor (Karaoglu *et al.*, 2001; Kelleher and Gilmore, 2006).

Purification and protein-protein interactions of OST. Complete heterooctomeric OST complexes have been affinity purified (Karaoglu *et al.* 1997; Spirig *et al.* 1997; Karaoglu *et al.* 2001; Chavan *et al.* 2006), and the subunits appear to be present in stoichiometric amounts (Karaoglu *et al.* 1997). The OST complexes themselves may themselves function as dimers (Chavan *et al.* 2006). The results of genetic interaction studies and coimmunoprecipitation- and chemical cross-linking experiments suggest the existence of three sub-complexes i) Swp1-Wbp1-Ost2, ii) Stt3-Ost4-Ost3, and iii) Ost1-Ost5 (Spirig *et al.* 1997; Karaoglu *et al.* 1997; Reiss *et al.* 1997; Li *et al.* 2003; Kim *et al.* 2003; reviewed by Knauer and Lehle, 1999; Kelleher and Gilmore, 2006). It has been noted, however, that treatment of OST with non-ionic detergents does not yield these three subcomplexes (Kelleher and Gilmore, 2006). Furthermore, additional interactions between OST subunits have been detected using chemical cross-linking approaches and membrane protein two-hybrid analyses (Yan *et al.* 2003, 2005). OST also interacts with the Sec61 translocon complex and large ribosomal subunit (Chavan *et al.* 2005; Harada *et al.* 2009), suggesting that the complex is poised to act on nascent, freshly translocated proteins. However, protein O-mannosyltransferases can compete for the hydroxyamino acids in a freshly translocated sequon (Ecker *et al.* 2003; see *O-mannosylation*).

Stt3 is the catalytic subunit of OST. There is strong evidence that Stt3, which has a soluble, lumenal domain towards its C-terminus preceded by 11 transmembrane domains (Kim *et al.* 2005), is the catalytic subunit of OST. First, it can be crosslinked to peptides derivatized with a photoactivatable group and containing an N-X-T glycosylation site, or to nascent polypeptide chains containing the sequon-mimicking, cryptic glycosylation site Q-X-T and a photoactivable side chain (Yan and Lennarz, 2002; Nilson *et al.* 2003). Second, Stt3 homologues are present in all eukarya, as well as in certain Bacteria and many Archaea, in which diverse types of glycan are transferred to protein (Kelleher and Gilmore, 2006; Kelleher *et al.* 2007). The Stt3 homologue from *Campylobacter jejuni*, PglB, was shown to be required for transfer of that bacterium's characteristic glycan to Asn in a substrate peptide when the *C. jejuni pgl* gene cluster was heterologously expressed in *E. coli* (Wicker *et al.* 2002). Third, Stt3 homologues from the protist *Leishmania major*, whose proteome contains no other OST subunits, complement the *S.*

cerevisiae stt3Δ mutants as well as null mutations in the genes for the essential OST subunits Ost1, Ost2, Swp1, and Wbp1, indicating that the protist Stt3 functions autonomously as an OST (Nasab *et al.* 2008; Hese *et al.* 2009). Stt3 has been assigned to GT Family 66.

Ost3 and Ost6: role of a thioredoxin domain. The other OST subunits for which catalytic activity has been demonstrated are the paralogues Ost3 and Ost6. *ost3Δ ost6Δ* double mutants have a more severe glycosylation defect than the single nulls (Knauer and Lehle, 1999b). The two proteins confer a degree of acceptor preference to the OST complexes that contain them (Schulz and Aebi, 2009) because they each have peptide binding grooves lined by amino acids whose side chains are complementary in hydrophobicity and charge to different substrate peptides (Jamaluddin *et al.* 2011). Ost3 and Ost6 are predicted to have four transmembrane domains at their C-termini and an N-terminal domain containing a thioredoxin fold with the CXXC motif common to proteins involved in disulfide bond shuffling during oxidative protein folding (Kelleher and Gilmore, 2006; Schulz *et al.* 2009). This domain most likely lies in the lumen (Kelleher and Gilmore, 2006). Mutations of the cysteines in the CXXC motifs of Ost3 and Ost6 lead to site-specific underglycosylation, indicating the importance of the thioreductase motif. This was confirmed by the demonstration that the thioredoxin domain of Ost6, expressed in *E. coli*, had oxidoreductase activity towards a peptide substrate (Schulz *et al.* 2009). These findings led to a model in which Ost3/Ost6 form transient disulfide bonds with nascent proteins and promote efficient glycosylation of more Asn-X-Ser/Thr sites by delaying oxidative protein folding (Schulz *et al.* 2009). Structural analyses of the thioredoxin domain of Ost6 showed that the peptide binding groove is present only when the CXXC motif is oxidized (Jamaluddin *et al.* 2011).

Recruitment of Ost3 or Ost6 to OST requires Ost4, a hydrophobic 36 amino protein (Kim *et al.* 2000, 2003; Spirig *et al.* 2005). Ost4 also interacts with Stt3 (Karaoglu *et al.* 1997; Spirig *et al.* 1997; Knauer and Lehle, 1999; Kim *et al.* 2003). *ost4Δ* strains are temperature-sensitive and severely underglycosylate protein (Chi *et al.* 1996).

Possible roles for other OST subunits. A sub-complex of Swp1p, Wbp1p, and Ost2p, has been suggested to confer the preference for $GlcNAc_2Man_9Glc_3$, possibly by providing the allosteric site (Kelleher and Gilmore, 2006). Evidence for a role of complex subunits other than Stt3 was obtained with *Trypanosoma cruzi* Stt3, which transfers $GlcNAc_2Man_{7\text{-}9}$ to protein *in vitro* as efficiently as it does glucosylated oligosaccharides. When expressed in *S. cerevisiae* in place of native Stt3, trypanosomal Stt3 now preferentially transferred $GlcNAc_2Man_9Glc_3$ to protein *in vitro* and *in vivo* (Castro *et al.* 2006). Similarly, when *Leishmania* Stt3 is expressed in the context of the other *S. cerevisiae* OST subunits, the *Leishmania* protein acquires a preference for transferring glucosylated oligosaccharides, rather than the non-glucosylated oligosaccharides that it transfers in the protist itself (Hese *et al.* 2009). Wbp1 may be involved in recognition of Dol-PP-$GlcNAc_2Man_9Glc_3$, because alkylation of a key cysteine

residue in this subunit inactivates OST, whereas inactivation is prevented by prior incubation with Dol-PP-$GlcNAc_2$ (Pathak *et al.* 1995). The protein's single transmembrane domain contains sequences important for incorporation into the OST complex, possibly by making interactions with Ost2 and Swp1 (Li *et al.* 2003).

Other than their membership in proposed OST subcomplexes and interactions with other OST subunits, little is known about the function of Swp1, Ost1, Ost2, and Ost5, although it has been suggested that Ost1 has a role in funneling nascent polypeptides to Stt3 (Lennarz, 2007).

Regulation of OST by the CWI pathway. Oligosaccharyltransferase may be regulated by the PKC-dependent CWI pathway or by Pkc1 itself, a notion that arose from the identification of *STT3* in a screen for mutants sensitive to the PKC inhibitor staurosporine and to elevated temperature (Yoshida *et al.* 1995). Although this suggested that adequate levels of N-glycosylation are needed for cells to overcome defects in CWI signaling, staurosporine sensitivity proved not to be a general consequence of deficient N-glycosylation, because only a subset of *stt3* alleles were sensitive to the drug, and mutants in most other OST subunits, with the exception of Ost4, were resistant (Chavan *et al.* 2003; Levin, 2005). A more direct link between Stt3 and the Pkc1-dependent signaling emerged from the findings that *STT3* mutations that lead to staurosporine sensitivity are located in N-terminal, predicted cytosolic domains of Stt3, and that *pkc1Δ* mutants have half of wild type OST activity *in vitro* (Chavan *et al.* 2003; Park and Lennarz, 2000). This led to the suggestion that CWI pathway regulates OST via an interaction between Pkc1 or components of the PKC pathway with the N-terminal domain of Stt3, and perhaps Stt3-interacting Ost4 as well (Chavan *et al.* 2003).

N-glycan processing in the ER and glycoprotein quality control:

Glucosidase II. This is a heterodimer of catalytic Gls2/Rot2 and Gtb1, the latter of which is necessary for, and influences the rate of, Glc trimming (Trombetta *et al.* 1996; Wilkinson *et al.*, 2006; Quinn *et al.* 2009).

Glycoprotein recognition by Pdi1 and the Pdi1-Htm1 complex. Unfolded or misfolded proteins are bound by protein disulfide isomerase Pdi1, a subset of which is in complex with Mns1 homolog Htm1. A stochastic model has been proposed in which both Pdi1 and the Pdi1-Htm1 complex recognize un- or misfolded proteins, but persistently misfolded proteins stand an increased chance of encountering Pdi1-Htm1 whose Htm1 component trims a Man from N-linked glycans, yielding a $GlcNAc_2Man_7$ structure bearing a terminal α1,6 Man (Clerc *et al.* 2009; Gauss *et al.* 2011).

Mannan elaboration in the Golgi:

Formation of core type N-glycan and mannan outer chains:

Elucidation of the pathway for formation of mannan outer chains. Two groups of proteins, the Mnn9/Anp1/Van1 trio, and the Mnn10 and Mnn11 pair, had been implicated in formation of the poly-α1,6-linked mannan backbone, but because strains deficient in these proteins retained mannosyltransferase activity and still made mannan containing α1,6 linkages, these proteins were considered more likely to affect mannan formation indirectly (reviewed by Orlean, 1997; Dean, 1999). Two key sets of findings led to clarification of mannan biosynthesis. First, co-immunoprecipitation and colocalization experiments established that Mnn9, Anp1, and Van1 occurred in two different protein complexes in the *cis*-Golgi, one containing Mnn9 and Van1 (subsequently named M-Pol I), the other, Mnn9, Anp1, Hoc1 (homologous to Och1), and the related Mnn10 and Mnn11 proteins (M-Pol II) (Hashimoto and Yoda, 1997; Jungmann and Munro, 1998; Jungmann *et al.* 1999). Second, both immunoprecipitated protein complexes had α1,6 mannosyltransferase activity, indicating that one or more of the Mnn9/Anp1/ Van1 group was an α1,6 mannosyltransferase (Jungmann and Munro, 1998; Jungmann *et al.* 1999). Consistent with their being glycosyltransferases, all five proteins have the GT-A fold protein topology and a "DXD motif" common to enzymes that have sugar nucleotides as donors and use the aspartyl carboxylates to coordinate divalent cations and the ribose of the donor (Wiggins and Munro, 1998; Lairson *et al.* 2008).

The contributions of the individual subunits to α1,6 mannan synthesis by each complex, and the roles of the two complexes in mannan formation, were explored in deletion mutants and in point mutants abolishing catalytic activity but otherwise preserving complex stability. The sizes of the mannans and the residual *in vitro* activities of the M-Pol complexes in these mutants led to the current model for mannan synthesis (Jungmann *et al.* 1999; Munro, 2001; **Figure 3** in main text). In it, M-Pol I, a heterodimer, acts first to extend the Och1-derived Man with further α1,6-linked mannoses. Analyses of mutants in the DXD motifs of Mnn9 and Van1 indicated that Mnn9 likely adds the first α1,6-liked Man, which is extended with 10-15 α1,6 mannoses in Van1-requiring reactions (Stolz and Munro, 2002; Rodionov *et al.* 2009). This α1,6 backbone is then elongated with 40-60 α1,6 Man by M-Pol II. Assays of M-Pol ll from strains lacking Mnn10 or Mnn11 indicated that these proteins are responsible for the majority of the α1,6 mannosyltransferase activity in that complex (Jungmann *et al.*, 1999). The contribution of Hoc1, a homologue of the Och1 α1,6-Man-T is not clear, for *HOC1* deletion neither alters M-Pol II activity nor impacts mannan size.

Localization of Och1 and Man-Pol complexes. The localization dynamics of Mnn9-containing M-Pol complexes and Och1 seem inconsistent with the order in which they act in mannan assembly, with Mnn9 showing a steady state localization in the *cis*-Golgi and continuously cycling between that compartment and the ER, but with Och1 cycling between the ER and *cis*- and *trans*-Golgi (Harris and Waters, 1996; Todorow *et al.* 2000; Karhinen and Makarow, 2004). It has been suggested that

substrate specificity, rather than transferase localization, determines their order in which the enzymes act (Okamoto *et al.* 2008). The size of N-linked mannan can be impacted by deficiencies in proteins required for localization of Golgi mannosyltransferases. For example, deletion of *VPS74*, also identified as *MNN3*, eliminates a protein that interacts with the cytoplasmic tails of certain transferases normally resident in the *cis* and *medial* Golgi compartments. The resulting mislocalization of several mannosyltransferases would explain the underglycosylation phenotype of *mnn3* mutants (Schmitz *et al.* 2008; Corbacho *et al.* 2010). Mutations in *SEC20*, which encodes a protein involved in Golgi to ER retrograde transport, also result in diminished Golgi mannosyltransferase activity, even though this glycosylation defect is not correlated with the secretory pathway defect (Schleip *et al.* 2001). The reason for this is not clear.

