The Sur7 Protein Regulates Plasma Membrane Organization and Prevents Intracellular Cell Wall Growth in *Candida albicans*

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The *Candida albicans* plasma membrane plays important roles in cell growth and as a target for antifungal drugs. Analysis of Ca-Sur7 showed that this four transmembrane domain protein localized to stable punctate patches, similar to the plasma membrane subdomains known as eisosomes or MCC that were discovered in *S. cerevisiae*. The localization of Ca-Sur7 depended on sphingolipid synthesis. In contrast to *S. cerevisiae*, a *C. albicans* *sur7* Δ mutant displayed defects in endocytosis and morphogenesis. Septins and actin were mislocalized, and cell wall synthesis was very abnormal, including long projections of cell wall into the cytoplasm. Several phenotypes of the *sur7* Δ mutant are similar to the effects of inhibiting β -glucan synthase, suggesting that the abnormal cell wall synthesis is related to activation of chitin synthase activity seen under stress conditions. These results expand the roles of eisosomes by demonstrating that Sur7 is needed for proper plasma membrane organization and cell wall synthesis. A conserved Cys motif in the first extracellular loop of fungal Sur7 proteins is similar to a characteristic motif of the claudin proteins that form tight junctions in animal cells, suggesting a common role for these tetraspanning membrane proteins in forming specialized plasma membrane domains.

INTRODUCTION

The plasma membrane carries out many complex functions in addition to acting as a protective layer around the cell. It is a highly regulated zone that must interface with the external environment and also interact with intracellular components to mediate processes such as endocytosis, secretion, and morphogenesis. Studies on plasma membrane organization in animal cells indicate that it is composed of discrete domains, including fluid domains where components readily diffuse and also specialized subdomains, such as lipid rafts and protein-organized barriers to diffusion (Brown and London, 2000; Nakada *et al.*, 2003; Hemler, 2005). The plasma membrane of the budding yeast *Saccharomyces cerevisiae* is composed of at least two distinct domains (Malinska *et al.*, 2003, 2004). One domain contains proteins that readily diffuse, such as the plasma membrane ATPase Pma1. Another plasma membrane domain appears as 300-nm punctate patches that are immobile. This latter domain was termed MCC (membrane compartment occupied by Can1), since it contains the Can1 arginine permease (Malinska *et al.*, 2003, 2004). Subsequent studies in *S. cerevisiae* have shown that these immobile punctate plasma mem-

brane domains also correspond to static sites of endocytosis that have been named eisosomes (Walther *et al.*, 2006).

The role of the eisosomes/MCC in endocytosis was elucidated in part by the discovery that these patches also include the Pil1 and Lsp1 proteins that function in endocytosis (Walther *et al.*, 2006). Pil1 and Lsp1 are paralogous cytoplasmic proteins that attach to the inner surface of the plasma membrane in patches that colocalize with the Sur7 transmembrane protein in the MCC (Walther *et al.*, 2006). Detection of other proteins that localize to eisosome/MCC patches indicates that these domains also carry out additional functions. For example, the sphingolipid-responsive Pkh1/2 protein kinases were found to associate with Pil1 and Lsp1 in eisosomes (Walther *et al.*, 2007; Luo *et al.*, 2008). The Pkh1/2 protein kinases are homologues of mammalian phosphoinositide-dependent kinases, which influence a variety of processes including endocytosis, cell wall integrity, actin localization, and response to heat stress (Dickson *et al.*, 2006; Dickson, 2008). Recent studies have also shown that the Pkh1/2 protein kinases appear to phosphorylate Pil1 and Lsp1 and to mediate the effects of sphingolipids on the regulation of the assembly and disassembly of eisosomes (Walther *et al.*, 2007; Luo *et al.*, 2008).

Other proteins that have been localized to eisosomes/MCC are proton symporters, such as Can1 (Malinska *et al.*, 2003, 2004). The proton symporters are distinct in that they are present in eisosomes in a manner that depends on the membrane potential (Grossmann *et al.*, 2007). The membrane potential was also required to observe more intense filipin staining of the eisosomes, which suggests that these domains have a special lipid composition in which ergosterol is either enriched or is more accessible to staining. It was suggested that segregation of the inward proton pumping activity of the symporters away from the outward proton pumping activity of the plasma membrane H⁺ATPase Pma1

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Abbreviations used: MCC, membrane compartment occupied by Can1; TMD, transmembrane domain.

may have physiological significance (Malinska *et al.*, 2003; Grossmann *et al.*, 2007).

Sur7 is thus far the only known transmembrane protein that is continuously localized to eisosomes/MCC, even in the absence of membrane potential (Grossmann *et al.*, 2007). The Sur7 sequence contains four putative transmembrane domains (TMDs). It lacks other obvious motifs, except for a Cys-containing sequence in extracellular loop 1 that is similar to a motif present at the same position in the claudin family of membrane proteins that will be described in this study (see *Discussion*). The claudin family members form membrane barrier domains in animal cells, such as tight junctions (Furuse and Tsukita, 2006). *SUR7* has been implicated in endocytosis because its overexpression partially suppresses the defects caused by mutation of *RV5161* or *RV5167* (Sivadon *et al.*, 1997), which encode BAR domain proteins that function in the scission phase of endocytosis (Toret and Drubin, 2006). Surprisingly, although Sur7 is thought to be a significant component of eisosomes in *S. cerevisiae*, present at an estimated several hundred copies per patch, its function is not known. Deletion of *SUR7* in *S. cerevisiae* caused only minor effects (Young *et al.*, 2002). Deletion of two other *SUR7*-related genes (*FMP45* and *YNL194C*), either singly or in combination, also did not have strong effects. The triple mutant showed a slight reduction in sporulation and an altered pattern of membrane sphingolipids (Young *et al.*, 2002). It is also not clear why Sur7 and the other components of eisosomes/MCC are immobile. The restricted mobility of Sur7 is not dependent on actin, microtubules, or the cell wall (Malinska *et al.*, 2004), although an earlier study suggested a possible role for the cell wall (Young *et al.*, 2002).

Candida albicans Sur7 was detected previously in the detergent-resistant fraction of the plasma membrane (Alvarez and Konopka, 2007) and was therefore investigated further in this study to better understand plasma membrane organization in this human fungal pathogen. *C. albicans* is an

interesting system for analysis of plasma membrane function, because it responds to environmental signals to grow in a variety of morphologies ranging from round budding cells to highly polarized hyphae (Sudbery *et al.*, 2004). Studies on the *C. albicans* plasma membrane also have important implications for understanding the mechanisms of the commonly used antifungal drugs (i.e., fluconazole, amphotericin, and echinocandins), which function by altering membrane sterol production, forming pores in the plasma membrane, and blocking cell wall growth (Odds *et al.*, 2003). Another potential advantage is that, in contrast to *S. cerevisiae*, the *C. albicans* genome appears to contain only two *SUR7*-related genes (orthologues of *SUR7* and *FMP45*). The results of these studies demonstrate that Sur7 affects a broad range of functions in *C. albicans* including endocytosis, localization of actin and septins, and is also required to prevent abnormal intracellular growth of the cell wall.

