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Hong Wang, Lois M Douglas, Vishukumar Aimanianda, Jean-Paul Latge, James Konopka. The *Candida Albicans* Sur7 Protein Is Needed for Proper Synthesis of the Fibrillar Component of the Cell Wall That Confers Strength. *Eukaryotic Cell*, 2011, 10 (1), pp.72-80. 10.1128/EC.00167-10 . pasteur-02531510

**HAL Id: pasteur-02531510**

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1     **The *C. albicans* Sur7 protein is needed for proper synthesis of the fibrillar component of**  
2   **the cell wall that confers strength**

3

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21    Running title: *C. albicans* Sur7 is needed for cell wall morphogenesis

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23

1 **ABSTRACT**

2           The *Candida albicans* plasma membrane plays important roles in interfacing with the  
3 environment, morphogenesis, and cell wall synthesis. The role of the Sur7 protein in cell wall  
4 structure and function was analyzed, since previous studies showed that this plasma membrane  
5 protein is needed to prevent abnormal intracellular growth of the cell wall. Sur7 localizes to  
6 stable patches in the plasma membrane, known as MCC, that are associated with eisosome  
7 proteins. The *sur7Δ* mutant cells displayed increased sensitivity to factors that exacerbate cell  
8 wall defects, such as detergent (SDS) and the chitin-binding agents Calcofluor White and Congo  
9 Red. The *sur7Δ* cells were also slightly more sensitive to inhibitors that block the synthesis of  
10 cell wall chitin (nikkomycin Z) and  $\beta$ -1,3 glucan (caspofungin). In contrast, Fmp45, a paralog of  
11 Sur7 that also localizes to punctate plasma membrane patches, did not have a detectable role in  
12 cell wall synthesis. Chemical analysis of cell wall composition demonstrated that *sur7Δ* cells  
13 contain decreased levels of  $\beta$ -glucan, a glucose polymer that confers rigidity to the cell wall.  
14 Consistent with this, *sur7Δ* cells were more sensitive to lysis, which could be partially rescued by  
15 increasing the osmolarity of the medium. Interestingly, Sur7 is present in static patches, whereas  
16  $\beta$ -1,3-glucan synthase is mobile in the plasma membrane and often associated with actin patches.  
17 Thus, Sur7 may influence  $\beta$ -glucan synthesis indirectly, perhaps by altering the function of the  
18 cell signaling components that localize to the MCC and eisosome domains.  
19

## 1 INTRODUCTION

2 *Candida albicans* is the most common human fungal pathogen. Although it is a  
3 commensal organism of humans, under certain conditions it causes life-threatening infections in  
4 susceptible individuals (18, 27). One feature that underlies the pathogenesis of *C. albicans* is its  
5 ability to grow in different morphologies including buds, elongated pseudohyphae, and long  
6 filamentous hyphal cells (35). Induction of the hyphal form correlates with increased expression  
7 of virulence factors, including adhesin proteins that promote biofilm formation and attachment to  
8 human cells, secreted hydrolytic enzymes that facilitate invasive growth and inactivate  
9 components of the complement pathway, and anti-oxidant enzymes that counteract the immune  
10 system (5, 7, 20, 40). The role of the plasma membrane has been under investigation in *C.*  
11 *albicans* pathogenesis because of the critical roles that it plays in the production of virulence  
12 factors and in transducing information and nutrients from the extracellular environment. The  
13 plasma membrane is also involved in dynamic cellular processes, such as secretion, endocytosis,  
14 and cell wall biogenesis that are essential for proper morphogenesis and viability. The critical  
15 role of the plasma membrane is highlighted by the fact that it is the target of the most commonly  
16 used antifungal drugs (28).

17 The *S. cerevisiae* plasma membrane is composed of at least two different subdomains  
18 (24, 25). One domain termed MCP (Membrane Compartment occupied by Pma1) contains  
19 proteins that readily diffuse, such as the plasma membrane ATPase Pma1. Another domain  
20 appears as 300 nm-sized patches that are immobile in the membrane. This latter domain was  
21 termed MCC (Membrane Compartment occupied by Can1), since it contains the Can1 arginine  
22 permease (24, 25). These static MCC domains are distinct from the mobile cortical actin patches  
23 or the finger-like invaginations that form at sites of endocytosis. Subsequent studies identified

1 other symporters in the MCC and also two families of proteins that are thought to contain four  
2 transmembrane domains (TMDs) (15, 17). The best-known examples of these two different  
3 families of tetra-spanning proteins are Nce102 and Sur7. Nce102 has been implicated in  
4 sphingolipid signaling that regulates formation of the MCC domains (15). The function of Sur7  
5 in *S. cerevisiae* is not clear, perhaps because of genetic redundancy since there are at least three  
6 paralogs of Sur7 (Fmp45, Ylr414c, and Pun1/Ynl194c) (2, 44). Mutation of the *SUR7* family  
7 members does not cause strong phenotypes under standard growth conditions (2, 44), although  
8 Pun1 was recently shown to be important for proper response to nitrogen stress (43).  
9 Pun1/Ynl194c is more divergent than the other members of the Sur7 family in *S. cerevisiae*,  
10 including differences in the Cys-containing motif in extracellular loop 1 of Sur7 family proteins  
11 and at the analogous position in the mammalian Claudin proteins found at tight junctions (2). In  
12 contrast, *C. albicans* contains just two members of this family, Sur7 and Fmp45. This suggests  
13 that the functions of Sur7 family members may have diverged between *S. cerevisiae* and *C.*  
14 *albicans*.