Mannan side branching and mannose phosphate addition:

Roles of the Ktr1 Man-T family members in mannan side branching. Five members of the Ktr1 family of Type II membrane proteins, Kre2/Mnt1, Yur1, Ktr1, Ktr2, Ktr3, also contribute to N-linked outer chain synthesis, as judged by the impact of null mutations on the mobility of reporter proteins (Lussier *et al.* 1996; 1997a; 1999). Of these proteins, Kre2/Mnt1, Ktr1, Ktr2, and Yur1 have been shown to have α1,2 Man-T activity. These Ktr1 family members, perhaps along with uncharacterized homologues Ktr4, Ktr5, and Ktr7 (Lussier *et al.* 1999) have a collective role in adding the second, and perhaps subsequent α1,2-mannoses to mannan side branches. Members of the Ktr1 family have been assigned to GT Family 15.

Addition and function of mannose phosphate. Both core type N-glycans and mannan can be modified with mannose phosphate on α1,2-linked mannoses in the context of an oligosaccharide containing at least one α1,2-linked mannobiose structure. Mannose phosphates confer a negative charge, an attribute exploited early on to isolate mannan synthesis mutants on the basis of their inability to bind the cationic dye Alcian Blue (Ballou, 1982; 1990). Mnn6/Ktr6, a member of the Ktr1 family, is the major activity responsible for transferring Man-1-P from GDP-Man to both mannan outer chains and, *in vitro*, to core N-glycans, generating GMP. However, because deletion of *MNN6* did not eliminate *in vivo* mannose phosphorylation in *och1Δ* strains that make only core type N-glycans, additional, as yet unidentified, core phosphorylating proteins must exist (Wang *et al.* 1997; Jigami and Odani, 1999). The Mnn4 protein is also involved in Man-P addition, but its role differs from Mnn6's in that deletion of Mnn4 reduces Man-P on core-type glycans (Odani *et al.* 1996). Mnn4 does not resemble glycosyltransferases, but does have a LicD domain found in nucleotidyltransferases and phosphotransferases involved in lipopolysaccharide synthesis. The *mnn4Δ* mutation is dominant, and Mnn4 has been proposed to have a positive regulatory role (Jigami and Odani, 1999). Levels of mannan phosphorylation are highest in the late log and stationary phases, when *MNN4* expression is elevated (Odani *et al.* 1997). Transcriptional regulation may involve the RSC chromatin remodeling complex because strains lacking Rcs14, a

subunit of that complex, show drastically reduced Alcian Blue binding and down-regulated expression of *MNN4* and *MNN6* (Conde *et al.* 2007).

A Golgi GlcNAc-T. *S. cerevisiae* also has the capacity to add GlcNAc to the non-reducing end of N-linked glycans. Heterologously expressed lysozyme received a $GlcNAc_2Man_{8\text{-}12}$ glycan additionally bearing a GlcNAc residue, and the responsible GlcNAc transferase proved to be Gnt1, whose localization mostly coincides with that of Mnn1 in the medial Golgi (Yoko-o *et al.* 2003). *GNT1* disruptants have no discernible phenotype, and Gnt1 may rarely act on native yeast glycans; its activity would require that UDP-GlcNAc be transported into the Golgi lumen (Yoko-o *et al.* 2003).

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File S3

O-Mannosylation

This Supporting File contains additional information related to **Biosynthesis of Wall Components Along the Secretory Pathway, *O-mannosylation***. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Protein O-mannosyltransferases in the ER:

Substrate proteins for different Pmt complexes. Analyses of glycosylation of individual proteins in *pmtΔ* strains showed that Pmt1/Pmt2 complexes are primarily involved in O-mannosylation of Aga2, Bar1, Cts1, Kre9, and Pir2, whereas homodimeric Pmt4 modifies Axl2, Fus1, Gas1, Kex2 (Gentzsch and Tanner 1997; Ecker *et al.* 2003; Proszynski *et al.* 2004; Sanders *et al.* 1999). However, some proteins, including Mid2, the WSC proteins, and Ccw5, are modified by both complexes, although the Pmt1/Pmt2 and Pmt4/Pmt4 dimers modify different domains of these target proteins (Ecker *et al.* 2003; Lommel *et al.* 2004).

Mutations in substrate proteins can cause them to be O-mannosylated by a different PMT, and PMTs can also have a role in quality control of protein folding in the ER (see *N-glycan processing in the ER and glycoprotein quality control*). Thus, wild type Gas1 is normally O-mannosylated by Pmt4, whereas $Gas1^{G291R}$, a model misfolded protein, is hypermannosylated by Pmt1-Pmt2 as well as targeted to the HRD-ubiquitin ligase complex for degradation by the ERAD system (Hirayama *et al.* 2008; Goder and Melero, 2011). The latter, chaperone-like function of Pmt1-Pmt2 may be distinct from Pmt1-Pmt2's O-mannosyltransferase activity (Goder and Melero, 2011).

Extension and phosphorylation of O-linked manno-oligosaccharide chains:

Extension with α-linked mannoses. The Ser- or Thr-linked Man is extended with up to four α-linked Man that are added by GDP-Man-dependent Man-T of the Ktr1 and Mnn1 families (Lussier *et al.* 1999; **Figure 4** in main text). The contributions of these proteins was deduced from the sizes of the O-linked chains that accumulated in strains in which Man-T genes had been deleted singly or in different combinations. Transfer of the first two α1,2-Man is carried out by Ktr1 sub-family members Ktr1, Ktr3, and Kre2, which have overlapping roles in the process, although Kre2 has the dominant role in addition of the second, α1,2-Man (Lussier *et al.* 1997a). The major O-linked glycan made in the *ktr1Δ ktr3Δ kre2Δ* triple mutant consists of a single Man (Lussier *et al.* 1997a). Ktr1, Ktr3, and Kre2 are also involved in making α1,2-branches to mannan outer chains (see *Mannan elaboration in the Golgi*).

Extension of the trisaccharide chain with one or two α1,3-linked Man is the shared responsibility of Mnn1 family members Mnn1, Mnt2, and Mnt3, with Mnn1 having the major role in adding the fourth Man but Mnt2 and Mnt3 dominating when the fifth is added (Romero *et al.* 1999). Mnn1 also transfers Man to N-linked outer chains. The α1,2 Man-T have been localized to the medial Golgi, and the Mnn1 α1,3 Man-T to the medial and trans-Golgi (Graham *et al.* 1994). Because protein-bound O-mannosyl glycans pulse-labeled in mutants defective in ER to Golgi transport such as *sec12*, *sec18*, and *sec20* contain two, sometimes more mannoses, GDP-Man-dependent O-glycan extension can occur at the level of the ER (Haselbeck and Tanner, 1983; Zueco *et al.* 1986; D'Alessio *et al.* 2005). The process is independent of nucleotide sugar diphosphatases (see *Sugar nucleotide transport*; D'Alessio *et al.* 2005), but presumably mediated in the ER by Man-T *en route* to the Golgi.

Importance and function of O-mannosyl glycans:

Importance of O-mannosylation for function of specific proteins. Analyses of single and conditionally lethal double *pmt* mutants show that O-mannosylation can be important for function of individual O-mannosylated proteins. For example, *pmt4Δ* haploids show a unipolar, rather than the normal axial budding pattern, which is due to defective O-mannosylation and resulting instability and mislocalization of Axl2, which normally marks the axial budding site (Sanders *et al.* 1999). Pmt4-initiated O-mannosylation is also necessary for cell surface delivery of Fus1, because the unglycosylated protein accumulates in the late Golgi (Proszynski *et al.* 2004). Defects in Pmt4-dependent O-glycosylation of Msb2 (as well as N-glycosyation) of osmosensor Msb2 lead to activation of the filamentous growth signaling pathway (Yang *et al.* 2009). In this case, underglycosylation may unmask a domain that normally is exposed and makes interactions when the signaling pathway is activated legitimately. O-mannosylation of Wsc1, Wsc2, and Mid2 is necessary for these Type I membrane proteins to fulfill their functions as sensors that activate the CWI pathway. Underglycosylation of the CWI pathway-triggering mechanosensor Wsc1 in a *pmt4Δ* mutant eliminates the stiffness of this rod-like glycoprotein and abolishes its "nanospring" properties, impairing Wsc1's function as a mechanosensor (Dupres *et al.* 2009). Further, in *pmt2Δ pmt4Δ* mutants, which, like CWI pathway mutants, require osmotic stabilization, deficient O-mannosylation results in incorrect proteolytic processing and instability of the sensors (Philip and Levin, 2001; Lommel *et al.* 2004).

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File S4

GPI anchoring

This Supporting File contains additional information related to **Biosynthesis of Wall Components Along the Secretory Pathway, *GPI anchoring***. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Assembly of the GPI precursor and its attachment to protein in the ER:

Steps on the cytoplasmic face of ER membrane:

Gpi3. Gpi3 is a member of GT Family 4 and has an EX_7E motif conserved in a range of glycosyltransferases (Coutinho *et al*. 2003). Mutational analyses indicate that the glutamates are be important for function of Gpi3 and certain EX_7E motif glycosyltransferases, although the comparative importance of the two glutamates varies between different transferases (Kostova *et al*. 2003). However, in the case of Alg2, the EX_7E motif is not important for protein function (Kämpf et al. 2009).

Formation of GlcNAc-PI by GPI-GnT. The acyl chains of the PI species that receive are the same length as those in other membrane phospholipids (Sipos *et al*. 1997). Evidence that GlcNAc transfer occurs at the cytoplasmic face of the ER membrane is that i) the catalytic domain of Gpi3's human orthologue faces the cytoplasm (Watanabe *et al*. 1996; Tiede *et al*. 2000), and ii) GlcNAc-PI can be labeled with membrane topological probes on the cytoplasmic side of the mammalian ER membrane (Vidugiriene and Menon, 1993).

Significance of Ras2 regulation of GPI-GnT. A clue to the significance of Ras2 regulation of GPI-GnT came from the observation that conditional mutants in GPI-GnT subunits show the phenotype of hyperactive Ras mutants, filamentous growth and invasion of agar. This led to the suggestion that Ras2-mediated modulation of GPI synthesis may be involved in the cell wall and morphogenetic changes that occur in the dimorphic transition to filamentous growth (Sobering *et al*. 2003; 2004).

Location of GlcNAc-PI de-N-acetylation. The de-acetylase reaction likely occurs at the cytoplasmic face of the ER membrane, because the bulk of Gpi12's mammalian orthologue is cytoplasmic, and because newly synthesized GlcN-PI is accessible on the cytoplasmic face of intact ER vesicles (Vidugiriene and Menon, 1993).

Transmembrane translocation of GlcN-PI. GlcN-PI is the precursor species most likely to be translocated to the lumenal side of the ER membrane. Flipping of GlcN-PI as well as GlcNAc-PI has been reconstituted in rat liver microsomes, but the protein involved has not been identified, and the possibility has been raised that GlcN-PI translocation may be mediated by a generic ER phospholipid flippase (Vishwakarma and Menon, 2006).

Lumenal steps in GPI assembly:

Inositol acylation. The acyl chain transferred to GlcN-(acyl)PI *in vivo* is likely palmitate, although a range of different acyl chains can be transferred from their corresponding CoA derivatives *in vitro* (Costello and Orlean, 1992; Franzot and Doering, 1999). Because mutants blocked in formation of all mannosylated GPIs accumulated inositol-acylated GlcN-PI (Orlean, 1990; Costello and Orlean, 1992), and because mannosylated GPI intermediates lacking an inositol acyl chain have not been reported, it is likely that inositol acylation precedes mannosylation *in vivo*. Gwt1, the acyltransferase, is likely to be catalytic because its affinity-purified mammalian orthologue transfers palmitate from palmitoyl CoA to a dioctanoyl analogue of GlcN-PI (Murakami *et al*. 2003). The protein has 13 transmembrane domains (Murakami *et al*. 2003; Sagane *et al*. 2011), and amino acid residues critical for function all face the lumen, indicating acyl transfer is a lumenal event (Sagane *et al*. 2011), although it is not yet known how acyl CoAs enter the ER lumen. Despite Gwt1's multispanning topology, the possibility that this inositol acyltransferase is also a GlcN-PI transporter is unlikely, because non-acylated, mannosylated GPIs can be formed in cell lines deficient in Gwt1's mammalian orthologue (Murakami *et al*. 2003).

GPI Man-T-I. The α1,4-Man-T Gpi14 shows greatest similarity to Alg3, is predicted to have 12 transmembrane segments (Oriol *et al*. 2002), and is assigned to GT Family 50. Two additional proteins, Arv1 and Pbn1, are involved in the GPI-Man-T-I step along with Gpi14. *arv1Δ* cells grow at 30°C but not at 37°C, and are delayed in ER to Golgi transport of GPI-anchored proteins, and accumulate GlcN-(acyl)PI *in vitro* (though not *in vivo*) (Kajiwara *et al*. 2008). Further, their temperature sensitivity is suppressed by overexpression the genes for most of the subunits of GPI-GnT, suggesting a functional link between *ARV1* and GPI assembly (Kajiwara *et al*. 2008). However, *arv1Δ* cells were not defective in Dol-P-Man synthase activity or in N-glycosylation, nor were mild detergent-treated *arv1Δ* membranes defective in GPI-Man-T-I activity, suggesting that Arv1 is not a Dol-P-Man flippase or directly involved in mannosyltransfer, and leading to the proposal that Arv1 is involved in delivering GlcN-(acyl)PI to GPI-Man-T-I (Kajiwara *et al*. 2008). Essential Pbn1 has been implicated at the GPI-Man-T-I step in yeast because expression of both *GPI14* and *PBN1* is necessary to complement mammalian cell lines defective in Pbn1's mammalian homologue Pig-X, and likewise, co-expression of *PIG-X* and the gene for Gpi14's mammalian homologue, *PIG-M*, partially rescues the lethality of *gpi14Δ* (Ashida *et al*. 2005; Kim *et al*. 2007). Repression of *PBN1* expression leads to accumulation of some of the ER form of the GPI protein Gas1, a phenotype seen in GPI precursor assembly mutants (Subramanian *et al*. 2006). However, it has not been reported whether *pbn1* mutants accumulate the predicted GPI intermediate GlcN-(acyl)PI. Because Pbn1 is also involved in processing a number of non-GPI proteins that pass though the ER to the vacuole, the vacuolar membrane, and the plasma membrane, it must have additional functions in the ER (Subramanian *et al*. 2006).

