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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