MATERIALS AND METHODS

Strains and Media

The *C. albicans* and *S. cerevisiae* strains used in this study (described in Table 1) were propagated on rich YPD medium or on synthetic medium essentially as described (Sherman, 1991). Uridine, 80 mg/l, was added to *C. albicans* cultures. The *C. albicans* *SUR7*-GFP strain was created by homologous recombination of green fluorescent protein (GFP) sequences into the 3' end of the *SUR7* open reading frame (ORF). The DNA used for the transformation was created by PCR using primers that contain ~70 base pairs of sequence homologous to the 3' end of the *SUR7* open reading frame to amplify a cassette containing GFP and a *URA3* selectable marker (Gerami-Nejad *et al.*, 2001). The colonies resulting from the transformation into *C. albicans* were then screened for GFP-positive cells by fluorescence microscopy. A similar approach was used to create an *LSP1*-GFP fusion in *C. albicans*. A *STE2*-GFP fusion expressed under the control of ADH promoter in plasmid pADH-*STE2*-GFP was linearized by digestion with *NotI* and then introduced into *C. albicans* using selection for *URA3*. Strains containing *CDC12*-GFP were created by linearizing plasmid pAW-*CDC12*-GFP by digestion with *BsaBI*, which was then integrated into the *CDC12* locus and selected for using the *URA3* marker as described previously (Warenda and Konopka, 2002).

A homozygous *sur7Δ* mutant (YJA10) was constructed in *C. albicans* strain BWP17 as described previously (Wilson *et al.*, 1999). In brief, PCR primers

Table 1. Strains used in this study

Strain	Parent	Genotype
<i>C. albicans</i> strains		
BWP17	Sc5314	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
DIC185	BWP17	<i>ura3Δ::λimm434/URA3 his1::hisG/HIS1 arg4::hisG/ARG4</i>
YJA10	BWP17	<i>sur7Δ::ARG4/sur7Δ::HIS1 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
YJA11	YJA10	<i>sur7Δ::ARG4/sur7Δ::HIS1 URA3/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
YJA12	YJA10	<i>sur7Δ::ARG4/sur7Δ::HIS1 SUR7::URA3 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
YJA13	YJA10	<i>sur7Δ::ARG4/sur7Δ::HIS1 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG ADH-STE2-GFP::URA3</i>
YJA14	YJA10	<i>sur7Δ::ARG4/sur7Δ::HIS1 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CDC12-GFP::URA3</i>
YJA15	BWP17	BWP17, except <i>SUR7</i> -GFP::URA3
YJA16	BWP17	BWP17, except <i>PIL1</i> -GFP::URA3
YJA17	YJA10	<i>sur7Δ</i> strain YJA10, except <i>PIL1</i> -GFP::URA3
YAW44	BWP17	BWP17, except <i>CDC12</i> -GFP::URA3
YAW54	BWP17	BWP17, except <i>pADH-STE2</i> -URA3
<i>S. cerevisiae</i> strains		
BY4741		<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>
BY4742		<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>
YJA24	BY4741	<i>BY4741 plus sur7::kanR ylr414c::HIS3 fmp45::LEU2 ynl194c::URA3</i>
YLD38	BY4742	<i>BY4742 plus SUR7-RFP::HIS3</i>
Ynlr414c-GFP	BY4741	<i>BY4741 plus YLR414C-GFP::HIS3</i>
YLD39		<i>BY4741/BY4742 diploid. MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 SUR7-GFP::HIS3 YLR414C-GFP::HIS3</i>

containing ~70 base pairs of sequence homologous to the sequences flanking the open reading frame of *SUR7* were used to amplify either the *ARG4* or *HIS1* selectable marker genes. Integration of the deletion cassettes at the appropriate sites was verified by PCR using combinations of primers that flanked the integration and also primers that annealed within the introduced cassettes. A strain in which the *sur7Δ* mutations were complemented by reintroduction of one wild-type copy of *SUR7* was constructed by PCR amplification of the genomic sequence from 1000 base pairs upstream of the initiator ATG to 300 bases downstream of the terminator codon of *SUR7*. This DNA fragment was then inserted between the *SacI* and *SacII* restriction sites of the *URA3* plasmid pDDB57 (Wilson *et al.*, 2000). The resulting plasmid was linearized in the promoter region by digestion with *BsrGI*, and integrated into the *sur7Δ* strain YJA10 using *URA3* selection to create a complemented strain called YJA12.

S. cerevisiae strains deleted for orthologues of *SUR7* were constructed by a successive set of gene deletions. The initial strain was obtained from the *S. cerevisiae* genome deletion strain collection and contained a *sur7::kanR* deletion. Next, PCR amplification cassettes were used to replace the YLR414C with *Schizosaccharomyces pombe his5* (Longtine *et al.*, 1998), FMP45 with *Candida glabrata LEU2* (plasmid template kindly provided by Dr. Linda Huang, University of Massachusetts, Boston, MA), and YNL194C with *C. albicans URA3* (Goldstein *et al.*, 1999). Heterologous genes were used as selectable markers to help promote integration at the targeted locus instead of at the position of the chromosomal copy of the marker gene. The presence of all four deletions mutations and the absence of the corresponding wild-type genes was verified by PCR. Similar results were obtained with independently derived mutants. Colocalization of Sur7-red fluorescent protein (RFP) and Ylr414c-GFP was carried out using strain YLD39, which was constructed by mating the Ylr414c-GFP strain from the yeast GFP-tagged collection (Huh *et al.*, 2003) to a Sur7-RFP strain (YLD38).

Sensitivity to antifungal drugs was assayed using Etest strips according to the directions of the manufacturer (AB Biodisk, Piscataway, NJ). Cells, 1×10^6 , were spread on a plate containing RPMI 1640 medium (Sigma, St. Louis, MO), 0.165 M MOPS, pH 7, and 2% dextrose. The plate was allowed to dry, and then an Etest strip was applied, and the plates were incubated at 37° for 48 h.

Fluorescence Microscopy

Cells used for analysis of GFP-tagged fusion proteins by microscopy were grown overnight to log phase. Fluorescence microscopy was used to detect GFP and differential interference contrast (DIC) optics were used to detect cell morphology. Endocytosis of the fluorescent dye FM 4-64 was carried out essentially as described previously (Vida and Emr, 1995). FM 4-64 (Invitrogen, Carlsbad, CA) was added to cells to a final concentration of 20 μ M, samples were incubated on ice for 15 min to stain the plasma membrane. The cells were washed and incubated for the indicated time at 30°, the internalization was stopped by adding sodium azide and sodium fluoride to a final concentration of 10 mM, and then the cells were analyzed by fluorescence microscopy. Ste2-GFP endocytosis was examined in cells incubated in the presence or absence of 5×10^{-7} M *C. albicans* α -factor mating factor pheromone (synthesized by Research Genetics, Huntsville, AL, and purified by Stony Brook University Proteomics Facility). Actin localization was determined using rhodamine-phalloidin essentially as described (Adams and Pringle, 1991). In brief, samples of cells were fixed for 1 h with 5% paraformaldehyde, incubated with 5 U of rhodamine-phalloidin (Invitrogen) for 1.5 h, and then viewed by fluorescence microscopy. Chitin staining was carried out by incubating cells with 20 ng/ml Calcofluor White as described (Pringle, 1991). To visualize the intracellular growth of cell wall in the *sur7Δ* mutant, cells were first permeabilized by washing with methanol and acetone and then stained with Calcofluor White, Aniline Blue (0.05%), or concanavalin A-FITC (20 μ g/ml). Filipin staining of *C. albicans* was performed essentially as described previously (Martin and Konopka, 2004) by treating cells with 200 μ g/ml filipin and then analyzing them immediately by fluorescence microscopy. Calcofluor, concanavalin FITC, and Filipin were purchased from Sigma, and Aniline Blue was purchased from J. T. Baker Chemical (Phillipsburg, NJ). Images were captured using an Olympus BH2 microscope (Melville, NY) equipped with a Zeiss AxioCam digital camera (Thornwood, NY).