GPI Man-T-II. Unlike the other Dol-P-Man-utilizing transferases of the GPI assembly and dolichol pathways, the α1,6-Man-T Gpi18 is predicted to have 8 transmembrane domains (Fabre *et al*. 2005; Kang *et al*. 2005). This protein and its orthologues have been assigned to GT Family 76.

GPI Man-T-III and IV. These two α1,2-Man-T, together with their homologues in the dolichol pathway, Alg9 and Alg12, are predicted to have 12 transmembrane domains and are assigned to GT Family 22 (Oriol *et al*. 2002). Overexpression of *GPI10* does not rescue the lethal *smp3Δ* null mutation, and *vice versa*, indicating that the two α1,2-Man-T have very strict acceptor specificities (Grimme *et al*. 2001).

Phosphoethanolamine addition: origin of Etn-P from Ptd-Etn. There is good evidence that the Etn-Ps, at least those on Man-1 and Man3, originate from Ptd-Etn. Yeast mutants unable to make CDP-Etn or CDP-Cho from exogenously supplied Etn, but still capable of making Ptd-Etn by decarboxylation of Ptd-Ser, do not incorporate [^{3}H]Etn into protein-bound GPIs or into a Man_2-GPI precursor that otherwise receives Etn-P on Man-1. However, radioactivity supplied as [^{3}H]Ser is incorporated into the Man_2-GPI after formation and decarboxylation of Ptd-[^{3}H]Ser (Menon and Stevens, 1992; Imhoff *et al*. 2000). The importance of Ptd-Ser decarboxylation for GPI anchoring is underscored by the finding that the combination of a conditional *gpi13* mutation, defective in the EtnP-T-III, with *psd1Δ* and *psd2Δ*, nulls in the two Ptd-Ser decarboxylase genes, are inviable (Toh-e and Oguchi, 2002). Direct transfer of Etn-P from Ptd-Etn to a GPI remains to be demonstrated *in vitro*.

Phosphoethanolamine addition: importance of the alkaline phosphatase domain of Mcd4, Gpi7, and Gpi13. These three proteins all have a large lumenal loop of some 400 amino acids that contains sequences characteristic of the alkaline phosphatase superfamily (Gaynor *et al*. 1999; Benachour *et al*. 1999, Galperin and Jedrzejas, 2001), consistent with involvement in formation or cleavage of a phosphodiester. This domain is important for function, because the $G^{227}E$ substitution that results in temperature-sensitive growth and a conditional block in GPI precursor assembly in the *mcd4-174* mutant (Gaynor *et al*. 1999) lies in one of the two metal-binding sites in alkaline phosphatase family members (Galperin and Jedrzejas, 2001). The metal is commonly zinc, and *in vitro* Etn-P addition from an endogenous donor is zinc dependent (Sevlever *et al*. 2001) and Zn^{2+} suppresses the temperature sensitivity of a *gpi13* allele.

Phosphoethanolamine addition: Man_2-GPI may be Mcd4's preferred substrate. Three sets of findings suggest that Mcd4 may act preferentially on Man_2-GPI: i) treatment of wild type cells with the terpenoid lactone YW3548, which inhibits addition of Etn-P to Man-1, leads to accumulation of Man_2-GPI (Sütterlin *et al*. 1997, 1998), ii) Man_2-GPI is the most abundant of the accumulating GPIs in *mcd4-174*, and iii) Man_2-GPI is the largest GPI formed *in vitro* by *mcd4* membranes (Zhu *et al*. 2006).

Phosphoethanolamine addition: importance of the Etn-P added to Man-1 by Mcd4 and additional possible functions for Mcd4. The finding that *mcd4* mutants accumulate unmodified Man_2-GPI suggests that the presence of Etn-P on Man-1 is important for GPI-Man-T-III to add the third Man. The requirement, though, is not absolute because *mcd4Δ* cells can be partially rescued by overexpression of Gpi10 (Wiedman *et al*. 2007). In addition to enhancing the efficiency of mannosylation by Gpi10, the Etn-P moiety on Man-1 may be important for additional reasons. *mcd4Δ* cells expressing human or trypanosomal Gpi10 orthologues, Man-T known to mannosylate Man_2-GPIs lacking Etn-P on Man-1 efficiently, still grow slowly (Zhu *et al*. 2006; Wiedman *et al*. 2007). Further, *mcd4Δ* cells expressing trypanosomal Gpi10 are retarded in export of GPI-proteins from the ER, unable to remodel their GPI lipid moiety to ceramide, and are defective in selection of axial budding sites (Zhu *et al*. 2006). How the presence of Etn-P on Man-1 influences these processes is not yet known.

Mutations in *MCD4* also impact cellular processes that are not directly connected with GPI biosynthesis. Cells expressing the Mcd4-P^{301}L variant, but not G^{227}E, are defective in the transport of Ptd-Ser to the Golgi and vacuole for decarboxylation, but unaffected in GPI anchoring suggesting an additional role for Mcd4 in transport dependent Ptd-Ser metabolism (Storey *et al*. 2001). Further, yeast overexpressing Mcd4 (as well as Gpi7 and Gpi13) release ATP into the medium, and Golgi vesicles from the Mcd4 overexpressers were enriched in that protein and showed elevated levels of ATP uptake (Zhong *et al*. 2003). It was suggested that Mcd4 normally mediates symport of ATP and Ptd-Etn into the ER lumen, and that overexpression of the protein leads ATP to accumulate in secretory vesicles, which eventually fuse with the plasma membrane (Zhong *et al*. 2003).

Phosphoethanolamine addition to Man-2 and its possible functions. GPI-Etn-P-II consists of catalytic Gpi7 and non-catalytic Gpi11. *Both gpi7Δ* and temperature-sensitive *gpi11Δ* disruptants complemented by the human Gpi11 orthologue *PIG-F* accumulate a Man_4-GPI bearing Etn-P on Man-1 and Man-3 but missing one on Man-2 (Benachour *et al*. 1999; Taron et al. 2000). Because loss of GPI-Etn-P function leads to accumulation of a Man_4-GPI with Etn-Ps on Man-1 and Man-3, GPI-Etn-P-II may normally add Etn-P to Man-2 after GPI-Etn-P-T-III has modified Man-3. However, because Man_3- and Man_4-GPIs with a single Etn-P on Man-2 accumulate in the *smp3* mutants and in temperature-sensitive *gpi11Δ* strains complemented by the human Gpi11 orthologue (Taron *et al*. 2000; Grimme *et al*. 2001), GPI-Etn-P-II has the capacity to act on Etn-P-free GPIs.

Diverse phenotypes of *gpi7Δ* cells indicate that the Etn-P moiety on Man-2 is important for a number of reasons. First, the combination of *gpi7Δ* with the GPI transamidase mutation *gpi8* leads to a synthetic growth defect, indicating that an Etn-P on Man-2 enhances transfer of GPIs to protein (Benachour *et al*. 1999). Second, *gpi7Δ* cells have defects in ER to Golgi transport of GPI-proteins and GPI lipid remodeling to ceramide (Benachour *et al*. 1999). Third, *GPI7* deletion leads to cell wall defects and

shedding of GPI-proteins, indicating defective transfer of such proteins into the wall (Toh-e and Oguchi, 1999; Richard *et al*., 2002). Lastly, *gpi7Δ* cells show a cell separation defect that results from mistargeting of Egt2, a GPI protein expressed in daughter cells and implicated in degradation of the septum (Fujita *et al*. 2004). These phenotypes suggest that the Etn-P group on Man-2 is recognized by GPI transamidase, the intracellular transport machinery, GPI lipid remodeling enzymes, and cell wall crosslinkers. An inability to remove Etn-P from Man-2 also leads to phenotypes (see *Remodeling of protein bound GPIs*).

Phosphoethanolamine addition to Man-3 by Gpi13 and the role of Gpi11. Gpi13 is the catalytic subunit of GPI-Etn-P-T-III, and, as expected from the fact that it adds the Etn-P that participates in the GPI transamidase reaction, *GPI13* is essential. The major GPI accumulated by yeast strains depleted of Gpi13 is a Man_4-GPI with a single Etn-P on Man-1 (Flury *et al*. 2000; Taron *et al*. 2000). Gpi11 is likely involved in the GPI-Etn-P-T-III reaction as well, because a recently isolated *gpi11*-Ts mutant also accumulates a Man_4-GPI with its Etn-P on Man-1 (K. Willis and P. Orlean, unpublished results), and human Gpi11 interacts with and stabilizes human Gpi13 (Hong *et al*. 2000). Human Gpi11 (Pig-F) also interacts with human Gpi7 (Shishioh *et al*. 2005). The lipid accumulation phenotypes observed in various types of *gpi11* mutants may prove to be explainable in terms of differential abilities of wild type Gpi11, mutant Gpi11, and human Gpi11 to interact with Gpi7, Gpi13, and possibly even Mcd4, and permit varying extents of Etn-P modification. Because GPIs with the same chromatographic mobilities may be isoforms modified with Etn-P at different positions, and because accumulating GPIs may be mixtures of isoforms, detailed structural analyses should give a clearer picture of the role of Gpi11 in Etn-P modification.

GPI transfer to protein:

Depletion of Gab1 and Gpi8 leads to actin bar formation. Additional functions for Gab and Gpi18 are suggested by the finding that depletion of Gab1 or Gpi8 from yeast, but not of Gaa1, Gpi16, or Gpi17, leads to accumulation of bar-like structures of actin that associate with the perinuclear ER and are decorated with cofilin (Grimme *et al*. 2004). This phenotype, which is not a general result of defective GPI anchoring, might reflect disruption of some functional interaction between resident ER membrane proteins and the actin cytoskeleton and consequent collapse of the ER around the nucleus (Grimme *et al*. 2004).

Remodeling of protein-bound GPIs:

Roles of Bst1, Per1, and Gup1 in ER exit and transport of GPI proteins. Modifications of the GPI lipid by Bst1, Per1, and Gup1 are necessary for efficient transport of GPI proteins from the ER to the Golgi. Loss of Bst1 function leads to retarded transport of GPI-proteins from the ER to the Golgi (Vashist *et al*. 2001), and delayed ER degradation of misfolded GPI proteins, suggesting that inositol deacylation generates sorting signals for ER exit of GPI proteins and for recognition by a quality control

mechanism for GPI-proteins (Fujita *et al*. 2006; Fujita and Jigami, 2008). *per1Δ* and *gup1Δ* cells also show significantly delayed ER to Golgi transport of GPI-proteins (Bosson *et al*. 2006; Fujita *et al*. 2006b). Lipid remodeling events generate a GPI able to associate with and be concentrated in membrane microdomains at ER exit sites prior to their export from the ER (Castillon *et al*. 2009). At these sites, the p24 complex of membrane proteins then serves as an adapter between GPI-proteins and the COP II machinery to promote incorporation of GPI proteins into COP II vesicles specialized for transport of GPI-proteins from the ER. Remodeled GPIs may bind p24 with higher affinity, therefore promoting export of the proteins bearing them (Castillon *et al*. 2011). In the Golgi, GPI-proteins with remodeled anchors are released and proceed onwards along the secretory pathway. However, p24 complexes, which cycle between the ER and Golgi, again monitor the remodeling status of GPIs and exert a quality control function in the Golgi by sensing and retrieving proteins with unmodified GPIs to the ER, where they may encounter the resident ER remodeling enzymes (Castillon *et al*. 2011).

Remodeling of the GPI lipid moiety to ceramide by Cwh43. Cwh43, which replaces the diacylglycerol moiety of GPIs with ceramide, is a large protein with 19 predicted transmembrane domains (Martin-Yken *et al*. 2001; Ghugtyal *et al*. 2007; Umemura *et al*. 2007). *cwh43Δ* cells accumulate GPI-proteins whose lipids are diacylglycerols with a very long acyl chain similar to the lipid generated after action of Bst1, Per1, and Gup1. Because ceramide remodeling requires prior action of Bst1, and *per1Δ* and *gup1Δ* strains show severe defects in remodeling, the exchange reaction seems to take place after the first three lipid modification steps. The mechanism is so far unknown, but could involve a phospholipase-like reaction that replaces diphosphatidic acid with ceramide phosphate or diacylglycerol with ceramide (Ghugtyal *et al*. 2007; Fujita and Kinoshita, 2010). However, alternatives to such a linear remodeling pathway, in which Cwh43 acts instead on the Bst1 or Per1 products, have been discussed (Umemura *et al*. 2007). The C-terminal domain of Cwh43 contains a motif that may be involved in recognition of inositol phosphate (Umemura *et al*. 2007). Because *mcd4* and *gpi7*, mutants defective in addition of Etn-P to Man-1 and Man-2, are affected in ceramide remodeling, Cwh43 may also recognize Etn-P side-branches. Cwh43 appears to act in the ER, where it remodels GPIs with a ceramide consisting of phytosphingosine bearing a C_{26} acyl chain, as well as in the Golgi, where the ceramide it introduces contains phytosphingosine with a hydroxy-C_{26} acyl group (Reggiori *et al*. 1997).