15         The structure of the MCC domains was broadened by identification of a complex of  
16 cytoplasmic proteins known as eisosomes that associate with the inner surface of the plasma  
17 membrane at MCC sites (39). Eisosomes have been implicated in endocytosis, but it is not clear  
18 how these static domains relate to sites of endocytosis mediated by actin patches, since these  
19 regions appear to be distinct in the plasma membrane (39, 44). However, there is likely to be at  
20 least an indirect link. Two proteins that localize to eisosomes, Pil1 and Lsp1, are needed for  
21 proper rates of endocytosis (39). Pil1 and Lsp1 associate with the Pkh1/2 protein kinases (23,  
22 38) that influence endocytosis and a variety of other processes including cell wall integrity, actin  
23 localization, and response to heat stress (10, 23). Pkh1/2 also mediate the effects of

1 sphingolipids on the regulation of the assembly and disassembly of eisosomes by  
2 phosphorylating Pil1 and Lsp1 (23, 38).

3         Analysis of a *C. albicans sur7Δ* (*Ca-sur7Δ*) mutant has implicated the MCC domains in  
4 the regulation of cell wall synthesis. In contrast to *S. cerevisiae*, a *Ca-sur7Δ* mutant displayed  
5 obvious defects in bud and hyphal morphogenesis (1-3). Interestingly, the mutant cells showed  
6 an unusual phenotype in that there were large intracellular regions of cell wall growth. The  
7 abnormal cell wall synthesis was suggested to be due in part to a defect in  $\beta$ -1,3-glucan  
8 synthesis, since the *sur7Δ* mutant showed changes in gene expression that overlapped with those  
9 observed when cells are treated with caspofungin, an inhibitor of cell wall  $\beta$ -1,3-glucan synthase  
10 (2). In addition, septins were mislocalized in *sur7Δ* cells, analogous to the altered localization of  
11 septins caused by treatment of cells with caspofungin (4). Therefore, *C. albicans sur7Δ* and  
12 *fmp45Δ* mutants were analyzed in this study to determine how the structure and function of the  
13 cell wall was altered. The results demonstrated that Sur7 is important for resistance to conditions  
14 that perturb cell wall function and also for the proper synthesis of the  $\beta$ -1,3-glucan component of  
15 the cell wall.

16

## 1 MATERIALS AND METHODS

2           **Strains and Media.** The *C. albicans* strains were propagated on rich YPD medium  
3 (glucose 2%, peptone 1% and yeast extract 2%) or on SD (Yeast Nitrogen Base synthetic  
4 medium with dextrose), essentially as described (34). 80 mg/liter uridine was added to cultures  
5 of *ura3Δ* cells. Hyphal morphogenesis was monitored by growing cells at 37°C in media  
6 containing 10% bovine calf serum. Invasive growth was assayed by spotting  $3 \times 10^3$  cells onto  
7 agar medium containing 4% bovine calf serum and then incubating the plates for 7 days at 37°C.

8           The *C. albicans sur7Δ* homozygous deletion mutant and the *sur7Δ + SUR7*  
9 complemented strain were constructed previously in *C. albicans* strain BWP17 (2). The *fmp45Δ*  
10 homozygous deletion mutant was constructed using similar methods. In brief, PCR primers that  
11 contain ~70 bp of sequence homologous to the 5' and 3' ends of the *FMP45* open reading frame  
12 were used to amplify a cassette containing either the *ARG4* or the *HIS1* selectable marker genes  
13 (41). The *sur7Δfmp45Δ* double mutants were constructed using the UAU method (13). The  
14 starting strain for this procedure was YHXW8 (*fmp45::HIS1 fmp45::NAT*), which was  
15 constructed in part by using the SAT-flipper (32). One allele of *SUR7* was then deleted by  
16 homologous recombination with the UAU cassette and identified after selection on medium  
17 lacking arginine. The UAU cassette consists of *ARG4* gene flanked by the 5' and 3' portions of  
18 the *URA3* gene (13). The mutant cells were then grown non-selectively to permit the  
19 endogenous recombination pathways to replace the other allele of *SUR7* to generate a  
20 *sur7::UAU/sur7::UAU* homozygous deletion. Segregants were then isolated on medium lacking  
21 Arg and Ura to identify cells that underwent a further recombination event that converts one  
22 UAU allele into a functional *URA3* gene by looping out the *ARG4* sequences. More than 30% of  
23 the Arg<sup>+</sup> Ura<sup>+</sup> colonies contained a homozygous deletion *sur7::UAU/sur7::URA3* and lacked the



1 wild-type *SUR7* gene, which is similar to the frequency reported for other non-essential genes  
2 (13). Integration of the deletion cassettes at the appropriate sites was verified by PCR using  
3 combinations of primers that flanked the integration site and also primers that annealed within  
4 the introduced cassettes.