Electron Microscopy

Samples used for transmission electron microscopy (TEM) were processed using standard techniques. Briefly, samples were collected in centrifuge tubes and fixed with 3% electron microscopy (EM) grade glutaraldehyde in 1× sodium cacodylate buffer (pH 7.4) at room temperature. After fixation, samples were washed in buffer and resuspended in an aqueous 4% permanganate solution, washed, stained with uranyl acetate, dehydrated through a graded series of ethyl alcohol and embedded in Epon resin. Ultrathin sections of 80 nm were cut with a Reichert-Jung (Heidelberg, Germany) UltracutE ultramicrotome and placed on formvar-coated slot copper grids. Sections were viewed with a FEI Tecnai12 BioTwinG2 electron microscope (Eindhoven, The Netherlands). Digital images were acquired with an AMT XR-60 CCD Digital Camera System. The TEM analyses were carried out at the Central Microscopy Imaging Center at Stony Brook University.

Microarray Analysis of Gene Expression

RNA was extracted from 3×10^8 cells using a RiboPure-Yeast RNA isolation kit (Ambion, Austin, TX). cDNA was synthesized using a Superscript II kit (Invitrogen) in a poly(T)-primed reaction containing a 3:2 mixture of amino-allyl-dUTP:dTTP plus dATP, dCTP, and dGTP. After RNase treatment, samples were purified on a silica gel column (Qiagen, Chatsworth, CA), coupled to NHS-reactive Cy3 or Cy5 dyes (GE Healthcare Bio-Sciences, Piscataway, NJ), and then dye incorporation was assayed spectrophotometrically. Control and experimental samples were mixed and hybridized in a Tecan (Durham, NC) HS4300 ProHybridization station to custom arrays from the Washington University Genome Sequencing Center that are spotted in triplicate with one 70-mer oligonucleotide for each open reading frame in the *C. albicans* ORF19 database (Brown *et al.*, 2006). Arrays were scanned using an Agilent G2505B microarray scanner.

Analysis of Sur7 Homologues

BLAST searches (Altschul *et al.*, 1990) were carried out to identify Sur7 homologues in the genome sequences of other organisms present at the Web site of the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov/BLAST/>) or the *S. cerevisiae* Genome Database (<http://www.yeastgenome.org/>). Multiple sequence alignments of the predicted Sur7 proteins and an evolutionary tree of their relatedness were carried out using Clustal W (Thompson *et al.*, 1994).

RESULTS

C. albicans Sur7 Localizes to Static Patches in the Plasma Membrane

To determine if the properties of Sur7 were conserved in *C. albicans*, Sur7-GFP was examined and found as expected to localize in punctate patches (Figure 1A). The Sur7-GFP patches appeared to be less prominent in the buds and emerging hyphae (germ tubes). This could be due to the delay in GFP folding (Malinska *et al.*, 2003), or it could indicate that the formation of new patches is regulated in buds and germ tubes (Walther *et al.*, 2007; Luo *et al.*, 2008). Similar to MCC/eisosome proteins in *S. cerevisiae*, the Ca-Sur7-GFP patches were extremely stable, with little or no mobility observed over 30 min (Figure 1B). The role of sphingolipids in the stability of the Sur7-GFP patches was examined by treating cells with myriocin to block an early step in sphingolipid synthesis (Figure 1C). Previous studies in *S. cerevisiae* showed that myriocin treatment shifted the localization of the Pil1 eisosome protein from membrane patches to the cytoplasm (Walther *et al.*, 2007; Luo *et al.*, 2008). Myriocin treatment of *C. albicans* disrupted the Sur7-GFP patches, but the Sur7-GFP remained in the plasma membrane as the fluorescence was maintained at the cell periphery (Figure 1C).

sur7Δ Mutant Is Defective in Endocytosis

The function of *SUR7* was examined by creating a homozygous deletion strain in which both copies of the gene were deleted from the diploid *C. albicans* genome (see *Materials and Methods*). Cells were then tested for ability to undergo endocytosis by following the trafficking of the lipophilic dye FM 4-64. Wild-type *SUR7* cells showed the expected result that FM 4-64 fluorescence was readily observed in the vacuolar membrane at 15 min, and trafficking to the vacuole was nearly complete by 30 min (Figure 2A). In contrast, the *sur7Δ* cells showed a slight delay in loss of FM 4-64 from the plasma membrane at 15 min and a more pronounced delay in the trafficking of small vesicle-sized particles to a larger vacuolar structure, because it took ~60 min before vacuolar staining was readily detected. This defect was specific for the *sur7Δ* mutation, because it could be largely complemented by reintroduction of one copy of the wild-type *SUR7* (Figure 2A). Thus, in contrast to *S. cerevisiae*, Ca-*SUR7* has a readily detectable function in endocytosis.

Figure 1. Sur7-GFP is present in static patches in the plasma membrane. *C. albicans* strain YJA15 carrying a *SUR7-GFP* fusion gene was analyzed by fluorescence microscopy. (A) Cells were grown in YPD medium to promote budding growth (left panel) and in GlcNAc medium to induce hyphal morphogenesis (middle and right panels). (B) Stability of the Sur7-GFP patches was examined by comparing pictures of the same cells taken 30 min apart. The merged images demonstrate the high degree of overlap of the patches. (C) Cells were treated with 5 or 25 μ M myriocin for 90 min to block sphingolipid synthesis, and then Sur7-GFP fluorescence was recorded. Bars, 10 μ m.

The *sur7 Δ* mutant was examined further by assaying ligand-induced endocytosis of Ste2, the α -factor mating pheromone receptor. *STE2-GFP* was expressed in the *MATa*/ α *C. albicans* cells under the control of the constitutive ADH promoter (see *Materials and Methods*). Treatment of wild-type *SUR7* cells with α -factor caused a dramatic shift in the localization of Ste2-GFP from the plasma membrane to the vacuole (Figure 2B). This shows that ligand-induced internalization of Ste2 can be used as a marker for endocytosis in *MATa*/ α *C. albicans* cells that lack a functional pheromone signal pathway, as has been done in *S. cerevisiae* (Zanolari *et al.*, 1992). The *sur7 Δ* cells were partially defective in internalizing Ste2-GFP in response to α -factor, indicating that they are defective in ligand-induced endocytosis in addition to their defect in steady-state internalization of FM 4-64 (Figure 2A). However, the ability to assess Ste2-GFP endocytosis in the *sur7 Δ* cells was affected by the altered localization of Ste2-GFP even in the absence of α -factor, which may be due to altered plasma membrane structure that will be described below.