Removal of Etn-P moieties from Man-1 and Man-2. The ER-localized Ted1 and Cdc1 proteins are homologous to mammalian PGAP5, which removes EtN-P moieties from Man-2 (Fujita *et al*. 2009), and genetic interactions connect these two proteins processing and export of GPI-proteins. Export of Gas1 is retarded in *ted1Δ* cells, and *ted1Δ*'s buffering genetic interactions with *emp24Δ* and *erv5Δ*, mutants deficient in two components of the p24 complex involved in maturation and trafficking of GPI proteins, indicate a functional relationship between the three proteins (Haass *et al*. 2007). Further, *cdc1*

mutations are suppressed by *per1*/*cos16* and *gup1* mutations (Paidhungat and Garrett, 1998; Losev *et al*. 2008). Ted1 and Cdc1 contain a lumenal metallophosphoesterase domain (Haass *et al*. 2007; Losev *et al*. 2008), and, consistent with this, *cdc1*'s temperature-sensitivity is suppressed by Mn^{2+}, the cation required by PGAP5 (Fujita *et al*. 2009). These findings are in turn consistent with Ted1 and Cdc1 being GPI-Etn-P phosphodiesterases, but this possibility awaits biochemical confirmation.

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File S5

Sugar nucleotide transport

This Supporting File contains additional information related to **Biosynthesis of Wall Components Along the Secretory Pathway, *Sugar nucleotide transport***. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

GDP-Man transport:

The GDP-Man transporter, Vrg4/Vig4. This protein forms homodimers (Abe *et al.* 1999; Gao and Dean, 2000), shows a wide distribution in the Golgi, and contains a GALNK motif involved in GDP-Man binding (Gao *et al.* 2001).

Gda1 and Ynd1. Evidence these proteins have partially overlapping functions is as follows. i) Deletion of either *GDA1* or *YND1* impacts mannosylation of N- and O-glycans, ii) high-level expression of *YND1* corrects some of *gda1Δ*'s glycosylation defects, and iii) *gda1Δ ynd1Δ* double mutants have a synthetic phenotype and show growth and cell wall defects (Gao *et al.* 1999). However, *gda1Δ ynd1Δ* double mutants are viable and capable of some mannosylation of N- and O-linked glycans, indicating that GDP-Man can enter the Golgi in their absence, and suggesting there may be a mechanism for GDP exit independent of GDP hydrolysis (D'Alessio *et al.* 2005).

GMP generated upon Man-P transfer to glycoproteins could also be a source of antiporter, but it is not a significant one because because the glycans made *gda1Δ* or *gda1Δ ynd1Δ* strains are not affected by disruption of *MNN4* or *MNN6* (Jigami and Odani, 1999; D'Alessio *et al.* 2005).

Other sugar nucleotide transport activities:

Transport activities for UDP-Glc, UDP-GlcNAc, and UDP-Gal also occur in *S. cerevisiae* (Roy *et al.* 1998; 2000 Castro *et al.* 1999), and there are eight further candidate transporters (Dean *et al.* 1997; Esther *et al.* 2008), a couple of which have been associated with these transport activities. Some of the transporters may have specificity for more than one sugar nucleotide. In the case of UDP-Glc, transport activity was present in the ER (Castro *et al.* 1999), but the responsible protein for that activity has yet to identified, although broad specificity Yea4 and Hut1 (see below) may transport UDP-Glc (Esther *et al.* 2008). One possible need for UDP-Glc transport into the ER might be for a glucosylation reaction at an early stage of β1,6-glucan assembly (**Section VI**). The Hut1 protein is a candidate for the UDP-Gal transporter (Kainuma *et al.* 2001), but whether that is Hut1's primary role *in vivo* is unclear because galactose has not been detected on *S. cerevisiae* glycans. Yea4 was characterized as an ER-localized UDP-GlcNAc transporter and its deletion impacts chitin synthesis (Roy *et al.* 2000; **Section V**). Of the other

transporter homologs, Hvg1 resembles Vrg4 most closely, but *hvgΔ* cells have neither a mannosylation nor a GDP-Man transport defect (Dean *et al.* 1997). The roles of the other proteins in sugar nucleotide transport, if any, is unknown. One or more transporters may supply the Golgi GlcNAc-T Gnt1 with its substrate (**Section IV.1.c.ii**).

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File S6

Chitin

This Supporting File contains additional information and discussion related to **Biosynthesis of Wall Components at the Plasma Membrane, *Chitin*.** The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Septum formation:

Phenotypes of *chs1Δ chs2Δ chs3Δ* triple mutants. *chs1Δ chs2Δ chs3Δ* strains grew very slowly but acquired a suppressor mutation that conferred a growth rate as fast as that of a *chs2Δ* mutant, although over a third of suppressed or unsuppressed cells in a culture were dead (Schmidt, 2004). Membranes from the triple mutants had no detectable chitin synthase activity. Unsuppressed triple mutants formed chains of up to eight cells that appeared to be connected by "cytoplasmic stalks", whereas suppressed strains formed shorter chains. Nuclear division continued in the mutant, but in some cells, nuclear segregation was unsuccessful. Ultrastructural analysis showed that in both suppressed and unsuppressed mutants, a bulky remedial septum arises upon thickening of the lateral walls in the mother cell-bud neck region. The suppressor was not identified, but its effect was to allow the remedial septa to be formed more efficiently. The phenotypes of the triple chitin synthase mutants indicate that although it is possible for *S. cerevisiae* to grow without chitin, Chs3-dependent chitin synthesis is nonetheless important for remedial septum formation in *chs2Δ* cells.

Chitin synthase biochemistry:

Directionality and mechanism of extension of β1,4-linked polysaccharide chains. Although the bacterial chitin synthase homologue NodC extends chito-oligosaccharides at their non-reducing ends (Kamst et al. 1999), both reducing- and non-reducing end extension has been reported for Chs-related vertebrate Class I hyaluronate synthases (Weigel and DeAngelis, 2007), and extension by insertion of Glc at the reducing end of a glycan chain has also been proposed for a bacterial cellulose synthase (Han and Robyt, 1998). The latter mechanism was suggested to involve a lipid pyrophosphate intermediate. However, no evidence has been obtained for any lipid-linked intermediate in chitin synthesis. The growing glycan chain may be extruded through the plasma membrane through a pore made up by a bundle of transmembrane helices, which occur towards the C-terminus of chitin synthases (Delmer, 1999; Guerriero *et al*. 2010; Merzendorfer, 2011; Carpita, 2011). Separate proteins might mediate chitin translocation, but no candidates have been identified. With non-reducing end extension, a nascent chitin chain would be extruded into the cell wall reducing end first, which would be compatible with the formation of linkages between

chitin and non-reducing ends of β-glucans (see *Cross-linkage of chitin to β1,6- and β1,3-glucan*; Kollar *et al.* 1995, 1997; Cabib and Duran, 2005; Cabib, 2009).

The stereochemical challenge in formation of β1,4-linked polysaccharides. Each sugar in a β1,4-linked polymer is rotated by about 180° relative to its neighbor, which presents the synthase with a steric challenge, because with successive rounds of addition of a β1,4-linked GlcNAc, the new acceptor 4-OH would alternate between two positions relative to incoming substrate and catalytic residues. Various ways of overcoming this, without invoking movements of the enzyme or the acceptor glycan, have been considered. The first possibility, that UDP-di-*N*-acetylchitobiose is the donor, has been ruled out by the finding that yeast membranes make no chitin when supplied with synthetic UDP-$GlcNAc_2$ (Chang *et al.* 2003). The second possibility is that β1,4-linked polysaccharide synthases have two UDP-sugar binding sites that orient the monosaccharides such that neither enzyme nor polymer needs to rotate, then catalyzes two glycosyltransfers (Saxena *et al.* 1995; Guerriero *et al*. 2010; Carpita, 2011). Evidence supportive of a two active site mechanism came from the finding that a bivalent UDP-GlcNAc analog consisting of two tethered uridine mimetics, envisaged to bind in both active sites, was a better inhibitor than the monomeric analog (Yaeger and Finney, 2004). The observation that the NodC protein, Chs1, and Chs2 all synthesize odd- as well as even-numbered chito-ooligosaccharides *in vitro* (Kang *et al.* 1984; Yabe *et al.* 1998; Kamst *et al.* 1999) is consistent with extension by addition with single GlcNAcs, but extension of GlcNAc, $GlcNAc_3$, or $GlcNAc_5$ by two GlcNAcs at a time would also generate odd-numbered chito-oligosaccharides, if these oligosaccharides are indeed used as primers. Third, it is possible that a chain is extended by a dimeric synthase whose subunits alternately add GlcNAcs, as discussed for cellulose synthase (Carpita, 2011). Consistent with this notion, a two-hybrid analysis indicated that Chs3 can interact with itself (DeMarini *et al.* 1997). The molecular weight of purified native Chs1 was estimated to be around 570,000, approximately consistent with a tetramer, but the authors noted the result may have been due to protein aggregation (Kang *et al.* 1984).

In vitro properties of yeast chitin synthases. Chitin synthase assays typically detect the transfer of [^{14}C]GlcNAc from UDP[^{14}C]GlcNAc to insoluble chitin that is then collected on filters, but a high-throughput method that relies on product binding to immobilized wheat germ agglutinin has also been described (Lucero *et al.* 2002). Of the two procedures, the filtration method would not detect chito-oligosaccharides (Yabe *et al.* 1998). CS I, CS II, and CS III activities differ in their pH optima and their responses to divalent cations (Sburlati and Cabib, 1986; Orlean, 1987; Choi and Cabib, 1994). The three chitin synthase activities have K_ms for UDP-GlcNAc in the range of 0.5-1.3 mM (Kang *et al.* 1984; Sburlati and Cabib, 1986; Orlean, 1987; Uchida *et al.* 1996). At low substrate concentrations relative to K_m (0.03-0.1 mM), purified Chs1 and membranes from cells overexpressing CHS2 make chito-oligosaccharides (Kang *et al.* 1984; Yabe *et al.* 1998). Whether these are *bona fide* chitin

synthase products whose formation reflects low rates of chain extension, or whether the oligosaccharides are generated by chitinase activity on longer nascent chains is not clear (Kang *et al.* 1984).

Effects of free GlcNAc and chitin oligosaccharides on chitin synthesis. *S. cerevisiae*'s three chitin synthases are all stimulated up to a few fold *in vitro* by high concentrations of free GlcNAc (e.g. 32 mM; Sburlati and Cabib, 1986; Orlean, 1987). Neither the mechanistic basis nor the physiological relevance of this are clear, but possible explanations are that GlcNAc serves as a primer or allosteric activator in the chitin synthetic reaction. Results of a kinetic analysis of the chitin synthase activity in wild type membranes led to the proposal that GlcNAc participates along with UDP-GlcNAc in a two substrate reaction with an ordered mechanism in which UDP-GlcNAc binds first (Fähnrich and Ahlers, 1981). Consistent with the idea that GlcNAc serves as a primer or co-substrate, the bacterial NodC chitin synthase homologue incorporates free GlcNAc at the reducing end of chito-oligosaccharide chains that are extended at their non-reducing end by GlcNAc transfer from UDP-GlcNAc (Kamst *et al.* 1999). However, were free GlcNAc to serve as a co-substrate or activator of chitin synthases *in vivo*, there would have to be a mechanism to generate it, for example from GlcNAc-1-P or GlcNAc-6-P (see *Precursors and Carrier Lipids*) or by turnover of GlcNAc-containing molecules.

Growing chitin chains presumably serve as acceptors for further GlcNAc addition, but such a primer function has not been shown using short oligosaccharides. NodC did not use short chito-oligosaccharides as GlcNAc acceptor from UDP-GlcNAc (Kamst *et al.* 1999), nor did purified Chs1 elongate chitotetraose into insoluble chitin in the presence of UDP-GlcNAc (Kang *et al.* 1984). However, inclusion of 1 mM $GlcNAc_5$ and $GlcNAc_8$ in assays of membrane preparations expressing predominantly Chs1 led to about a 1.25-fold increase in incorporation of GlcNAc into chitin from UDP-GlcNAc in the presence of free GlcNAc (Becker *et al*. 2011), suggesting a primer function for longer chito-oligosaccharides. The initiation and early elongation steps in chitin synthesis clearly still need to be defined.

S. cerevisiae's chitin synthases and auxiliary proteins:

Chitin synthase classes. Fungal chitin synthases can be classified into five to seven classes on the basis of amino acid sequence similarity, with *S. cerevisiae* Chs1, Chs2, and Chs3 being assigned to Classes I, II, and IV respectively (Roncero, 2002; Ruiz-Herrera *et al.* 2002; Van Dellen *et al.* 2006; Merzendorfer, 2011). Members of the other classes are found in filamentous fungi. *S. cerevisiae*'s chitin synthases show most amino acid sequence divergence in their amino terminal halves, and these non-homologous regions may make interactions with proteins involved in regulation or trafficking of the individual synthases (Ford *et al.* 1996). Deletion analyses have shown that amino acids in Chs3's hydrophilic C-terminal region are also important for function (Cos *et al.* 1998).

Chitin synthase I:

Activity of N-terminally truncated Chs1. N-terminally truncated forms of Chs1 lacking up to 390 amino acids show a gradual lowering of both specific activity and their ability to be activated by trypsin (Ford *et al.* 1996).