5 The 3' ends of the *SUR7* and *FMP45* open reading frames were fused to GFP via  
6 homologous recombination using previously described methods (45). PCR primers containing  
7 ~70 bp of sequence homologous to the 3' end of the *SUR7* or *FMP45* open reading frames were  
8 used to amplify a cassette containing a more photostable version of eGFP (CaGFP $\gamma$ ) and a *URA3*  
9 selectable marker (45). The Ura<sup>+</sup> colonies resulting from the transformation of the cassette into  
10 *C. albicans* were then screened for GFP-positive cells by fluorescence microscopy and  
11 confirmed by PCR.

12 **Microscopy.** Cells used for microscopic analysis of Sur7-GFP and Fmp45-GFP were  
13 grown overnight to log phase. Fluorescence microscopy was used to detect GFP and Differential  
14 Interference Contrast (DIC) optics was used to detect cell morphology. Chitin staining was  
15 carried out by incubating cells with 40 ng/ml Calcofluor White (Sigma-Aldrich) as described  
16 (29). In order to visualize the intracellular growth of cell wall, cells were first permeabilized by  
17 washing with methanol and acetone, and then stained with Calcofluor White. Covalent labeling  
18 of the cell walls with the fluorescent dye Fluorescein was carried out by washing cells 3 times in  
19 phosphate buffered saline (PBS), resuspending at 10<sup>8</sup> cells/ml in PBS, and then adding  
20 Fluorescein-5-maleimide (FM) (Invitrogen) to a final concentration of 0.2  $\mu$ g/ml. The cells were  
21 incubated for 15 min, washed 3 times with PBS, and then resuspended in YPD medium.  
22 Labeling the cells with FM in this manner did not have detectable effects on growth. At different  
23 times after FM labeling, the cells were permeabilized as described above and then stained with 4

1  $\mu\text{g/ml}$  Calcofluor White. Cell integrity was analyzed by mixing equal volumes of cell culture  
2 and a solution of 0.4% Trypan Blue (Sigma-Aldrich), incubating for 5 min, and then visualizing  
3 by light microscopy to identify lysed cells that stained blue. Images were captured using an  
4 Olympus BH2 microscope equipped with a Zeiss AxioCam digital camera.

5 **Drug and chemical sensitivity.** Sensitivity to SDS, Congo Red, Calcofluor White, and  
6 hygromycin was tested by spotting dilutions of cells onto YPD plates prepared with the indicated  
7 concentration of the corresponding chemical. Three  $\mu\text{l}$  of a ten-fold dilution series of cells was  
8 spotted on the surface of the plates. The plates were then incubated at  $37^\circ\text{C}$  for the indicated  
9 time and photographed. The sensitivity of cells to nikkomycin Z, rapamycin, and tunicamycin  
10 was assayed by testing the effects of a serial dilution series of drug concentrations in 96 well  
11 plates. Cells were adjusted to  $1 \times 10^4$  cells/ml in SD medium, plated in the wells, and then a two-  
12 fold dilution series of the indicated drug was applied. The plates were incubated at  $37^\circ\text{C}$  for two  
13 days, and then the extent of growth was quantified with a spectrophotometer. Sensitivity to  
14 fluconazole, amphotericin and caspofungin was assayed using Etest strips according to the  
15 manufacturer's directions (bioMérieux, Inc., Durham, N.C.). In brief,  $1 \times 10^6$  cells were spread  
16 on an agar plate containing RPMI 1640 medium (Sigma-Aldrich), 0.165 M MOPS pH 7, and 2%  
17 dextrose, an Etest strip was applied, and then the plates were incubated at  $37^\circ\text{C}$  for 48 hrs.

18  
19 **Chemical analysis of cell wall composition.** Strains were adjusted to 0.01 O.D. 600 nm  
20 and then grown in YPD at  $30^\circ\text{C}$  for 15 h. Cells were washed twice with water and boiled in 50  
21 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, 2% SDS and 40 mM  $\beta$ -mercaptoethanol  
22 for 1 h, twice. The sediment obtained after centrifugation (3600 rpm, 10 min) was washed five  
23 times with water and then incubated in 1 M NaOH containing 0.5 M  $\text{NaBH}_4$  at  $65^\circ\text{C}$  for 1 h,

1 twice. The insoluble pellet obtained upon centrifugation (3600 rpm, 10 min) was washed with  
2 water to neutrality and freeze-dried (alkali-insoluble (AI) fraction). The supernatant fraction was  
3 then neutralized, dialyzed against water, and freeze-dried (alkali-soluble (AS) fraction).  
4 Monosaccharide composition was determined by gas-liquid chromatography (GLC, Perichrom  
5 PR2100 Instrument (Perichrom, Saulx-les-Chartreux, France) equipped with flame ionization  
6 detector (FID) and fused silica capillary column (30 m x 0.32 mm id) filled with BP1) using  
7 meso-inositol as the internal standard. Derivatized hexoses (alditol acetates) were obtained after  
8 hydrolysis (4N trifluoroacetic acid/8N hydrochloric acid, 100°C for 4h) of the AI- and AS-  
9 fractions followed by reduction and per-acetylation. Monosaccharide composition (percent) was  
10 calculated from the peak areas with respect to that of internal standard (14).