Figure 2. *sur7 Δ* is defective in endocytosis. A control *C. albicans* strain (DIC185), a *sur7 Δ* strain (YJA11), and a complemented version of this strain in which one copy of *SUR7* was reintroduced (YJA12) were assayed for ability to undergo endocytosis in two different assays. (A) Cells were treated with the lipophilic dye FM4-64 and then monitored after 15, 30, and 60 min by fluorescence microscopy for the accumulation of the fluorescence in the vacuolar membrane. (B) Ligand-induced endocytosis of the α -factor mating pheromone receptor was examined in wild-type (YAW54) or *sur7 Δ* (YJA13) cells engineered to express *STE2-GFP* from the constitutive *ADH* promoter to avoid the need to convert the cells to the homozygous *MATa*/*MATa* genotype. Cells were incubated in the presence or absence of 5×10^{-7} M *C. albicans* α -factor for 60 min.

Lsp1 Localization Is Not Dependent on Sur7

S. cerevisiae Sur7 has been implicated in binding to the Lsp1 and Pil1 proteins that localize to eisosomes and function in endocytosis (Walther *et al.*, 2006, 2007). We therefore examined the localization of *C. albicans* Lsp1-GFP in the *sur7 Δ* mutant to determine if Sur7 is required to organize Lsp1 into eisosome patches. (We will follow the nomenclature of the *Candida* Genome Database and refer to orf19.3149 as the Lsp1 ortholog, even though it actually aligns slightly better with *S. cerevisiae* Pil1. Note that Lsp1 and Pil1 are very closely related in *C. albicans* [81% identity], making it difficult to determine which of these proteins is the ortholog of *S. cerevisiae* Lsp1). Lsp1-GFP showed the expected punctate distribution in wild-type *C. albicans*. Surprisingly, Lsp1-GFP

Figure 3. Lsp1-GFP localizes to plasma membrane patches in *sur7Δ* cells. (A) The localization of Lsp1-GFP was analyzed in wild-type *SUR7* cells (YJA16) and *sur7Δ* mutant cells (YJA17) by fluorescence microscopy. The middle of the cells was mainly in the focal plane, so the Lsp1-GFP appears primarily as patches around the periphery of the cell. (B) The stability of the Lsp1-GFP patches in the *sur7Δ* strain YJA17 was assessed by comparing images of the same cells recorded 30 min apart. The merge shows that the Lsp1-GFP patches remain essentially unchanged. The tops of the cells were in the focal plane to more readily observe the stability of the Lsp1-GFP patches.

also showed a punctate localization pattern similar to that in the *sur7Δ* cells (Figure 3A). To determine if the lack of Sur7 affected the immobile nature of the patches, images were recorded of the same cells after various times of incubation (Figure 3B). The Lsp1-GFP patches did not appear to shift position over a 30-min time course of analysis in either the wild-type or the mutant cells, indicating that Sur7 is not needed to restrict the mobility of the Lsp1-GFP patches. Preliminary studies using FRAP (fluorescence recovery after photobleaching) indicated there was no obvious effect of the *sur7Δ* mutation on the exchange of Lsp1-GFP subunits between eisosome patches (not shown). The rate of recovery of Lsp1-GFP into the photobleached regions was very slow in both the wild-type and *sur7Δ* mutant cells. Thus, although mutation of *SUR7* causes a phenotype in *C. albicans*, Sur7 is not required for clustering of Lsp1-GFP into eisosome patches on the plasma membrane.

Morphogenesis and Actin Localization Defects in *sur7Δ* Cells

Cultures of the *sur7Δ* mutant exhibited a variety of different morphologies ranging from relatively normal-looking cells to large cells with broad bud necks (Figure 4B). Time-lapse studies revealed that the large cells resulted at least in part from continued growth of mother cells during bud morphogenesis (not shown), indicating a breakdown in the mechanisms that usually polarize new growth to the bud. Consistent with this, rhodamine-phalloidin staining revealed that actin was mislocalized in the *sur7Δ* mutants. The cortical patches of actin that lie on the inner surface of the plasma membrane were primarily restricted to the buds of wild-type cells, but were present in both mother and daughter cells in the *sur7Δ* mutant (Figure 4B). Wild-type cells also contained a series of actin cables that span from the bud to the mother cell, which facilitate polarized growth by mediating transport of secretory vesicles to the bud (Figure 4A).

Figure 4. Actin polarization defect in *sur7Δ* cells. The control strain (DIC185; A) and the *sur7Δ* mutant (YJA11; B) were grown to log phase and then fixed and stained with rhodamine-phalloidin to detect actin localization (see *Materials and Methods*). Note that essentially all *sur7Δ* cells showed abnormal actin polarization, even those that appeared to have relatively normal morphology.

Obvious cables were not detected in the *sur7Δ* cells, but they did appear to contain faint structures that resembled a network of thin filaments.

Sur7 Is Needed to Restrict Septin Proteins to the Bud Neck

The wide bud necks and continued growth of the mother cells suggested that septin function might be compromised in the *sur7Δ* mutant. Septin proteins localize to the bud neck where they carry out several functions, including the recruitment of proteins that function in cytokinesis (Longtine and Bi, 2003; Douglas *et al.*, 2005; Gladfelter, 2006). Septins also function as a boundary domain that helps to maintain cell polarization by restricting plasma membrane proteins and cortical actin patches to the bud (Barral *et al.*, 2000; Takizawa *et al.*, 2000). Control *SUR7* cells showed expected localization of a GFP-tagged septin protein (Cdc12-GFP) to the bud neck (Figure 5). The septins appear as a single ring at early stages of the cell cycle and at later stages appear as a split ring. Septum formation occurs between the rings. In contrast, Cdc12-GFP was mislocalized in the *sur7Δ* cells and was found in patches throughout the bud and mother cell plasma membranes (Figure 5). Interestingly, in ~14% of the *sur7Δ* cells ($n = 407$) the septins appeared to form small ring structures at ectopic sites away from an existing bud neck, and some cells contained multiple rings. Cdc12-GFP detected at the bud neck in *sur7Δ* cells formed rings that appeared to be fainter and disrupted. Consistent with altered septin localization, the cells also showed an uneven distribution of chitin detected by Calcofluor staining (Figure 5).