Chitin synthase II and proteins impacting its localization and activity:

Detection of Chs2's activity. Studies of Chs2 enzymology use membranes from strains overexpressing the protein because the activity of genomically encoded Chs2 in membranes of cells grown in minimal medium is negligible (Nagahashi *et al.* 1995). The high amounts of *in vitro* activity obtained by overexpressing Chs2 indicate that levels of Chs2 activity are not tightly limited by endogenous activating or regulatory proteins, in contrast to Chs3.

Effects of proteolysis on wild type and truncated forms of Chs2. Although endogenously activated, processed forms of Chs2 have not been identified, trypsin treatment of partially purified, full-size and N-terminally truncated Chs2 generated a range of discrete protein fragments. The smallest of these, a 35 kDa protein containing the amino acid sequences proposed to be involved in catalysis, was suggested to be sufficient for catalysis, although the instablity of this form prevented its purification to test this notion (Uchida *et al.* 1996). Some 220 amino terminal amino acids of Chs2 are dispensable for *in vivo* function (Ford *et al.* 1996), and moreover, Chs2 versions lacking these amino terminal amino acids have higher *in vitro* activity than the full-length protein, and this activity is stimulated by trypsin (Uchida *et al.* 1996; Martínez-Rucobo *et al.* 2009). Other truncated forms of Chs2, or forms with amino acid substitutions, also vary in their extent of activation by trypsin (Ford *et al.* 1996; Uchida *et al.* 1996). It has been noted that amino acid deletions or substitutions in Chs2 could perturb interactions with native mechanisms for activation and localization of the protein (Ford *et al.* 1996).

Chitin synthase III and proteins impacting its localization and activity:

Relationship between Pfa4 and Chs7 and their roles in Chs3 exit from the ER. Chs3 interacts with Chs7 and is palmitoylated by Pfa4. The Chs3-Chs7 interaction also occurs in *pfa4Δ* cells, though to a slightly reduced extent, and Chs3 can still be palmitoylated, likewise to a lesser extent, in *chs7Δ* cells, indicating that Chs3 palmitoylation is not obligatory for Chs3 recognition by Chs7 (Lam *et al.* 2006). Pfa4 does not palmitoyate Chs7. It seems that Pfa4 and Chs7 act in parallel, though not wholly independently, to promote folding of Chs3 prior to the synthase's exit from the ER. These roles of Pfa4 and Chs7 are specific to Chs3, for neither is required for exit of Chs1 and Chs2 from the ER (Trilla *et al.* 1999; Lam *et al.* 2006).

Rcr1 and Yea4 in Chs3-dependent chitin synthesis. These proteins have both been localized to the ER membrane. Rcr1 has a slight negative regulatory effect on Chs3-dependent chitin synthesis. High copy *RCR1* confers resistance to Congo Red, a dye that binds chitin (as well as β1,3-glucan (Kopecká and Gabriel, 1992)), whereas *rcr1Δ* cells showed slightly increased

sensitivity to Congo Red and CFW (Imai *et al.* 2005). Wild type cells overexpressing *RCR1* have 70% of the chitin in control cells, and *rcr1Δ* cells make 115% of wild type levels of chitin. However, *RCR1* overexpression affects neither the amount nor localization of Chs3, Chs5, and Chs7, nor do Rcr1 and Chs7 physically interact (Imai *et al.* 2005). The role of Rcr1 in Chs3-dependent chitin synthesis is therefore not clear, but the protein has also been reported to act after the ER and have a role in an endosome-vacuole pathway that impacts trafficking of plasma membrane nutrient transporters (Kota *et al.* 2007). The second ER membrane protein, Yea4, was identified through its homology to the *Kluyveromyces lactis* UDP-GlcNAc transporter (Roy *et al.* 2000). Membrane vesicles from cells overexpressing Yea4 have 8-fold elevated levels of UDP-GlcNAc transport activity, consistent with Yea4's function as a transporter (Roy *et al.* 2000). *yea4Δ* cells contain 65% of wild type levels of chitin, implicating Yea4 in chitin synthesis, but whether and how Yea4's transport activity contributes to this process is unclear.

Role of exomer in transport of wall related proteins other than Chs3. Exomer has roles in polarized transport of other wall related proteins to the cell surface. Thus, transport of Fus1, which promotes cell fusion during mating, requires Chs5 for transport to the shmoo tip (Santos and Snyder, 2003), along with the ChAPs Bch1 and Bus7, but not Chs6 (Barfield *et al.* 2009). Further, much of the GPI-anchored chitin-β1,3-glucan cross-linker Crh2 (see *Cross-linkage of chitin to β1,6- and β1,3-glucan*) fails to reach sites of polarized growth and accumulates intracellularly in *chs5Δ*, although another GPI-protein, Cwp1, was unaffected (Rodriguez-Pena *et al.* 2002). Co-transport of Chs3 and Crh2 would ensure colocalization of these proteins for efficient cross linking of nascent chitin to β1,3-glucan.

Role of Chs4 farnesylation in the activation and localization of Chs3. Chs4 has a C-terminal farnesylation site (Bulawa *et al.* 1993; Trilla *et al.* 1997) that is used (Grabinska *et al.* 2007) and the consensus of studies of the importance of the prenyl group is that the modification has roles in Chs4 function and localization. Mutants expressing a non-farnesylatable Cys to Ser variant of Chs4 make one third of normal amounts of chitin, have lower *in vitro* CS III activity, and show CFW resistance (Grabinska *et al.* 2007; Meissner *et al.* 2010). In two of three studies, the prenylation site mutant of Chs4 was found in the cytoplasm, suggesting that lipidation is important for membrane localization of the protein (Reyes *et al.* 2007; Meissner *et al.* 2010). Chs4 reaches the plasma membrane in mutants affected in Chs3 transport, indicating it is transported there independently of Chs3 (Reyes *et al.* 2007), but two sets of findings raise the possibility that Chs3 interacts with Chs4 at the level of the ER. First, two-hybrid analyses established that cytoplasmic domains of Chs3 and the ER-localized CAAX protease Ste24 interact. Second, *ste24Δ* cells exhibit moderate CFW resistance, chitin content is reduced, and less Chs3 was localized at the bud neck. *Vice versa*, high-copy expression of *STE24* leads to CFW sensitivity and some increase in cellular chitin (Meissner *et al.* 2010). Chs4 localization, though, was not affected in *ste24Δ,* nor was an interaction detected between Chs4 and Ste24. It was

suggested that Chs3 recruits farnesylated Chs4 in the ER for processing by Ste24, and that the modification contributes to subsequent correct localization of Chs3 and activation of CS III (Meissner *et al.* 2010).

Chitin synthase III in mating and ascospore wall formation:

Regulation of Chs3 during chitosan synthesis. The Chs4 homologue Shc1, which is 43% identical to Chs4 but expressed only during sporulation, has a role in chitosan synthesis, because homozygous *shc1Δ shc1Δ* diploids make ascospores with very little chitosan (Sanz *et al.* 2002). Shc1 and Chs4 are functionally related because when Shc1 is expressed in vegetative cells, it can activate CS III, and when Chs4 is overexpressed in *shc1Δ shc1Δ* diploids, it partially corrects the sporulation defect (Sanz *et al.* 2002). However, although Shc1 serves as CS III activator in *chs4Δ* cells, it does so without properly localizing Chs3 to septins as Chs4 does in vegetative cells, likely because it cannot interact with Bni4 (Sanz *et al.* 2002). Haploid *chs4Δ shc1Δ* cells do not show a synthetic growth defect, indicating they are not an essential redundant pair, and indeed, analyses of the *SHC1* genetic interaction network suggests Shc1 may have additional roles distinct from those of Chs4 that are not directly related to chitin synthesis (Lesage *et al.* 2005). Sporulation-specific kinase Sps1, regulates mobilization of Chs3 as well as sporulation-specific β1,3-glucan synthase Fks2/Gsc2 (see *β1,3-glucan*) to the prospore membrane (Iwamoto *et al.* 2005).

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File S7

β1,3-glucan

This Supporting File contains additional information and discussion related to **Biosynthesis of Wall Components at the Plasma Membrane, *β1,3-glucan*.** The subheadings used in the main text are retained, and new subheadings are underlined.

Fks family of β1,3-glucan synthases:

Identification of Fks1, Fks2, and Fks3. Fks1 (Cwh53/Etg1/Gsc1/Pbr1) was identified in screens for hypersensitivity to the calcineurin inhibitors FK506 and cyclosporin A and to CFW, for resistance to echinocandin and papulocandin, and following purification of β1,3-glucan synthase activity (reviewed by Orlean, 1997 and Lesage and Bussey, 2006). Cross-hybridization with *FKS1* and copurification with Fks1 led to identification of Fks2/Gsc2, which is 88% identical to Fks1 (Inoue *et al.* 1995; Mazur *et al.* 1995). The *S. cerevisiae* proteome also contains Fks3, which is 55% identical to Fks1 and Fks2 (Dijkgraaf *et al.* 2002). The Fks proteins are assigned to GT Family 48, and a strong case can be made for them being processive β1,3-glucan synthases themselves, although roles as glucan exporters cannot yet be excluded (Mazur *et al.* 1995; Dijkgraaf *et al.* 2002; Lesage and Bussey, 2006).

Functional domains of Fks1. Fks1 is predicted to have an N-terminal cytoplasmic domain of some 300 amino acids that is followed by six transmembrane helices, a second cytoplasmic domain of about 600 amino acids, then 10 transmembrane helices (Inoue *et al.* 1995; Mazur *et al.* 1995; Qadota *et al.* 1996; Dijkgraaf *et al.* 2002; Okada *et al.* 2010). Three functional domains have been distinguished (Okada *et al.* 2010). Amino acids important for β1,3 glucan synthesis *in vivo* are located in the first cytoplasmic domain. Mutations here have little impact on *in vitro* activity and do not affect the protein's interaction with Rho1, but cells have a lowered β1,3 glucan content. Mutations in the second cytoplasmic domain that lie close to the C-terminus of the sixth helix lead to a loss of cell polarity as well as defects in endocytosis, but have little effect on *in vitro* and *in vivo* b-glucan synthesis, and this part of Fks1 may interact with factors involved in cell polarity (Okada *et al.* 2010). Mutations in Fks1 in residues more distal to the sixth helix lead to low *in vitro* glucan synthase activity and large decreases in *in vivo* incorporation of [^{14}C]glucose into β1,3 glucan, suggesting that if Fks1 is a synthase, this part of the protein contains the catalytic site (Dijkgraaf *et al.* 2002; Okada *et al.* 2010).

Fatty acid elongases and phytosphingosine and Fks1 function. The ER-localized fatty acid elongase Elo2/Gns1 may impact Fks1 at the level of that organelle, because *gns1* mutants, isolated on account of their resistance to a papulocandin analogue, have very low *in vitro* β1,3-glucan synthase activity (el-Sherbeini and Clemas, 1995) and accumulate

phytosphingosine in the ER membrane (Abe *et al.* 2001). Phytosphingosine inhibits β1,3 glucan synthase *in vitro*, leading to the idea that this sphingolipid synthetic intermediate is a negative regulator of β1,3-glucan synthesis at the level of the ER (Abe *et al.* 2001).

Roles of the Fks proteins in β1,3-glucan synthesis

Roles of Fks3 and Fks3 in sporulation. Fks2 is important in sporulation because *fks2Δ fks2Δ* diploids have a severe defect in this process (Mazur *et al.* 1995; Huang *et al.* 2005), and form disorganized ascospore walls with lower relative amounts of hexose in their alkali-insoluble fraction and a lower alkali soluble β1,3-glucan content (Ishihara *et al.* 2007). Homozygous *fks3Δ fks3Δ* diploids also form abnormal spores, indicating a role for the third Fks homologue in ascopore wall formation, but showed no alteration in the distribution of hexoses between alkali soluble- and insoluble fractions (Ishihara *et al.* 2007). However, the walls of ascospores formed in diploids lacking both Fks2 and Fks3 were more disorganized than those of ascospores made by *fks2Δ fks2Δ* diploids (Ishihara *et al.* 2007). Expression of *FKS2* or *FKS1* under the control of the *FKS2* promoter, but not the *FKS1* promoter, corrected the sporulation defect of homozygous *fks1Δ fks2Δ* diploids, suggesting that the function of Fks2 in sporulating diploids resembles that of Fks1 in vegetative cells. In contrast, overexpression of *FKS3* did not suppress the phenotype of *fks2Δ* spores, and *FKS1* or *FKS2* overexpression does not correct the defect in *fks3Δ* spores, indicating Fks3's function in sporulation does not overlap with that of Fks2. It was proposed that Fks2 is primarily responsible for synthesis of β1,3-glucan in the ascospore wall, and that Fks3, rather than functioning as a synthase, modulates glucan synthesis by interacting with glucan synthase regulators such as Rho1 (Ishihara *et al.* 2007).