## 1 RESULTS

2           **Sur7-GFP and Fmp45-GFP localize to punctate plasma membrane domains in *C.***  
3 ***albicans*.** Analysis of the *C. albicans* genome detected only one paralog of Sur7, named Fmp45,  
4 which shows 37% identity and 54% similarity to the Sur7 sequence. In contrast, *S. cerevisiae*  
5 contains at least three paralogs of Sur7 (Fmp45, Ylr414c, and Pun1/Ynl194c). Although the *C.*  
6 *albicans* paralog of Sur7 was previously named Fmp45, it shows approximately equal amino  
7 acid sequence relatedness to *S. cerevisiae* Fmp45 and Ynl194c (31% identity). To determine if  
8 Ca-Fmp45 was present in punctate patches in the plasma membrane, we examined the  
9 localization of proteins that were fused to GFP. For this analysis, a recently described version of  
10 GFP (GFP $\gamma$ ) that is more photostable (45) was fused to the C-terminal coding sequences of *SUR7*  
11 and *FMP45*. As expected, Sur7-GFP was detected in a punctate pattern in the plasma membrane  
12 in mother cells, but was only weakly detected in new buds (Fig. 1A). Fmp45-GFP was also  
13 detected in punctate structures in mother cells, in agreement with previous reports for Fmp45-  
14 GFP in *S. cerevisiae* and *C. albicans* (3, 44). In contrast to Sur7-GFP, however, we also detected  
15 Fmp45-GFP in new buds. The Fmp45-GFP present in small buds was distributed in a more  
16 homogeneous fashion and was not concentrated into punctate domains as it was in mature cells  
17 (Fig. 1A). Thus, Sur7-GFP and Fmp45 give similar, but not identical localization patterns.

18  
19           **Analysis of cell wall localization in *sur7 $\Delta$*  and *fmp45 $\Delta$*  cells.** To determine whether  
20 Fmp45 functions in cell wall morphogenesis, a homozygous *fmp45 $\Delta$*  deletion mutant was  
21 constructed in the *C. albicans* BWP17 strain. Microscopic analysis of live cells did not reveal  
22 obvious morphological defects for the *fmp45 $\Delta$*  cells; they formed buds (Fig. 2A) and hyphae  
23 (data not shown) that were similar to the wild type control strain. The *fmp45 $\Delta$*  cells also did not

1 contain the intracellular growth of the cell wall seen in the *sur7Δ* mutant (Fig. 2A, lower panels)  
2 and Sur7-GFP localized properly in the *fmp45Δ* cells (Fig. 1B). The *fmp45Δ* mutant also was not  
3 defective in invasive growth into agar, as was the *sur7Δ* mutant (Fig. 2B). In contrast, as  
4 reported previously (2), about half the *sur7Δ* mutant cells showed obvious morphological  
5 defects, including enlarged cells and bud necks, and many cells displayed ectopic intracellular  
6 growth of the cell wall (Fig. 2A). Fmp45-GFP was detected at greatly reduced levels in *sur7Δ*  
7 cells, demonstrating that Sur7 is required for normal organization of MCC domains (Fig. 1B).

8 Older cells were examined to search more sensitively for a weak phenotype caused by  
9 *fmp45Δ*. The rationale for this was based on a previous suggestion that older *sur7Δ* mutant cells  
10 may have more severe defects than younger cells, since most of the new buds appeared to be  
11 relatively normal (2). To identify older cells in the population, the cells were covalently labeled  
12 with fluorescein-maleimide (FM) so that they could be detected by fluorescence microscopy  
13 whereas any new cells that formed subsequent to the labeling procedure would not be  
14 fluorescent. The FM-labeled cells were then grown for 6 hrs at 30°C, permeabilized, and  
15 analyzed by staining the cell wall with Calcofluor White. The older FM-labeled *fmp45Δ* cells  
16 did not show any obvious differences relative to the wild-type control strain (Fig. 2C). In  
17 contrast, nearly all of the older *sur7Δ* mutant cells showed altered Calcofluor White staining of  
18 the outer cell wall and intracellular growth of the cell wall (Fig 2C). Time course studies  
19 demonstrated that the percentage of FM-labeled *sur7Δ* cells with cell wall abnormalities  
20 increased over time from ~ 45% to ~ 90% at the 6 hrs time point (Fig. 2D). Interestingly, the  
21 newer cells that were not fluorescent developed altered cell wall structures as soon as 2 hrs after  
22 FM labeling (Figs. 2C and 2D). The altered cell wall localization in the newer (non-FM-labeled)  
23 cells increased over time to a steady-state level of about 45% by 6 hrs. Thus, the *sur7Δ* mutant

1 accumulates cell wall abnormalities over time, indicating defects in both the spatial and temporal  
2 regulation of wall synthesis.