Sur7 Prevents Intracellular Growth of Cell Wall

The altered morphology and cell wall organization of the *sur7Δ* mutant was analyzed in more detail by TEM. Sections of wild-type *SUR7* cells showed the expected smooth cell wall layer evenly surrounding the cell (Figure 6A). The

Figure 5. Abnormal septin localization at sites distal to the bud neck in *sur7Δ* cells. Septin localization was monitored by analysis of a Cdc12-GFP fusion protein in a wild-type control strain (YAW44; A) and *sur7Δ* strain (YJA14; B). (C) A higher magnification image of Cdc12-GFP localization in the *sur7Δ* strain. Top panels, Cdc12-GFP; middle panels, Calcofluor White stain of cell wall chitin; bottom panels, DIC images. Bars, 5 μ m.

sur7Δ mutant was dramatically different in that the cell wall was thicker and more uneven than in wild-type cells (Figure 6B). The septa were also abnormal looking and thicker, but appeared to contain the expected chitin-rich primary septum that is characteristically observed as a thin clear zone in EM analysis (Figure 6C). More significant, however, was the appearance of cell wall material in the cytoplasm of the *sur7Δ* mutant. In most cases the intracellular cell wall ap-

peared as torus-shaped structures, suggesting that they may represent tubes of cell wall growth through the cytoplasm. These structures were most frequently detected in mother cells, and to a lesser degree in buds and emerging hyphae. Comparison of cells with different amounts of abnormal cell wall suggested the following trends (Figure 6D). In cells with relatively minor cell wall abnormalities, the unusual cell wall growth was restricted to the periphery in the form of small invaginations into the cytoplasm. Cells with intermediate levels of unusual wall growth contained torus-shaped structures in the middle of the cell and occasionally spikes, as well as abnormalities around the cell periphery. Cells with high levels of unusual wall growth contained more of the abnormal structures that were also thicker, suggesting that they continue to grow over time.

Closer inspection of the cell wall suggested a possible sequence of events in formation of the unusual structures (Figure 6E). In some *sur7Δ* cells, the plasma membrane appeared to overlap and form convoluted structures. Similar plasma membrane abnormalities were also detected in which the space between the overlapping membranes is partially or completely filled in with cell wall material. Continued growth of these structures could lead to the formation of the larger cell wall abnormalities.

The structure of the abnormal cell wall growth in the *sur7Δ* cells was characterized further in cells that were permeabilized by methanol treatment to permit staining of intracellular cell wall chitin with Calcofluor. A variety of abnormal structures were stained in the *sur7Δ* cells, including long tubes that in some cases span most of the length of the cell (Figure 7B). These structures were never seen in the wild-type cells analyzed in the same way (Figure 7A). The structure of the intracellular cell wall growth was analyzed further by deconvolution microscopy to create a reconstructed three-dimensional image (see Supplementary Movie). This analysis indicated that at least some of the tubes in the *sur7Δ* mutant appeared to arise from previous sites of septum formation. Other structures were also detected, including some that resembled a wide sheet of cell

Figure 6. EM analysis of abnormal cell wall growth in *sur7Δ* cells. The wild-type strain DIC185 (A) and the *sur7Δ* strain YJA11 (B) were analyzed by TEM. Note in the *sur7Δ* mutant the abnormal appearance of torus-shaped structures of cell wall material and also the variations in cell wall thickness. Arrows indicate sites of invaginations of cell wall growth. (C) Higher magnification view of the septum of a *sur7Δ* mutant cell. Primary septum indicated by an arrowhead. (D) Representative *sur7Δ* cells exhibiting different amounts of abnormal intracellular wall growth. (E) Higher magnification images of abnormalities associated with the cell wall. Cells were analyzed by TEM as described in *Materials and Methods*. (A–D) black bars, 1 μ m; (E) white bars, 100 nm.

Figure 7. Characterization of intracellular cell wall growth in *sur7Δ* cells. The cell wall composition of wild-type strain DIC185 (A), *sur7Δ* strain YJA11(B), or the *sur7Δ* mutant strain YJA12 (C), in which one copy of *SUR7* was reintroduced, was assessed by staining as indicated with Calcofluor, Aniline Blue, and concanavalin A-FITC. Cells were washed with methanol and acetone to permeabilize the plasma membrane and allow the intracellular cell wall structures to be stained.

wall growth. To our knowledge, such extreme intracellular cell wall structures have not been reported previously and

may therefore reveal new aspects in the control of the spatial and temporal regulation of cell wall formation (see *Discussion*).

*Similarities between the Effects of *sur7Δ* and caspofungin*

To gain insight into the pleiotropic effects of the *sur7Δ* mutant, microarray analysis of gene expression was carried out to determine which gene pathways are affected (Table 2 and Supplementary Table). Nine of the 22 genes that were induced more than fivefold in the *sur7Δ* mutant relative to a control strain are predicted to encode cell wall-related proteins, including five glycosylphosphatidylinositol (GPI)-anchored proteins that should localize to the cell wall (*ECM331*, *PGA23*, *PGA13*, *RBR1*, and *PGA31*). The *CHT2* chitinase gene was repressed more than fivefold. Interestingly, the overall expression profile of the *sur7Δ* mutant shows significant overlap with the genes that are induced or repressed in response to caspofungin, an antifungal drug that blocks β -glucan synthase from forming the major β -1,3-glucan component of the cell wall. The changes in gene expression caused by caspofungin vary, depending on the conditions used. However, a core set of caspofungin-responsive genes was identified by comparing different data sets (Bruno *et al.*, 2006). Of the 23 genes that were induced more than or equal to twofold in the caspofungin core response, 17 genes were also induced more than or equal to twofold in the *sur7Δ* cells (Supplementary Table). This included 11 of the 12 most highly induced genes in the caspofungin core response; most differences involved genes that were only weakly induced by caspofungin (less than threefold).

Analysis of cells treated with Aniline Blue, a fluorescent dye that binds cell wall β -1,3-glucan, showed the expected staining of the extracellular cell wall layer in both wild-type and *sur7Δ* cells (Figure 7). Both the wild-type and mutant cells also stained with concanavalin A-FITC, which binds to

Table 2. Genes whose expression is altered ≥ 5 -fold in *sur7Δ* cells

ORF designation	Gene name	Fold change	Description
orf19.4082	DDR48	43.2	Stress-associated protein, DNA repair
orf19.4255	ECM331	26.1	Putative GPI-anchored protein
orf19.6420	PGA13	19.3	Putative GPI-anchored protein
orf19.3740	PGA23	18.4	Putative GPI-anchored protein
orf19.2125		13.6	
orf19.2060	SOD5	13.2	Superoxide dismutase
orf19.4477	CSH1	13.0	Member of aldo-keto reductase family
orf19.4688	DAG7	12.6	α -specific transcription, α -factor induced
orf19.756	SAP7	11.2	Secreted aspartyl proteinase
orf19.6202	RBT4	11.1	Similar to plant pathogenesis-related protein
orf19.5302	PGA31	9.6	Putative GPI-anchored protein
orf19.4279	MNN1	9.1	Putative α -1,3-mannosyltransferase
orf19.3548.1	WH11	8.5	Cytoplasmic protein expressed in white phase
orf19.24	RTA2	7.7	Stress-associated protein
orf19.535	RBR1	7.0	Putative GPI-anchored protein
orf19.1120	FAV2	6.9	Induced by mating factor in MTL α /MTL α opaque cells
orf19.675		6.5	Similar to cell wall proteins
orf19.2062	SOD4	6.4	Superoxide dismutase
orf19.6595	RTA4	6.2	Lipid transport to plasma membrane
orf19.251		5.4	Putative cell wall protein
orf19.5557	MNN4-4	5.0	Putative mannosyltransferase
orf19.909	STP4	5.0	Putative transcription factor
orf19.3895	CHT2	-5.3	Chitinase, GPI anchor protein
orf19.4777	DAK2	-5.8	Dihydroxyacetone kinase
orf19.111	CAN2	-5.9	Putative amino acid permease
orf19.5305	RHD3	-7.0	Putative GPI-anchored protein
orf19.2475	PGA26	-8.0	Putative GPI-anchored protein
orf19.4450.1		-10.0	
orf19.3707	YHB1	-21.8	Nitric oxide dioxygenase