File S8
β1,6-Glucan

This Supporting File contains additional information and discussion related to **β1,6-Glucan.** The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Proteins involved in β1,6-glucan assembly

ER proteins:

Fungus-specific ER chaperones required for β1,6-glucan synthesis:

Evidence for the chaperone function of Rot1, Big1, and Keg1 in β1,6-glucan synthesis. Rot1, Big1, and Keg1, which do not resemble known carbohydrate-active enzymes, seem unlikely to catalyze formation of β1,6-glucan (Lesage and Bussey, 2006). Rather, they seem to function as ER chaperones with varying degrees of importance for the stability of proteins involved in β1,6-glucan synthesis, and in some cases, they may cooperate. Observations supporting this notion, and indicating a relationship to Kre5, are as follows. Analyses of levels of β1,6-glucan synthesis-related proteins in a *rot1*-Ts mutant indicate that Kre6 has the strongest dependence on Rot1 for stability, although Kre5 and Big1 show appreciable dependence as well (Takeuchi *et al.* 2008). Keg1, a protein essential for growth in osmotically supported medium, physically interacts with Kre6 in the ER membrane, and a *keg1*-Ts mutant is suppressed at high copy by *ROT1*, though not *BIG1*; however, a physical interaction between Keg1 and Rot1 could not be detected (Nakamata *et al.* 2007). Because the *big1Δ rot1Δ* double mutant has the same growth rate as each single mutant, it was suggested that Rot1 and Big1 impact β1,6-glucan synthesis in the same way, and possibly function in the same compartment or even in a complex (Machi *et al.* 2004). However, although *rot1*, *big1*, and *kre5* mutations individually all lower β1,6-glucan levels to the same extent, the *kre5 big1* double mutant, but apparently not a *kre5 rot1* strain (Lesage and Bussey, 2006), shows a reduced growth rate and lowered β1,6-glucan content compared with each single mutant, suggesting the function of Rot1 is partly distinct from that of Kre5 (Azuma *et al.* 2002; Lesage and Bussey, 2006). Indeed, the non-conditional *rot1-1* mutant shows a synthetic growth and N-glycosylation defect in combination with *ost3Δ* (though not *ost6Δ*), as well as a partial defect in O-mannosylation of the chitinase Cts1, indicating a wider role for Rot1 in glycosylation (Pasikowska *et al*. 2012).

More widely distributed secretory pathway proteins:

Kre6 and Skn1:

Localization and transport of Kre6. Recent studies indicate that much of Kre6 is ER-localized, where it interacts with Keg1, but Kre6 is also detectable in secretory vesicles and at the plasma membrane at sites of polarized growth (Nakamata *et al.* 2007; Kurita *et al.* 2011). In addition to Kre6's lumenal domain, the protein's cytoplasmic tail is important for Kre6's function in β1,6-glucan assembly and its transport to the plasma membrane (Li *et al.* 2002; Kurita *et al.* 2011). A truncated form of Kre6 lacking its 230 N-terminal amino acids failed to be localized to the plasma membrane, and did not correct the β1,6-glucan synthetic defect of *kre6Δ*, although it appeared stable (Kurita *et al.* 2011). It was concluded that transport of Kre6 to the plasma membrane is necessary for the protein to fulfill its role in β1,6-glucan synthesis (Kurita *et al.* 2002). Localization of Skn1 has not been explored in detail.

Skn1 and plant defensin resistance. *skn1Δ*, but not *kre6Δ* strains, are defective in $M(IP)_2C$ synthesis and resistant to a plant defensin that interacts with this sphingolipid to exert its antifungal activity (Thevissen *et al.* 2005). Defensin-susceptibility is unconnected with cellular β1,6-glucan content because other β1,6-glucan synthesis mutants are defensin-sensitive (Thevissen *et al.* 2005).

Plasma membrane protein Kre1:

Kre1 as receptor for K1 killer toxin. Membrane anchored Kre1 has an additional role as receptor for K1 killer toxin. Spheroplasts of *kre1Δ* cells are resistant to this toxin, but expression of the C-terminal 63 amino acids of Kre1 was sufficient to make spheroplasts, but not intact cells, toxin sensitive again, leading to the proposal that Kre1's GPI-modified C-terminus serves as the membrane receptor for K1 toxin after initial toxin binding to β1,6-glucan (Breinig *et al.* 2002).

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File S9
Cell Wall-Active and Nonenzymatic Surface Proteins and Their Functions

This Supporting File contains additional information and discussion related to **Cell Wall-Active and Nonenzymatic Surface Proteins and Their Functions**. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Known and predicted enzymes

Chitinases:

S. cerevisiae's two chitinases, Cts1 and Cts2, are both members of GH Family 18, but of the two, Cts1 resembles plant-type chitinases, whereas the predicted Cts2 protein is more similar to the bacterial chitinase subfamily (Hurtado-Guerrero and van Aalten, 2007). Cts1 has endochitinase activity, a pH optimum of 2.5, and is more active on nascent than on preformed chitin (Correa *et al.* 1982). The structure of the catalytic domain, which has chitinase activity on its own, has been determined (Hurtado-Guerrero and van Aalten, 2007). Little is known about Cts2, but because *CTS2* complements a defect in the sporulation-specific chitinase of *Ashbya gossypii* (Dünkler *et al.* 2008), Cts2 may have a role in sporulation.

β1,3-glucanases:

Exg1, Exg2 and Ssg/Spr1 exo-β1,3-glucanases:

These proteins are members of GH Family 5 and were originally characterized biochemically as exo-β1,3-glucanases (Larriba *et al.* 1995). Exg1 is a soluble cell wall protein released upon treatment with dithiothreitol (Cappellaro *et al.* 1998), whereas Exg2 may normally be membrane- or wall-anchored because it has a potential GPI attachment site (Caro *et al.* 1997), whose deletion results in release of the protein into the medium (Larriba *et al.* 1995). Single or double null mutants in *EXG1* and *EXG2* have no obvious defects, although *exg1Δ* cells have slightly elevated levels of β1,6 glucan and *EXG1* overexpressers lower amounts of that polymer. This, together with the finding that the Exg proteins can act on the β1,6-glucan pustulan *in vitro* (Nebreda *et al.* 1986), raises the possibility that Exg1 and Exg2 have roles in β-glucan remodeling (Jiang *et al.* 1995; Lesage and Bussey, 2006). Ssg1/Spr1 is a sporulation-specific protein. Its mRNA is expressed late in sporulation, and homozygous null diploids show a delay in the onset of ascus formation (Muthukumar *et al.* 1993; San Segundo *et al.* 1993).

Bgl2, Scw4, Scw10 endo-β1,3-glucanases:

These proteins are members of GH Family 17. Scw4, Scw10, and Bgl2 can be extracted from the wall with dithiothreitol (Capellaro *et al.* 1998), suggesting wall association via disulfides. However, a population of Scw4 and Scw10

resists extraction by hot SDS and β-mercaptoethanol, and is released instead by mild alkali or by β1,3-glucanase digestion, indicating a covalent linkage to β1,3-glucan (Yin *et al.* 2005). However, Scw4 and Scw10 lack PIR sequences. Purified Bgl2 binds both β1,3-glucan and chitin (Klebl and Tanner, 1989), but whether these non-covalent interactions represent an additional mode of wall association, or reflect an enzyme-substrate interaction, is unexplored.

Levels of Bgl2 and Scw10 need to be balanced in order to ensure cell wall stability (Sestak *et al.* 2004). This proposal is based on the findings that deletion of *BGL2* in the *scw4Δ scw10Δ* background (but not of *SCW11*, *EXG1*, *CRH1*, or *CRH2*) alleviated many of the phenotypes of that double mutant, that overexpression of *BGL2* is lethal in a wild type background, and that high level expression of *SCW10* in *bgl2Δ* significantly increases the strain's CFW sensitivity (Klebl and Tanner, 1989; Sestak *et al.* 2004). Bgl2 and Scw10 may also contribute to compensatory responses to mutationally induced wall stress, because *BGL2* and *SCW10*, as well as *EXT1* and *CRH1*, are upregulated in *mnn9*, *kre6*, *mnn9*, and *gas1* mutants (Lagorce *et al.* 2003). What Bgl2 and Scw10's precise biochemical roles are, and how they antagonize one another, are intriguing questions.

Eng1/Dse4 and Eng2/Acf2 endo-β1,3-glucanases:

These two related proteins are members of GH family 81. *ENG1* expression is highest at the M to G_1-phase transition and shut down during sporulation. Eng1 localizes to the daughter side of the septum, consistent with a hydrolytic role during cell separation (see *Septum formation*; Baladron *et al.* 2002). Eng2 recognizes β1,3-glucans of at least five residues and releases trisaccharides from the non-reducing end of the substrate, but has no detectable transglycosidase activity (Martín-Cuadrado *et al.* 2008).

Gas1 family β1,3-glucanosyltransferases:

Domain organization and mechanism of Gas proteins. Gas1 and its four paralogues, Gas2, Gas3, Gas, 4, and Gas5 (Popolo and Vai, 1999), are members of the GH Family 72. The catalytic domain of Gas proteins lies in their N-terminal half, and in the case of Gas1 and Gas2, is followed by a cysteine-rich domain that is a member of the CBM43 group of carbohydrate binding modules. The other Gas proteins lack this module but have a serine and threonine-rich sequence instead, and Gas1 has both (Popolo and Vai, 1999).

The biochemical activity of Gas proteins was first defined for the *Aspergillus fumigatus* Gas1 homologue, Gel1, but *S. cerevisiae* Gas1, Gas2, Gas4, and Gas5 all proved to carry out the same reaction *in vitro* (Mouyna *et al.* 2000; Carotti *et al.* 2004; Ragni *et al.* 2007b; Mazan *et al.* 2011). The proteins have β1,3-glucanosyltransfer or "elongase" activity, which involves cleavage of a β1,3 glucosidic linkage within a β1,3-glucan chain, then transfer of the newly generated reducing end of the

cleaved glycan to the non-reducing end of another β1,3 glucan molecule, thus extending the acceptor β1,3-glucan chain (Mouyna *et al.* 2000). The structure of a soluble form of Gas2 in complex with β1,3-gluco-oligosaccharides revealed the presence of two oligosaccharide binding sites and led to a base-occlusion hypothesis for how transglycosylation could be favored over hydrolysis. In the hypothesized mechanism, one binding site is occupied by the donor glucan, which is hydrolyzed with formation of an enzyme-oligosaccharide intermediate, whereupon the other, acceptor, site is transiently filled by the second product of the hydrolysis reaction. Occupancy of the acceptor site has the effect of occluding the catalytic base on the enzyme, preventing any incoming water molecule from being activated for nucleophilic attack on the enzyme-saccharide intermediate. The gluco-oligosaccharide in the acceptor site is then displaced by a longer and tighter binding acceptor glucan with concomitant formation of the new β1,3-glucosidic linkage (Hurtado-Guerrero *et al.* 2009).

In the case of Gas1 and Gas2, the cysteine-rich domain is necessary for catalytic activity, being required for proper folding of the catalytic domain, for substrate binding, or for both (Popolo *et al.* 2008). This domain, however, is not necessary for activity of Gas4 or Gas5, which lack it, and, because Gas4 and Gas5 generate profiles of oligosaccharides from β1,3-gluco-oligosaccharide substrates that are different from those released by Gas1 and Gas2, it is possible that the cysteine-rich domain influences cleavage site preference (Ragni *et al.* 2007b). Nonetheless, expression of Gas4, but not Gas2, in a *gas1Δ* strain fully complemented the *gas1Δ* growth defect in media with a pH of 6.5 or above (Ragni *et al.* 2007a).

Localization of Gas1. Gas1 fused to GFP but retaining its N- and C-terminal signal sequences is detectable in the lateral wall, in the chitin ring in small-budded cells, and near the primary septum, and remains in the bud scar after cell separation (Rolli *et al.* 2009). Gas1 localization to the chitin ring and bud scars was abolished in cells lacking the chitin-β1,3-glucan cross-linkers Crh1 and Crh2, suggesting that Gas1 anchorage to chitin was dependent on linkage of a Gas1-β1,6-glucan-β1,3-glucan complex to chitin (Rolli *et al.* 2009). Consistent with this, Gas1 was shed into the medium from *chs3Δ* cells, which are unable to make the chitin known to be cross-linked to β-glucan (Cabib and Duran, 2005). Because the released Gas1 was not significantly larger than Gas1 in lysates of wild type cells (Rolli *et al.*, 2009), the β1,6-glucan-β1,3-glucan presumed to link the protein to chitin must be quite small. Some Gas1 was also released from *chs2Δ* cells, suggesting that localization of Gas1 near the primary septum requires Chs2-dependent chitin synthesis (Rolli *et al.* 2009). However, because the chitin made by Chs2 is free of cross-links (Cabib and Duran, 2005), its association with Gas1 would be indirect. Cell-associated Gas1 was distributed throughout the remedial septum made in *chs2Δ* cells (**Section V.1.a**). Intriguingly, Gas1 was also shed from *chs1Δ* cells, though at reduced levels when the medium was buffered to lower chitinase activity. Amounts and localization of cell-associated Gas1 appeared

unchanged, however, presumably because Chs2 and Chs3 still make chitin. Nonetheless, this observation indicates that Chs1 or its product contribute to wall association of some Gas1 (Rolli *et al.* 2009).

Functions of Gas2, Gas3, Gas4, and Gas5. The following findings indicate that Gas5 and Gas3 have wall-related functions in vegetative cells. *GAS5* is expressed during vegetative growth but repressed during sporulation, and *gas5Δ* strains are Calcofluor White sensitive (Caro *et al.* 1997). Purified Gas3 is inactive (Ragni *et al.* 2007b), and *gas3Δ* strains make no genetic interactions with strains with single or double deletions in other *GAS* genes (Rolli *et al.* 2010). Moreover, Gas3 cannot substitute for Gas1, but overexpression in *gas1Δ* of wild type *GAS3* or a *gas3* mutant encoding catalytically inactive Gas3 exacerbated the *gas1Δ* growth defect, indicating that high levels of Gas3 are toxic (Rolli *et al.* 2010).