3

4 ***sur7Δ* mutant is more sensitive to agents that exacerbate cell wall defects.** The *sur7Δ*  
5 mutant forms extra ectopic cell wall, but it is not clear whether this affects the function of the  
6 cell wall. To investigate this, the *sur7Δ* and *fmp45Δ* mutants were analyzed for ability to grow  
7 on agar medium containing compounds that exacerbate the growth defects of cell wall synthesis  
8 mutants including SDS, Calcofluor White, Congo Red, and hygromycin (9). Interestingly, the  
9 *sur7Δ* mutant showed greatly increased sensitivity to all four of these reagents, whereas the  
10 *fmp45Δ* mutant did not (Fig. 3). Calcofluor White and Congo Red are thought to perturb cell  
11 wall synthesis by binding to the chitin component of the cell wall. The basis for the sensitivity to  
12 SDS is not known, but it may be due to enhanced sensitivity of cell wall synthesis components or  
13 a general effect on the plasma membrane. Hygromycin is an aminoglycoside antibiotic whose  
14 ability to inhibit protein synthesis is increased in a wide range of cell wall mutants for unknown  
15 reasons. Altogether, these results indicate that the *sur7Δ* mutant forms defective cell walls.

16

17 **Sensitivity to antifungal drugs.** The mutant cells were analyzed further by testing their  
18 sensitivity to drugs that perturb cell wall synthesis. The Pkc1 cell wall stress pathway is  
19 expected to play an important role in cells that form abnormal cell walls. To test this, broth  
20 dilution assays in 96-well plates were used to examine the sensitivity to the drug  
21 cercosporamide, which is a selective inhibitor of the *C. albicans* Pkc1 protein kinase (36). The  
22 results demonstrated that *sur7Δ* cells were 8-fold more sensitive, consistent with the mutant cells  
23 forming defective cell walls (Fig. 4A). The *sur7Δ* mutant was also about 50% more sensitive to

1 nikkomycin Z, an inhibitor of chitin synthase (Fig. 4A), but showed normal sensitivity to  
2 tunicamycin, an inhibitor of N-linked glycosylation that is expected to perturb a wide range of  
3 glycosylated cell wall proteins. As reported previously, Etest assays showed that the *sur7Δ*  
4 mutant was two-fold more sensitive to caspofungin (2). Thus, the *sur7Δ* mutant is slightly more  
5 sensitive to inhibitors of both chitin and β-glucan synthases. The *sur7Δ* mutant was not  
6 hypersensitive to all compounds that exacerbate the defects of cell wall mutants. For example, it  
7 showed only a slight difference in sensitivity to rapamycin ( $\leq 2$ -fold) and was not sensitive to  
8 high calcium concentration (0.5 M) (data not shown).

9       The *fmp45Δ* mutant did not display increased sensitivity to cercosporamide or inhibitors  
10 of cell wall synthesis. Etest strip assays also showed that *fmp45Δ* did not show increased  
11 sensitivity to fluconazole (data not shown) as does *sur7Δ* (2). Furthermore, we did not detect  
12 increased sensitivity of the *fmp45Δ* homozygous deletion mutant to amphotericin (Fig 4B),  
13 which contrasts with a previous report from a high-throughput study that an *fmp45Δ/FMP45*  
14 heterozygote is more sensitive to amphotericin (42).

15  
16       **Altered cell wall composition of *sur7Δ* cells.** The carbohydrate composition of the  
17 *sur7Δ* cell wall was analyzed to determine if the constituents were altered compared to the wild-  
18 type control strain. The cell walls were divided into alkali insoluble (AI) and alkali soluble (AS)  
19 fractions (see Materials and Methods). Interestingly, the *sur7Δ* mutant showed a decrease in the  
20 AI-fraction of the cell wall that includes the important structural polymers, chitin and β-glucan  
21 (Fig 5A). The AI/AS ratio decreased from  $3.2 \pm 0.5$  for the wild type control (DIC185) to  $1.9 \pm$   
22  $0.2$  for the *sur7Δ* strain (Fig. 5B). Analysis of the monosaccharide composition in the AI-  
23 fraction from the wild type control strain DIC185 showed that glucose was the major component,

1 consistent with  $\beta$ -glucan being the most abundant fraction of the cell wall. There was a  
2 significant decrease ( $P < 0.01$ ) in the glucose content in the *sur7 $\Delta$*  cell wall AI-fraction compared  
3 to that of the wild type control strain (Fig. 5C). The level of glucosamine detected in the AI  
4 fractions, which corresponds to *N*-acetylglucosamine (GlcNAc) in the cell wall, was not  
5 significantly different for the wild type and *sur7 $\Delta$*  mutant strains (Fig. 5D), even though  
6 increased GlcNAc is often detected in cell wall mutants due to a compensatory mechanism that  
7 increases chitin levels. In contrast, the AS-fraction was increased in the *sur7 $\Delta$*  cells, mainly due  
8 to elevated mannose content (Fig. 5E). The reduced AI/AS ratio and the low level of  $\beta$ -glucan  
9 suggest that the *sur7 $\Delta$*  cells are likely to form weaker cell walls.