mannoproteins in the cell wall. In contrast, Aniline Blue and concanavalin A failed to stain the internal cell wall growth that stained strongly with Calcofluor. Concanavalin A forms a ~104-kDa tetramer that may not pass efficiently through the cell wall. However, differential staining with Aniline Blue is not likely because of its inability to pass through the cell wall, because it has a lower MW than Calcofluor (787 vs. 917 Daltons) or other commonly used cell stains (e.g., rhodamine-phalloidin MW is ~1306 Daltons). Thus, the unusual cell wall synthesis observed in the *sur7* Δ mutant appears to be enriched in chitin. The unusual chitin synthesis in the *sur7* Δ mutant is therefore similar to the increased chitin synthesis that occurs at septa in *C. albicans* cells treated with a sublethal dose of caspofungin (Walker *et al.*, 2008). Furthermore, caspofungin treatment causes delocalization of septins in *C. albicans* similar to what is observed in the *sur7* Δ mutant (Aaron Mitchell, personal communication). Thus, the phenotypes of the *sur7* Δ mutant share several similarities to the inhibition of β -glucan synthesis.

The sensitivity of the *sur7* Δ mutant to caspofungin and two other commonly used antifungal drugs, amphotericin and fluconazole, was analyzed using Etest strips (Figure 8). The *sur7* Δ mutant showed only a slight increase in sensitivity to caspofungin (less than or equal to twofold), indicating that β -glucan synthase activity is not strongly impaired by mutation of *SUR7*. The *sur7* Δ mutant showed the same sensitivity as the wild-type strain for amphotericin, a polyene antibiotic that binds ergosterol and disrupts membrane integrity (Odds *et al.*, 2003). In contrast, the *sur7* Δ mutant

showed greatly increased sensitivity to fluconazole (about fivefold), a drug that blocks the Erg11 step of ergosterol synthesis (Odds *et al.*, 2003). This raises the interesting possibility that drugs targeting Sur7 could enhance the sensitivity to fluconazole, which could be important in dealing with the emergence of drug resistant strains.

Altered Hyphal Morphogenesis by *sur7* Δ Mutant

C. albicans cells were induced with serum to examine the role of Sur7 during hyphal morphogenesis. Wild-type cells induced in liquid culture with 10% serum formed narrow hyphae with long parallel walls. Although the majority of

Figure 8. Altered sensitivity of *sur7* Δ mutant to antifungal drugs. Etest strips were used to test the sensitivity to the indicated antifungal drug in the wild-type strain DIC185, the *sur7* Δ strain YJA11, and the *sur7* Δ mutant strain in which one copy of *SUR7* was reintroduced (YJA12). Cells were spread on a plate containing RPMI 1640 medium, the Etest strip was applied, and then the plates were incubated at 37° for 48 h. The Etest strips release a gradient of drug causing a zone of growth inhibition. Note the increased sensitivity of the *sur7* Δ mutant to fluconazole.

Figure 9. Hyphal morphogenesis defects of *sur7* Δ mutant. (A) Calcofluor staining of hyphal cells induced with serum for 75 min. (B) Filipin staining of cells induced with 100 mM GlcNAc for 70 min to form hyphae. (C) Cells were spotted on solid medium agar plates containing 4% serum or 2.5 mM GlcNAc as indicated and grown for 7 d at 37°C, and then the plates were rinsed to remove surface growth and reveal invasive hyphal growth. The strains used were wild-type strain DIC185, *sur7* Δ strain YJA11, and the *sur7* Δ mutant strain in which one copy of *SUR7* was reintroduced (YJA12).

the *sur7Δ* cells induced with serum appeared to take on a filamentous morphology, the width of the hyphae was variable and many cells resembled pseudohyphae (Figure 9). Calcofluor staining showed that the initial hyphal cells formed by the *sur7Δ* cells did not contain the abnormal intracellular cell wall growth, but this could be observed in older hyphal cells after longer times of growth (not shown). When *C. albicans* cells were induced with the amino sugar *N*-acetylglucosamine (GlcNAc), which is a weaker inducer of hyphal growth, the majority of *sur7Δ* cells were defective in forming hyphae and instead grew as enlarged rounded cells or pseudohyphae (Figure 9B). The hyphae formed by the *sur7Δ* cells were typically wider, consistent with a defect in highly polarized morphogenesis. In contrast, >95% of wild-type cells formed hyphae in response to GlcNAc. Filipin staining was used to assess the polarization of the plasma membrane in the hyphae, because this fluorescent polyene antibiotic preferentially stains the tips of wild-type hyphae (Martin and Konopka, 2004; Alvarez *et al.*, 2007). Filipin binds ergosterol, suggesting that polarization of ergosterol occurs in this region or a distinct lipid composition that provides better access of filipin to ergosterol. Enriched filipin staining could be detected at the tips of the *sur7Δ* hyphal cells, although it was quite variable in intensity and usually not as bright as in wild-type cells (Figure 9B), consistent with a defect in cell polarization.

Analysis of hyphal induction on solid medium agar plates revealed a defect in invasive growth for the *sur7Δ* mutant. Cells spotted on the surface of an agar plate were grown for 7 d, and then the surface growth was rinsed away to reveal invasive growth into the agar. The *sur7Δ* mutant formed filamentous growth into the agar, but the invasive zone was less than half as long as for the wild-type control cells on agar containing serum or GlcNAc as the inducer (Figure 9C).

Analysis of SUR7-Homologous Genes in *S. cerevisiae*

Given the obvious phenotypes of the *C. albicans* *sur7Δ* mutant, we reexamined the failure to observe a strong phenotype for *S. cerevisiae* mutants lacking *SUR7* and its paralogs. In contrast to *C. albicans*, which appears to only contain one *SUR7* paralog (*FMP45*), *S. cerevisiae* has been reported to contain two other paralogs (*FMP45* and *YNL194C*). The triple *sur7Δ fmp45Δ ynl194cΔ* mutant did not display strong defects (Young *et al.*, 2002), so we searched for and found a fourth *SUR7* paralog (*YLR414C*) in the *S. cerevisiae* genome. Consistent with this gene encoding a Sur7 paralog, a GFP-tagged version of the Ylr414c protein localized to punctate patches in the plasma membrane that showed extensive colocalization with Sur7-RFP (Figure 10A). This contrasts with a genome-wide study of protein localization that detected Ylr414c-GFP generally in the plasma membrane and cytoplasm (Huh *et al.*, 2003). These previous results may have been affected by the very weak signal of Ylr414c-GFP relative to Sur7-GFP and other limitations of high throughput studies. Surprisingly, the quadruple *S. cerevisiae* mutant (*sur7Δ fmp45Δ ynl194cΔ ylr414cΔ*) showed only minor defects, such as a greater tendency to form rounded cells (Figure 10B). Chitin and actin localization appeared to be similar to wild type, as did endocytosis of the dye FM 4-64. Thus, either there are additional Sur7-related proteins in *S. cerevisiae* or there has been evolutionary divergence such that they are not needed in *S. cerevisiae*.