Gas2 and Gas4 have overlapping functions in ascospore wall assembly. Their genes are expressed only during sporulation, and although diploids homozygous for single *GAS2* or *GAS4* deletions sporulate normally, diploids lacking both Gas2 and Gas4 have a severe sporulation defect (Ragni *et al.* 2007a). The inner glucan layer of the spore wall from by double homozygous *gas2 gas4* nulls was disorganized and detached from chitosan, and dityrosine, though present, was less abundant and diffusely distributed. The absence of β1,3-glucanosyltransferase activity may result in shorter β1,3-glucan chains that are more loosely associated with chitosan. Gas2 and Gas4 likely need to be GPI anchored to fulfill their key roles in ascospore wall formation, which in part explains the severe sporulation defect of homozygous *gpi1/gpi1* and *gpi2/gpi2* diploids (Leidich and Orlean, 1996). Because such diploids lack dityrosine, additional GPI-proteins must normally be involved in ascospore wall assembly.

Yapsin aspartyl proteases:

Yapsin processing. Yapsins are synthesized as zymogens and undergo proteolytic processing to generate a mature active enzyme. The steps include removal of a propeptide and excision of an internal segment flanked by basic amino acids that separates the enzyme's two catalytic domains, which remain disulfide-linked (Gagnon-Arsenault *et al.* 2006, 2008). In the case of Yps1, the propeptide removal and excision steps are likely autocatalytic at an environmental pH of 3, but involve other proteases, including yapsins, at pH 6 (Gagnon-Arsenault *et al.* 2008).

Cell wall phenotypes of yapsin-deficient strains. Strains lacking individual yapsin genes are sensitive to various cell wall disrupting agents, though their sensitivity profiles differ. For example, *yps7Δ* is the only *yps* null hypersensitive to CFW, but *yps1Δ* the only mutant sensitive to the β1,3-glucan synthase inhibitor caspofungin (Krysan *et al.* 2005). The quintuple *yps1Δ yps2Δ yps3Δ yps6Δ yps7Δ* null mutant is viable, but undergoes osmotically remedial lysis at 30°C, as does the *yps1Δ yps2Δ*

yps3Δ triple deletion strain, and to a slightly lesser extent, the *yps1Δ yps2Δ* double null (Krysan *et al.* 2005). The temperature-sensitive lysis phenotype of strains lacking multiple yapsins is consistent with a role for these proteins when cell walls are stressed, and indeed, expression of *YPS1*, *YPS2*, *YPS3*, and *YPS6* is upregulated under such conditions (Garcia *et al.* 2004; Krysan *et al.* 2005).

Non-enzymatic CWPs

Structural GPI proteins:

Sps2 family:

Ecm33. Mannan outer chains produced by *ecm33Δ* cells are slightly smaller than normal, although O-mannosylation and core-type N-glycans are not affected. Epitope-tagged Pst1 is most abundant at the surface of buds, but Ecm33's localization is uncertain because tagging Ecm33 abolishes its *in vivo* function (Pardo *et al.* 2004). Ecm33 occurs in both plasma membrane and wall-anchored forms, but must retain its GPI anchor and plasma membrane localization for *in vivo* function (see *Incorporation of GPI proteins into the wall*; Terashima *et al.* 2003; Yin *et al*. 2005). Expression of a minimal amount of GPI-anchored Ecm33 may be necessary for growth at high temperature, because the temperature-sensitivity of *mcd4*, *gpi7*, *gpi13* and *gpi14* mutants is suppressed by overexpression of *ECM33* (Toh-e & Oguchi, 2002; A. Sembrano and P. Orlean, unpublished).

Tip1 family:

Localization of Cwp2 and Tip1 is influenced by the timing of their expression. A swap of the promoters of *CWP2* and *TIP1* caused these genes' products to exchange their cellular location, indicating that the localization of Cwp2 and Tip1, and perhaps that of other CWPs, is influenced by the timing of their expression in the cell cycle (Smits *et al.* 2006). Cwp1, however, is localized to the birth scar in a manner that depends on normal septum formation, but, because neither Tip1 nor Cwp2 is targeted to the birth scar when expressed behind *CWP1*'s promoter, additional *CWP1* sequences are required for Cwp1 localization (Smits *et al.* 2006).

Ccw12:

Structural features of Ccw12. Ccw12 has a predicted mass of 13 kDa but migrates on denaturing polyacrylamide gels with an apparent molecular weight of a least 200 kDa. Elimination of Ccw12's three N-linked sites shows that N-linked glycans are mostly responsible for this apparent size increase, but these modifications are not necessary for *in vivo* function, because Ccw12 lacking its N-linked sites complements *ccw12Δ* phenotypes (Ragni *et al.* 2007c). O-mannosylation contributes some 42 kDa to the apparent size of Ccw12 (Hagen *et al.* 2004). The protein is not obviously related to any known enzymes, but contains

two repeats of the sequence TTEAPKNGTSTAAP (Mrša *et al.* 1999). Deletion of one or both of these does not affect cross-linkage Ccw12 to the wall, but the repeats are nonetheless critical for *in vivo* function because proteins lacking them do not restore the growth and cell wall defects of *ccw12Δ* (Ragni *et al.* 2007c). Four sequences similar to the Ccw12 repeat are present in Sed1 (Mrša *et al.* 1999; Ragni *et al.* 2007c).

Certain Tip1 family members and Slr1 also migrate in denaturing polyacrylamide gels with much higher molecular weights than would be expected (van der Vaart *et al.* 1995; Terashima *et al.* 2002).

A new mechanism for compensating loss of multiple GPI-CWP uncovered in *ccw12Δ* . Deletion of additional genes for GPI-CWP in the *ccw12Δ* background uncovered a mechanism for compensating for loss of multiple GPI-CWPs. Rather than showing an exacerbated phenotype, the *ccw12Δ ccw14Δ* double null was less sensitive to CFW compared with *ccw12Δ*, and the *ccw12Δ ccw14Δ dan1Δ* mutant showed wild type levels of sensitivity to CFW and nearly normal levels of chitin. Moreover, additional deletion of *CWP1* and *TIP1* had no further effect on CFW sensitivity, although walls of the quintuple mutant had a thicker inner glucan layer and a thinner but more ragged outer mannoprotein layer (Hagen *et al.* 2004). It seems that although loss of Ccw12 alone activates the CWI pathway-mediated chitin stress response (Ragni *et al.* 2007c, 2011; see *Chitin synthesis in response to cell wall stress*), deletion of additional GPI-CWP genes forces cells over a threshold that leads to triggering of a new compensatory response, whereupon the chitin response becomes less important. This new response depends on Sed1 and the non-GPI-CWP Srl1. Not only is their expression upregulated in the *ccw12Δ ccw14Δ dan1Δ cwp1Δ tip1Δ* strain, but deletion of either in the *ccw12Δ ccw14Δ dan1Δ* background reverts the strain to the high-chitin phenotype of *ccw12Δ* (Hagen *et al.* 2004). In addition, the cell wall remodeling genes *SCW10* and *BGL2* are upregulated and *CRH2* downregulated, suggesting that the response involves alterations of the structure of the β-glucan layer (Hagen *et al.* 2004). More generally, the phenotypes of the multiple GPI-CWP mutants indicate that GPI-CWPs have a collective role in maintaining cell wall stability (Lesage and Bussey, 2006; Ragni *et al.* 2007c). Ccw12 and Slr1 also have parallel functions in a pathway that relieves defects in a polarized morphogenesis signaling network (see *Slr1*).

Other non-enzymatic GPI-proteins:

Ccw14/Ssr1/Icwp as an inner cell wall protein. A monoclonal antibody that recognizes Ccw14/Ssr1 on immunoblots does not detect the protein on intact cells, whereas it does have access to the glycoprotein in tunicamycin-treated cells or in *mnn1 mnn9* mutants (Moukadiri *et al.* 1997). Assuming that the antibody would have had access to its epitope on Ccw14/Ssr1 if the protein were at the surface of wild type cells, this finding is consistent with Ccw14/Ssr1 being a protein of the inner cell wall

(Moukadiri *et al.* 1997).

Flocculins and agglutinins:

Roles and interactions of Aga1 and Fig2 in mating. Deletion of *FIG2* in *MAT*a cells with the W303 background, but not *MAT***a** cells, increases the agglutinability of *MAT*α cells, suggesting a role for Fig2 in attenuating agglutination of *MAT*a cells (Erdman *et al.* 1998; Jue and Lipke, 2002). Both Aga1 and Fig2 have an additional, additive role in mating in *MAT*α strains that is unconnected with Aga2, because simultaneous deletion of *AGA1* and *FIG2* in certain *MAT*α *sag1Δ* backgrounds leads to a severe mating defect on solid medium, whereas individually deleting the *AGA1* and *FIG2* in those strain backgrounds does not (Guo *et al.* 2000). An explanation for the expanded roles for Aga1 and Fig2 in mating came from detection of heterotypic adhesive interactions between Aga1 and Fig2, and homotypic interactions between Fig2 and Fig2, which are mediated by WPCL and CX_4C domains present in both proteins (Huang *et al.* 2009).

Non-GPI-CWP:

PIR proteins:

PIR protein localization. Fusions of Pir1 and Pir2 with red fluorescent protein are found at bud scars of both haploid and diploid cells, with Pir1 being localized inside the chitin ring. This localization of Pir1 is independent of normal chitin ring and primary septum formation because the protein is still transported to the budding site in *chs2Δ* and *chs3Δ* cells, although in the absence of the chitin ring in *chs3Δ*, Pir1 no longer shows a ring-like distribution (Sumita *et al*. 2005). Some Pir1 and Pir2, and most Pir3, are also present in lateral walls, where these proteins can be detected by immunoelectron microscopy using antibody to Pir3 (Yun *et al.* 1997). Pir4 has been reported to show a uniform distribution at the cell surface, but in one study, this distribution was restricted to growing buds (Moukadiri *et al.* 1999; Sumita *et al.* 2005).

A Kex2 processing site in PIR proteins. The four PIR proteins contain a site for processing by the Kex2 protease, but although Kex2 acts on the PIR proteins *in vivo*, wall localization of these proteins is unaffected in *kex2Δ*, so the significance of this processing event is unclear (Mrša *et al.* 1997).

Scw3 (Sun4):

SUN proteins. Members of this family of highly glycosylated proteins have a common C-terminal domain of some 250 amino acids in which the spacing of four cysteines is conserved (Velours *et al.* 2002). The SUN proteins other than Scw3/Sun4 (Sim1, Uth1, and Nca3) have been implicated in various cellular functions unrelated to the cell wall, but SUN family members have been assumed to be glucanases because they are homologous to *Candida wickerhamii* BglA, an additional protein

identified in a screen of a cDNA expression library for proteins that reacted with an antibody to a cell-bound β-glucosidase (Skory and Freer, 1995). However, glycosidase activity has not been verified for BglA and the SUN proteins show no homology to any carbohydrate active enzymes, making it doubtful they are glycosidases.

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Table S1 Proteins involved in cell wall biogenesis in *Saccharomyces cerevisiae*

Process or protein type	Protein name	Activity or Function	CAZy Family[1]
Precursor supply			
	Ugp1	UDPGlc pyrophosphorylase	
	Pmi40	phosphomannose isomerase	
	Sec53	phosphomannomutase	
	Psa1/Srb1/Vig9	GDP-Man pyrophosphorylase	
	Gfa1	glutamine: Fru-6-P amidotransferase	
	Gna1	GlcN-6-P N-acetylase	
	Agm1/Pcm1	GlcNAc phosphate mutase	
	Uap1/Qri1	UDPGlcNAc pyrophosphorylase	
	Rer2	cis-prenyltransferase (Dol_{10-14})	
	Srt1	cis-prenyltransferase (Dol_{19-22})	
	Dfg10	dehydrodolichol reductase	
	Sec59	Dol-kinase	
	Cwh8/Cax4	Dolichyl pyrophosphate phosphatase	
	Dpm1	GDP-mannose:dolichyl-phosphate Man-T	GT2
	Alg5	UDP-glucose:dolichyl-phosphate Glc-T	GT2
	Yea4	UDP-GlcNAc transporter	
	Vrg4/Vig4	GDP-Man transporter	
	Gda1	GDPase	
	Ynd1	Apyrase	
N-glycosylation			
	Alg7	UDP-GlcNAc: Dol-P GlcNAc-1-P-T	
	Alg13 + Alg14	UDP-GlcNAc: Dol-PP-GlcNAc β1,4-GlcNAc-T	GT1