10

11 **Osmotic rescue of *sur7 $\Delta$*  cell lysis defect.** The altered cell wall composition suggested  
12 that *sur7 $\Delta$*  cells would be more sensitive to lysis. To test this, cells were grown at 42°C, since  
13 elevated temperature promotes cell wall stress. As expected, only a few percent of the cells of  
14 wild-type control strain DIC185 stained with Trypan Blue, a vital stain used to determine cell  
15 viability. In contrast, the *sur7 $\Delta$*  cells grew more slowly than the wild type at 42°C and Trypan  
16 Blue staining demonstrated that there was a large fraction of lysed cells. More than 40% of the  
17 *sur7 $\Delta$*  cells stained with Trypan Blue at 42°C in either rich YPD or synthetic SD medium,  
18 indicating that the cells had lysed and the Trypan Blue dye could gain access to the interior of the  
19 cell (Fig. 6). Increasing the osmolarity by adding 1 M sorbitol to the medium decreased the  
20 percent of Trypan Blue staining from 41.5% to 20.9% in rich medium and 45.7% to 14.9% in  
21 synthetic SD medium. Similar results were observed for medium with 1 M NaCl (data not  
22 shown). The observation that increased osmolarity decreased the number of Trypan Blue stained  
23 *sur7 $\Delta$*  cells confirms that the lysis defect is due at least in part to weakened cell walls.



1           Microscopic analysis of the cells also revealed that both large and small *sur7Δ* cells  
2 stained with Trypan Blue. When the *sur7Δ* mutant was grown in rich YPD medium at 30°C,  
3 most of the cells that stained with Trypan Blue were larger sized. It is not surprising that the  
4 larger cells observed in the *sur7Δ* mutant cultures would lyse more readily, since their increased  
5 volume places extra demands on the cell wall. However, it was interesting that some smaller  
6 cells also stained with Trypan Blue (Fig. 7A). The lysis of smaller cells was more obvious when  
7 cells were grown in synthetic SD medium, since the *sur7Δ* mutant formed fewer large cells under  
8 these conditions but still showed lysis of smaller cells (Fig. 7B). These results indicate that the  
9 *sur7Δ* mutation causes defects in cell wall synthesis in younger cells as well as the older larger  
10 cells that have accumulated more of the aberrant ectopic cell wall structures.

11

12           **Deletion of *FMP45* does not exacerbate the phenotype of *sur7Δ* cells.** Double mutant  
13 *sur7Δfmp45Δ* cells were analyzed to determine whether Fmp45 carries out a significant role in  
14 the absence of Sur7. In view of the possibility that the double mutation may cause lethality, we  
15 used the UAU method to create a *sur7Δfmp45Δ* mutant strain (13). The UAU method entails  
16 deleting one copy of a gene (or gene cluster) with the UAU cassette (*ARG4* gene flanked by the  
17 5' and 3' portions of a *URA3* gene) and then selecting on medium lacking Arginine and Uridine  
18 for cells in which both copies of the targeted gene are deleted (see Materials and Methods). This  
19 approach was successful in yielding *sur7Δfmp45Δ* mutants, three of which were picked for  
20 analysis. The double mutants showed ectopic staining of cell wall chitin (Fig. 8A), increased  
21 cercosporamide sensitivity (Fig. 8C), and lysis at 42°C (Fig. 8D) that was similar to the *sur7Δ*  
22 single mutant but not worse. The three *sur7Δfmp45Δ* mutants were also better able to form  
23 hyphae in response to serum than *sur7Δ* alone (Fig. 8B), suggesting the possibility that

1 production of Fmp45 in the absence of Sur7 may have negative effects. Altogether, these results  
2 indicate that Fmp45 does not contribute a significant function in the absence of Sur7, which is  
3 consistent with the observation that Fmp45-GFP is not stably produced in *sur7* $\Delta$  cells (Fig. 1)

4

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## 1 DISCUSSION

2 Cell wall synthesis plays a key role in *C. albicans* pathogenesis. The essential role of the  
3 cell wall for virulence is highlighted by the success of the drug caspofungin in treating infections  
4 by *C. albicans* and *A. fumigatus* (28). The cell wall also plays important roles by interacting  
5 with the immune system during infection (26). Many of the key steps of cell wall synthesis  
6 occur at the plasma membrane where Sur7 is localized. This includes the action of  $\beta$ -glucan and  
7 chitin synthases that extrude the elongating polymers of glucose and GlcNAc, respectively,  
8 through the plasma membrane (19, 33).  $\beta$ -glucan and chitin fibrils form the inner layer of the  
9 cell wall that gives it strength and rigidity. The outer layer is composed primarily of different  
10 types of mannoproteins. These various cell wall components must be regulated in concert to  
11 efficiently coordinate cell wall growth.