Ylr414c displays low overall sequence identity with Sur7 (~20%), but contains four TMDs spaced at intervals similar to Sur7 and the other paralogs. The Ylr414c protein also contains most of the highly conserved residues that are present in other Sur7-homologous proteins from a broad

Figure 10. YLR414C encodes a new Sur7 paralog in *S. cerevisiae*. (A) Colocalization of Ylr414c-GFP and Sur7-RFP to punctate membrane patches in *S. cerevisiae*. The YLR414c-GFP was constructed as part of a genome-wide analysis of protein localization (Huh *et al.*, 2003). (B) The function of a new *SUR7*-related gene, *YLR414C*, was tested by constructing a quadruple deletion mutant (*sur7::kanR ylr414c::HIS3 fmp45::LEU2 ynl194c::URA3*) in *S. cerevisiae*. The mutant cells were compared with the wild-type control (BY4741) for staining with Calcofluor (chitin), rhodamine-phalloidin (actin), and FM 4-64 (endocytosis). FM 4-64 was allowed to internalize for 30 min before recording the image. (C) Multiple sequence alignment of the region surrounding the Cys-containing motif in extracellular domain 1 of Sur7 and human claudin proteins. Complete multiple sequence alignment of a larger set of fungal Sur7-related proteins is shown in Supplementary Figure S1 and an evolutionary tree of their relatedness is shown in Supplementary Figure S2.

range of fungal species (Supplementary Figure S1). In particular, Ylr414c contains a cysteine motif in the middle of the first extracellular loop that is highly conserved in other Sur7 family proteins (Figure 10C). This consensus sequence, WxxW/YxxC(7–10 aa)C, is very similar to the conserved motif found in the similar position of extracellular loop 1 of the claudin family of plasma membrane proteins, GLWxxC(8–10 aa)C (Van Itallie and Anderson, 2004). The claudin proteins are also similar to Sur7 in that they contain four TMDs. Members of the claudin family form special plasma membrane domains in animal cells, such as tight junctions (Van Itallie and Anderson, 2004; Furuse and Tsukita, 2006). Similarity between Sur7 and the broader family of claudin/PMP-22/EMP/MP20 proteins has significant implications for understanding the role of these plasma membrane proteins from yeast to humans (see *Discussion*).

DISCUSSION

The plasma membrane forms an important barrier that is also involved in dynamic processes, such as interfacing with the extracellular environment, morphogenesis, and cell wall biogenesis. In addition, most pharmaceutical drugs, including antifungal drugs, affect plasma membrane components. In spite of its importance, plasma membrane structure is not well understood because of technical limitations because of its hydrophobicity. However, recent studies have begun to identify specialized subdomains that include lipid rafts and protein domains. Therefore, the role of Sur7 was analyzed in this study because it has been localized in *S. cerevisiae* to membrane patches termed eisosomes or MCC (Young *et al.*, 2002; Malinska *et al.*, 2003). Sur7 was implicated in endocytosis in *S. cerevisiae* based on its localization to eisosomes (Walther *et al.*, 2006) and its ability to partially suppress the defects of an *rvs167* mutant when overproduced (Sivadon *et al.*, 1997). Surprisingly, mutational analysis of *SUR7* and its paralogs in *S. cerevisiae* have not identified strong phenotypes (Young *et al.*, 2002). In contrast, analysis of a *C. albicans* *sur7Δ* mutant revealed a wide range of phenotypes including mislocalization of actin and septins and also defects in endocytosis, morphogenesis, and cell wall growth. Thus, the analysis of *SUR7* in *C. albicans* further broadens the range of eisosome functions.

Sur7 Prevents Abnormal Intracellular Growth of Cell Wall in C. albicans

Of all the phenotypes displayed by the *sur7Δ* mutant, the most surprising was the intracellular growth of cell wall material. To our knowledge, such extensive ectopic cell wall growth has not been reported previously. The intracellular wall growth in the *Ca-sur7Δ* mutant was frequently in the shape of an elongated rod, with one end emanating from the cell cortex (Figure 6 and Supplementary Movie). EM analysis suggested that the rods are typically hollow, as evidenced by the appearance of rings of wall material in thin sections (Figure 5). In some cases there were also wide sheets of wall growth within the cell (Figures 6 and 7 and Supplementary Movie). Other types of abnormal wall growth were also observed, including variations in cell wall width, and septa that were unusually thick and misshapen. Comparison of cells with different amounts of intracellular cell wall material suggested that the abnormal wall growth initiated with convolution of the plasma membrane that resulted in inward growth of the outer cell wall into the cytoplasm. Continued growth of these structures apparently forms the elongated structures seen in the *Ca-sur7Δ* mutant.

The internal cell wall material stained prominently with Calcofluor, but not Aniline Blue, indicating that it is chitin-rich. Elevated chitin synthesis has been reported previously in *S. cerevisiae* and *C. albicans* as a compensatory response to cell wall damage (Garcia-Rodriguez *et al.*, 2000; Smits *et al.*, 2001; Walker *et al.*, 2008). For example, treatment of *C. albicans* with sublethal doses of a β -glucan synthase inhibitor, caspofungin, induces increased chitin synthesis that rescues viability by forming unusually thick, chitin rich septa (Walker *et al.*, 2008). These results suggest that the abnormal wall synthesis seen in the *sur7Δ* mutant is due to activation of chitin synthesis as part of a compensatory pathway. Consistent with this, *sur7Δ* cells share two other similarities with caspofungin-treated cells. One is the mislocalization of septins to ectopic sites (Figure 5 and Aaron Mitchell, personal communication). Abnormal septin localization was also seen in *fks1Δ* *S. cerevisiae* cells that are partially deficient in β -1,3-glucan synthase activity (Garcia-Rodriguez *et al.*, 2000). In addition, the *sur7Δ* mutant and cells treated with caspofungin show similar changes in gene expression. Eleven of the twelve genes that are most highly induced in the core caspofungin response were also induced in the *sur7Δ* mutant. In addition, the *CHT2* chitinase gene was repressed in both conditions. The more extensive cell wall synthesis seen in the *sur7Δ* mutant but not in cells treated with sublethal doses of caspofungin could be due to the different types of cell wall damage or to the fact that *sur7Δ* mutants induce additional genes related to cell wall function that are not part of the caspofungin Core response, such as genes that encode GPI-anchored proteins (*PGA13* and *PGA31*) and mannosyltransferases (*MNN1* and *MNN4-4*).