Alg1	GDP-Man: Dol-PP-$GlcNAc_2$ β1,4-Man-T	GT33
Alg2	GDP-Man: Dol-PP-$GlcNAc_2Man$ α1,3-Man-T *and* GDP-Man: Dol-PP-$GlcNAc_2Man_2$ α1,6-Man-T	GT4
Alg11	GDP-Man: Dol-PP-$GlcNAc_2Man_3$ α1,2-Man-T *and* GDP-Man: Dol-PP-$GlcNAc_2Man_4$ α1,2-Man-T	GT4
Rft1	Candidate Dol-PP-oligosaccharide flippase	
Alg3	Dol-P-Man: Dol-PP-$GlcNAc_2Man_5$ α1,3-Man-T	GT58
Alg9	Dol-P-Man: Dol-PP-$GlcNAc_2Man_6$ α1,2-Man-T *and* Dol-P-Man: Dol-PP-$GlcNAc_2Man_8$ α1,2-Man-T	GT22
Alg12	Dol-P-Man: Dol-PP-$GlcNAc_2Man_7$ α1,6-Man-T	GT22
Alg6	Dol-P-Man: Dol-PP-$GlcNAc_2Man_9$ α1,3-Glc-T	GT57
Alg8	Dol-P-Man: Dol-PP-$GlcNAc_2Man_9Glc$ α1,3-Glc-T	GT57
Alg10	Dol-P-Man: Dol-PP-$GlcNAc_2Man_9Glc_2$ α1,2-Glc-T	GT59
Stt3	OST catalytic subunit	GT66
Wbp1	OST subunit	
Swp1	OST subunit	
Ost1	OST subunit	
Ost2	OST subunit	
Ost3	OST subunit; cysteine oxidoreductase	
Ost6	OST subunit; cysteine oxidoreductase	
Gls1/Cwh41	ER glucosidase I (α1,2 exoglucosidase); indirectly affects β1,6-glucan	GH63
Gls2/Rot2	ER glucosidase II (α1,3 exoglucosidase α-subunit); indirectly affects β1,6-glucan	GH31
Gtb1	ER glucosidase II (regulatory subunit)	
Mns1	ER α-mannosidase I	GH47
Htm1/Mnl1	ER-degradation enhancing a-mannosidase-like protein	GH47
Yos9	Lectin, recognizes α1,6-Man on glucosidase II product, targets misfolded protein for ERAD	
Png1	Cytosolic peptide N-glycanase	
Och1	Initiating α1,6-Man-T	GT32
Mnn9	M-Pol I α1,6-Man-T	GT62

	Van1	M-Pol I α1,6-Man-T	GT62
	Mnn9	M-Pol II α1,6-Man-T	GT62
	Anp1	M-Pol II α1,6-Man-T	GT62
	Mnn10	M-Pol II α1,6-Man-T	GT34
	Mnn11	M-Pol II α1,6-Man-T	GT34
	Hoc1	M-Pol II α1,6-Man-T	GT32
	Mnn2	α1,2-Man-T; Mnn1 subfamily; major role in mannan side chain branching	GT71
	Mnn5	α1,2-Man-T; Mnn1 subfamily; major role in mannan side chain branching	GT71
	Mnn4	Positive regulator of Man phosphorylation	
	Mnn6/Ktr6	α-Man-P-T; acts on N- and O-glycans in Golgi	GT15
	Mnn1	α1,3-Man-T; acts on N- and O-glycans in Golgi	GT71
	Kre2/Mnt1	α1,2-Man-T; acts on N- and O-glycans in Golgi	GT15
	Ktr1	α1,2-Man-T; acts on N- and O-glycans in Golgi	GT15
	Ktr2	α1,2-Man-T; acts on N-glycans in Golgi	GT15
	Ktr3	α1,2-Man-T; acts on N- and O-glycans in Golgi	GT15
	Yur1	α1,2-Man-T; acts on N-glycans in Golgi	GT15
	Ktr4	Putative α-ManT	GT15
	Ktr5	Putative α-ManT	GT15
	Ktr7	Putative α-ManT	GT15
	Gnt1	GlcNAc-T	GT8
	Vrg4	GDP-Man transporter	
	Gda1	GDPase	
	Ynd1	Apyrase	
O-mannosylation			
	Pmt1	Dol-P-Man: protein: O-Man-T; Pmt1 family	GT39
	Pmt2	Dol-P-Man: protein: O-Man-T; Pmt2 family	GT39

	Pmt3	Dol-P-Man: protein: O-Man-T; Pmt2 family	GT39
	Pmt4	Dol-P-Man: protein: O-Man-T; specific for membrane proteins	GT39
	Pmt5	Dol-P-Man: protein: O-Man-T; Pmt1 family	GT39
	Pmt6	Dol-P-Man: protein: O-Man-T; Pmt2 family	GT39
	Mnt2	α1,3-Man-T; Mnn1 subfamily; acts on O-glycans in Golgi	GT71
	Mnt3	α1,3-Man-T; Mnn1 subfamily; acts on O-glycans in Golgi	GT71
GPI anchoring			
	Gpi1	GPI-Gnt subunit	
	Gpi2	GPI-Gnt subunit	
	Gpi3	GPI-Gnt subunit, UDP-GlcNAc: Ptd-Ins α1,6-GlcNAc transferase	GT4
	Gpi15	GPI-Gnt subunit	
	Gpi19	GPI-Gnt subunit	
	Eri1	GPI-Gnt subunit	
	Ras2	Negative regulator of GPI-Gnt	
	Gpi12	GPI-Ins-deacetylase	
	Gwt1	GPI-Ins-acyltransferase	
	Gpi14	GPI-ManT-I: Dol-P-Man: GlcN-Ptd-(acyl)Ins α1,4-Man-T	GT50
	Pbn1	Putative subunit of GPI-Man-T-I	
	Arv1	Proposed to present GlcN-(acyl)PI to Gpi14	
	Mcd4	GPI-Etn-P-T-I	
	Gpi18	GPI-ManT-II: Dol-P-Man: Man-GlcN-Ptd-(acyl)Ins α1,6-Man-T	GT76
	Pga1	GPI-ManT-II subunit	
	Gpi10	GPI-Man-T-III: Dol-P-Man: Man_2-GlcN-Ptd-(acyl)Ins α1,2-Man-T	GT22
	Smp3	GPI-Man-T-IV: Dol-P-Man: Man_3-GlcN-Ptd-(acyl)Ins α1,2-Man-T	GT22
	Gpi13	GPI-Etn-P-T-III	
	Gpi11	Subunit of GPI-Etn-P-T-II and GPI-Etn-P-T-III	

Gpi7	GPI-Etn-P-T-II	
Gpi8	GPI transamidase catalytic subunit	
Gaa1	GPI transamidase subunit	
Gab1	GPI transamidase subunit	
Gpi16	GPI transamidase subunit	
Gpi17	GPI transamidase subunit	
Bst1	GlcN-(acyl)PI inositol deacylase	
Per1	Removes acyl chain at *sn*-2 position of protein-bound GPIs	
Gup1	MBOAT O-acyltransferase, transfers C_{26} acyl chain to *sn*-2 position of protein-bound GPIs	
Cwh43	Replaces GPI diacylglycerol with ceramide	
Cdc1	Homologue of mammalian PGAP5; possible GPI-Etn-P phosphodiesterase	
Ted1	Homologue of mammalian PGAP5; possible GPI-Etn-P phosphodiesterase	
Chitin and chitosan synthesis		
Chs1	Chitin synthase I catalytic protein	GT2
Chs2	Chitin synthase II catalytic protein	GT2
Chs3	Chitin synthase catalytic subunit	GT2
Cdk1	Mitotic protein kinase, phosphorylates Chs2	
Cdc14	Phosphoprotein phosphatase, dephosphorylates Chs2	
Dbf2	Mitotic exit kinase, phosphorylates Chs2	
Inn1	Localized to mother cell-bud junction with Chs2 and Cyk3, implicated in Chs2 activation	
Cyk3	Localized to mother cell-bud junction with Chs2 and Inn1, implicated in Chs2 activation	
Pfa4	Protein acyltransferase, palmitoylates Chs3	
Chs7	Chaperone required for ER exit of Chs3	
Rcr1	ER protein, small negatve effect on Chs3-dependent chitin synthesis	
Yea4	ER protein and UDP-GlcNAc transporter, *yea4Δ* has 65% of wild type levels of chitin.	
Chs5	Exomer component, involved in Chs3 trafficking	

Chs6	Exomer component, involved in Chs3 trafficking	
Chs4/Skt5	Prenylated protein that interacts with, activates, and anchors Chs3 to septin ring	
Bni4	Scaffold protein, tethers Chs3 and Chs4 to septins	
Shc1	Sporulation-specific Chs4 homologue	
Cda1	Chitin de-N-acetylase	
Cda2	Chitin de-N-acetylase	
β-1,3 glucan synthesis		
Fks1/Gsc1/Cwh53/ Etg1/Pbr1	Probable β1,3-glucan synthase, major role in vegetative cells	GT48
Fks2/Gsc2	Probable β1,3-glucan synthase, stress-induced, role in sporulation	GT48
Fks3	Probable β1,3-glucan synthase, role in sporulation	GT48
Rho1	GTPase; activator of Fks1 and Fks2	
β-1,6 glucan formation		
Kre5	Diverged UDP-Glc: glycoprotein Glc-T homologue	GT24
Rot1	Fungus-specific ER chaperone	
Big1	Fungus-specific ER chaperone	
Keg1	Fungus-specific ER chaperone	
Kre6	Resembles β-1,6/β-1,3 glucanases	GH16
Skn1	Sequence and functional Kre6 homologue; additional role in MIPC synthesis	GH16
Kre9	Fungus-specific O-mannosylated protein	
Knh1	Kre9 homologue	
Kre1	GPI-protein, secondary receptor for K1 killer toxin	
Glycosidases, cross-linking enzymes, and proteases		
Cts1	Endo-chitinase	GH18
Cts2	Chitinase	GH18
Exg1/Bgl1	Major exo-β-1,3-glucanase of the cell wall; soluble	GH5

	Exg2	GPI-anchored plasma membrane exo-β1,3-glucanase	GH5
	Ssg1/Spr1	Sporulation-specific exo-β-1,3-glucanase	GH5
	Bgl2	Endo-β1,3-glucanase; can make β1,6-linked Glc side branch	GH17
	Scw4	Endo-β1,3-endoglucanase-like	GH17
	Scw10	Endo-β1,3-endoglucanase-like	GH17
	Scw11	Endo-β1,3-endoglucanase-like	GH17
	Eng1/Dse4	Endo-β1,3-endoglucanase	GH81
	Eng2/Acf2	Endo-β1,3-endoglucanase	GH81
	Dcw1	GPI-protein, resembles α1,6-endomannanase	GH76
	Dfg5	GPI-protein, resembles α1,6-endomannanase; Dcw1 homologue	GH76
	Crh1	GPI-protein, chitin β-1,6/1,3-glucanosyltransferase	GH16
	Crh2/Utr2	GPI-protein, chitin β-1,6/1,3-glucanosyltransferase	GH16
	Crr1	GPI-protein, chitin β-1,6/1,3-glucanosyltransferase; sporulation-specific	GH16
	Gas1	GPI-protein, β-1,3-glucanosyltransferase	GH72
	Gas2	GPI-protein, β-1,3-glucanosyltransferase; sporulation specific	GH72
	Gas3	GPI-protein, β-1,3-glucanosyltransferase	GH72
	Gas4	GPI-protein, β-1,3-glucanosyltransferase; sporulation specific	GH72
	Gas5	GPI-protein, β-1,3-glucanosyltransferase	GH72
	Yps1	GPI-protein, yapsin aspartyl protease	
	Yps2/Mkc7	GPI-protein, yapsin aspartyl protease	
	Yps3	GPI-protein, yapsin aspartyl protease	
	Yps6	GPI-protein, yapsin aspartyl protease	
GPI-CWP			
	Ecm33	Sps2 family; structural/non-enzymatic	
	Pst1	Sps2 family; structural/non-enzymatic	
	Sps2	Sps2 family; structural/non-enzymatic; required for ascospore wall formation	

Sps22	Sps2 family; structural/non-enzymatic; required for ascospore wall formation
Cwp1	Tip1 family
Cwp2	Tip1 family
Tip1	Tip1 family; anaerobically induced
Tir1	Tip1 family; anaerobically induced
Tir2	Tip1 family; anaerobically induced
Tir3	Tip1 family; anaerobically induced
Tir4	Tip1 family; anaerobically induced
Dan1/Ccw13	Tip1 family; anaerobically induced
Dan4	Tip1 family; anaerobically induced
Sed1	Induced in stationary phase
Spi1	Induced by stress with weak organic acids; related to Sed1
Ccw12	Major role in stabilizing walls of daughter cells walls and mating projections
Ccw14/Ssr1	Inner cell wall protein
Dse2	Daughter cell specific, role in cell separation
Egt2	Daughter cell specific, role in cell separation
Fit1	Iron binding
Fit2	Iron binding
Fit3	Iron binding
Flo1	Flocculin
Flo5	Flocculin
Flo9	Flocculin
Flo10	Flocculin
Flo11/Muc1	Required for pseudohypha formation by diploids and agar invasion by haploids
Aga1	*MAT***a** agglutinin subunit, disulfide-linked to Aga2, which binds *MAT*α agglutinin Sag1
Fig2	Aga1-related adhesin

	Sag1	*MAT*α agglutinin
Non-GPI-CWP		
	Pir1/Ccw6	"Protein with internal repeat", ester-linked via Glu (originally Gln in repeats) to β1,3-glucan
	Pir2/Hsp150/Ccw7	"Protein with internal repeat", ester-linked via Glu (originally Gln in repeats) to β1,3-glucan
	Pir3/Ccw8	"Protein with internal repeat", ester-linked via Glu (originally Gln in repeats) to β1,3-glucan
	Pir4/Cis3/ Ccw5/Ccw11	One "internal repeat" sequence", ester-linked via Glu (originally Gln in repeats) to β1,3-glucan
	Scw3/Sun4	Member of SUN family
	Srl1	Acts in parallel with Ccw12 in pathway operative when regulation of Ace2 and polarized morphogenesis are defective

[1]CAZy glycosyltransferase (GT) and glycosylhydrolase (GH) families are defined in the Carbohydrate Active Enzymes database (http://www.cazy.org/) (Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., et al., 2009 The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res*. **37:** D233-238).