12

13 **Cell wall function.** Previous studies demonstrated that Sur7 was needed for proper  
14 spatial regulation of cell wall growth (2). This unusual ectopic cell wall phenotype of *sur7* $\Delta$   
15 cells also raised the question of whether the mutant cell walls could function properly. Several  
16 lines of evidence now confirm that the *sur7* $\Delta$  mutant produces defective cell walls. One is that  
17 the *sur7* $\Delta$  mutant was more sensitive to agents that exacerbate cell wall defects including SDS,  
18 Calcofluor White, and Congo Red (Fig. 3 and (3)). However, the *sur7* $\Delta$  cells were not sensitive  
19 to all conditions that can affect cell wall mutants. For example, they were not more sensitive to  
20 elevated calcium and were only weakly sensitive to rapamycin (data not shown). Furthermore,  
21 the *sur7* $\Delta$  mutant was only slightly more sensitive to caspofungin and nikkomycin Z, inhibitors  
22 of  $\beta$ -glucan and chitin synthases, respectively (Fig. 4 and (2)). One possibility for the relatively  
23 small effect of caspofungin and nikkomycin Z is that the *sur7* $\Delta$  mutation may alter the

1 coordination of cell wall synthesis but might not directly affect the enzymatic activity of the  $\beta$ -  
2 glucan and chitin synthases. Altogether, these results indicate that mutation of *SUR7* affects an  
3 important subset of cell wall functions.

4 As a test of cell wall strength, the *sur7* $\Delta$  mutant was examined for a cell lysis defect that  
5 could be suppressed by increased osmolarity. Growing the *sur7* $\Delta$  cells at 42°C resulted in a high  
6 proportion of lysed cells (>40%), consistent with previous observations that growth at elevated  
7 temperatures stresses the cell wall and enhances the defects of many cell wall mutants.  
8 Increasing the osmolarity of the medium by adding 1 M sorbitol partially rescued this lysis  
9 defect for the *sur7* $\Delta$  cells (Fig. 6). This confirms that the *sur7* $\Delta$  mutation causes defects in the  
10 functional properties of the cell wall, as well as in the spatial regulation of cell wall synthesis.

11 In contrast to Sur7, the Fmp45 protein did not have a detectable role in cell wall  
12 synthesis. The *fmp45* $\Delta$  mutant did not display altered morphogenesis or cell wall synthesis, and  
13 it was not more sensitive to a variety of conditions that were expected to exacerbate cell wall  
14 defects. Furthermore, the phenotype of a *sur7* $\Delta$ *fmp45* $\Delta$  mutant was not worse than the single  
15 *sur7* $\Delta$  mutant (Fig. 8). Thus, although Sur7 and Fmp45 show ~37% identity, they have distinct  
16 roles.

17  
18 **Cell wall synthesis.** Chemical analysis demonstrated that the cell wall composition was  
19 significantly altered in *sur7* $\Delta$  cells (Fig. 5). In particular, the AI/AS ratio of the alkali insoluble  
20 to alkali soluble fractions was greatly decreased, due to both a decrease in the AI fraction and an  
21 increase in the AS fraction. The increase in the AS fraction was primarily due to elevated  
22 mannan levels, which is consistent with the observation from microarray studies that the  
23 expression of the mannosyl transferase genes *MNN1* and *MNN4-4* was increased in *sur7* $\Delta$  cells

1 ~8-fold and ~5-fold , respectively (2). Altered mannosylation may also account for the increased  
2 sensitivity of the *sur7Δ* mutant to hygromycin, since defects in mannosylation have been linked  
3 to hypersensitivity to this drug (21). The biggest change in the AI fraction for the *sur7Δ* mutant  
4 was a decrease in  $\beta$ -glucan, the glucose polymer that constitutes the major component of the cell  
5 wall (19, 33). This decrease in  $\beta$ -glucan content is consistent with previous studies which  
6 showed that the *sur7Δ* mutant displayed changes in gene expression and altered septin  
7 localization that were similar to effects cause by caspofungin (2, 4) (6). Furthermore, the  
8 reduced  $\beta$ -glucan levels are consistent with the formation of weaker cell walls in *sur7Δ* cells,  
9 since  $\beta$ -glucan plays a major role in cell wall strength and rigidity (22).