The similarities between the *sur7Δ* mutant and cells treated with caspofungin raise the possibility that Sur7 is needed for proper function of β -glucan synthase. Previous studies have also suggested a link between eisosomes and cell wall synthesis. A chemically modified echinocandin inhibitor of cell wall β -1,3-glucan synthesis was cross-linked to *C. albicans* Pil1, and in a high-throughput study of the *S. cerevisiae* proteome the Fks1 subunit of β -1,3-glucan synthase was found in a complex with Pil1 (Radding *et al.*, 1998; Gavin *et al.*, 2002; Odds *et al.*, 2003; Edlind and Katiyar, 2004). However, it is not clear that Sur7 plays a major role in the regulation of β -glucan synthase. The *sur7Δ* mutant was only slightly more sensitive to caspofungin (less than two-fold), and the cell wall β -glucan stained evenly with Aniline Blue, suggesting that the loss of Sur7 does not cause an overall defect in β -glucan synthase activity. Perhaps Sur7 plays a role in the spatial or temporal regulation of cell wall synthesis. Alternatively, the absence of Sur7 may only cause a similar stress response, and Sur7 may not directly affect β -glucan synthase activity. In this case Sur7 may be needed to act as a scaffold to recruit cell wall regulators such as the Pkh1/2 kinases, and in the absence of Sur7 a signal is induced that mimics cell wall damage, leading to activation of a compensatory pathway.

The mislocalization of septins to ectopic sites away from the bud neck in the *sur7Δ* mutant is likely to be directly involved in the abnormal morphogenesis and cell wall synthesis (Figure 5). Septin localization in wild-type cells is tightly restricted to the bud neck, where the septins form a ring on the inner surface of the plasma membrane that carries out several functions (Gladfelter *et al.*, 2001; Douglas *et al.*, 2005; Gladfelter, 2006). One is to act as a barrier that restricts actin patches and plasma membrane proteins to the bud (Barral *et al.*, 2000; Takizawa *et al.*, 2000). Thus, abnormal septin ring function could help contribute to the mislocalization of actin seen in *sur7Δ* mutants. Another important

septin function is to recruit chitin synthase to the bud neck. This suggests that septins present at ectopic sites could act to nucleate cell wall growth at these sites away from the bud neck. In support of the link between altered septin localization and intracellular wall growth, certain *S. cerevisiae* septin mutants and a *cla4Δ* mutant, which is a regulator of septins, display ectopic cell wall growth that is similar to the spikes of wall growth seen in the *sur7Δ* mutant (Roh *et al.*, 2002; Schmidt *et al.*, 2003).

Role of Sur7 in Endocytosis

The *sur7Δ* mutant was partially defective in endocytosis, but surprisingly there was only a slight lag in internalization of the FM 4-64 dye from the plasma membrane. The more pronounced defect was a delay in vesicles trafficking to the vacuole (Figure 2). Because Sur7 is present at the plasma membrane, it seems likely that this latter defect was an indirect effect, perhaps caused by the mislocalization of actin or other components. Previous studies raised the possibility that Sur7 might act as a scaffold for the formation of eisosomes by interacting with the cytoplasmic Pil1 and Lsp1 proteins in *S. cerevisiae* (Walther *et al.*, 2006; Grossmann *et al.*, 2007). It was therefore surprising that Lsp1-GFP still localized to punctate patches in the *C. albicans sur7Δ* mutant. This indicates that other factors regulate the formation of these clusters and their recruitment to the plasma membrane.

S. cerevisiae Sur7 Family Members

In view of the strong phenotypes displayed by the *C. albicans sur7Δ* mutant, it was surprising that in *S. cerevisiae* a triple deletion of *SUR7* and two paralogous genes caused only mild defects in sporulation and a change in sphingolipid species (Young *et al.*, 2002). We identified an additional Sur7 paralog (YLR414C) that also localized to punctate patches in the plasma membrane (Figure 10) and is reportedly induced by cell wall damage (Rodriguez-Pena *et al.*, 2005). However, the *S. cerevisiae* quadruple mutant (*sur7Δ, fmp45Δ, ynl194cΔ, ylr414cΔ*) showed only a slight defect in morphology and did not display an obvious cell wall defect. Perhaps the roles for Sur7 have diverged in different fungal species or that there are yet other *SUR7*-related genes in *S. cerevisiae*. Homology searches detected only two Sur7-related proteins encoded in the *C. albicans* genome (Ca-Sur7 and Ca-Fmp45). Ca-FMP45 was not highly expressed in microarray analysis of gene expression of either wild type or the *sur7Δ* mutant. Thus, *C. albicans* may be more dependent on *SUR7* than *S. cerevisiae*.

Sur7 Protein Family

Alignment of fungal Sur7 proteins revealed a conserved Cys-containing motif WxxW/YxxC(7–10 aa)C present in the middle of extracellular loop 1 between TMDs 1 and 2 (Figure 10C and Supplementary Figure S1). A similar GLWxxC(8–10 aa)C motif is present in extracellular loop 1 of the claudin family of proteins that also contain four TMDs (Van Itallie and Anderson, 2004; Furuse and Tsukita, 2006), suggesting that there are important structural and functional similarities between Sur7 and claudin proteins. The claudin proteins associate to form tight junctions in animal cells, which are specialized membrane barrier domains (Anderson *et al.*, 2004). The GLWxxC(8–10 aa)C motif is also present in a broader range of plasma membrane proteins (pfam00822) that are all thought to function in part by interacting with other membrane proteins (Van Itallie and Anderson, 2004, 2006).

The conserved Cys motif in extracellular loop 1 helped to reveal the new Sur7 paralog in *S. cerevisiae* (Ylr414c). Inter-

estingly, Ylr414c contains a Cys-consensus motif in extracellular loop 1 that is an exact match to the claudin motif (Figure 10C). Ylr414c is also the Sc-Sur7 family member that is most similar to the only Sur7-family protein detected in *S. pombe* and in the more divergent Basidiomycete fungi (Supplementary Figure S2).

A distinct Cys-containing motif, GxxGxC(8–20 aa)C, was found in the Fig1 family of fungal proteins that also contain four TMDs (Zhang *et al.*, 2006). *S. cerevisiae* Fig1 is induced by mating pheromone and functions in a low-affinity calcium uptake system, cell fusion during mating, and in mating pheromone-induced cell death (Muller *et al.*, 2003; Zhang *et al.*, 2006). Fig1 localizes the plasma membrane, but is not detected in punctate patches.

The Sur7, Fig1, and claudin proteins differ structurally from the tetraspanins, which comprise another major family of proteins with four TMDs. Tetraspanins contain only a small first extracellular loop and a larger second extracellular loop with a distinct type of Cys-containing motif (Hemler, 2005). The Cys residues in the tetraspanin motif form disulfide bonds, and it is therefore predicted that the Cys residues in the consensus sequence in the Sur7, Fig1, and claudin proteins also form disulfide bonds. Tetraspanins are thought to recruit other proteins to specialized membrane domains and affect a wide range of processes in animal cells including signal transduction, cell adhesion, and formation of membrane microdomains (Hemler, 2005). There are yet other families of proteins that also contain four TMDs, including the connexins and occludins. Thus, recognition of the common functional and structural features of the various types of membrane proteins with four TMDs should help to define their plasma membrane function in a wide range of cell types from yeast to humans.

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