10         The next major question that needs to be resolved is whether Sur7 plays a direct or  
11 indirect role in cell wall synthesis. An indirect role is suggested by the very different patterns of  
12 subcellular localization for the static Sur7 patches and the mobile actin patches (2, 44). In *S.*  
13 *cerevisiae*,  $\beta$ -glucan synthase associates with mobile actin patches, which is thought to facilitate  
14 proper expansion of bud growth (11, 30, 37). In addition, Sur7-GFP is not readily detected in  
15 new buds (Fig. 1 and (44)), which are very active sites of cell wall synthesis. These differences  
16 in subcellular localization suggest that Sur7 is not directly involved in cell wall synthesis.  
17 However, other evidence raises the possibility that Sur7 or other MCC/eisosome proteins may  
18 directly regulate at least some aspects of cell wall synthesis. For example, an echinocandin class  
19 inhibitor of  $\beta$ -1,3-glucan synthesis was found to chemically crosslink primarily to the *C.*  
20 *albicans* Pil1 and Lsp1 proteins (12, 31), which are components of eisosomes that associate with  
21 Sur7 (39). In addition, a high-throughput study of *S. cerevisiae* proteins identified the Fks1  
22 subunit of  $\beta$ -1,3-glucan synthase in a complex with Pil1 (16).

1 Further support for the idea that MCC/eisosomes are involved in cell wall regulation  
2 comes from the analysis of the *S. cerevisiae* Pkh1/2 protein kinases, which localize to eisosomes  
3 (23, 38). In *S. cerevisiae*, Pkh1/2 regulate the Pkc1, Sch9 and Ypk1 protein kinases that act in  
4 part to control cell wall synthesis (8). Interestingly, the *C. albicans sur7Δ* cells were 8-fold more  
5 sensitive to the Pkc1 inhibitor cercosporamide (Fig. 4), similar to *C. albicans* cells with reduced  
6 Pkc1p activity (36). Since Pkc1 regulates many genes involved in cell wall synthesis, this raised  
7 the possibility that the cell wall defect of *sur7Δ* cells is due to decreased Pkc1 activity.  
8 However, other data indicate that Pkc1 is active in *sur7Δ* cells. For one, *PKC1* mutations cause  
9 the opposite effect of mutating *SUR7*. The *pkc1* mutant cells produce decreased cell wall and  
10 are not known to cause extra ectopic growth of the cell wall. Also, targets of the Pkc1-activated  
11 Rlm1 transcription factor (6) were not decreased in *sur7Δ* cells but were instead highly  
12 expressed. *PGA13* was induced 16-fold and *CRH11* was induced 4-fold (2). Expression of cell  
13 wall synthesis genes were also not decreased in the *sur7Δ* cells. *GSCI* (β-1,3-glucan synthase)  
14 as well as *KRE1* and *KRE7* (β-1,6-glucan synthases) were expressed at slightly increased levels,  
15 and the expression of the chitin synthase genes was not changed significantly in *sur7Δ* cells (2).  
16 In contrast, there were extensive changes in the expression of genes that encode a variety of  
17 other putative cell wall proteins, especially predicted GPI anchored proteins that are likely to  
18 directly interact with the cell wall. Many of these genes are thought to be activated by the Cas5  
19 transcription factor that is important for response to caspofungin treatment (6). These data  
20 indicate that the cell wall defects of the *sur7Δ* cells are not due to a general failure of cells to  
21 sense and respond to cell wall damage. Future studies on MCC/eisosomes will help to define the  
22 links between these novel domains and cell wall synthesis.  
23

1

2

3 **ACKNOWLEDGMENTS**

4           We thank the members of our labs for their helpful advice and comments on the  
5 manuscript, Dr. Francisco Javier Alvarez for providing strains, and Dr. Yoshikazu Ohya for  
6 advice. This research was supported by a grant awarded to J.B.K. from the National Institutes of  
7 Health (RO1 AI47837) and a grant awarded to J.-P.L. from ERA-NET PathoGenoMics  
8 ANTIFUN.

9

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- 3

1

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<br/>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<br/>his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>            |
| YJA11                      | YJA10  | <i>sur7Δ::ARG4/sur7Δ::HIS1 URA3/ ura3Δ::λimm434<br/>his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>                      |
| YJA12                      | YJA10  | <i>sur7Δ::ARG4/sur7Δ::HIS1 SUR7::URA3 ura3Δ::λimm434/<br/>ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i> |
| YHXW1                      | BWP17  | <i>fmp45Δ::ARG4/FMP45 ura3Δ::λimm434/ ura3Δ::λimm434<br/>his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>                 |
| YHXW2                      | YHXW1  | <i>fmp45Δ::ARG4/fmp45Δ::HIS1 ura3Δ::λimm434/ ura3Δ::λimm434<br/>his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>          |
| YHXW3                      | YHXW2  | <i>fmp45Δ::ARG4/fmp45Δ::HIS1 URA3/ ura3Δ::λimm434<br/>his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>                    |
| YHXW4                      | BWP17  | BWP17, except <i>SUR7-GFPγ::URA3</i>   |
| YHXW5                      | BWP17  | BWP17, except <i>FMP45-GFPγ::URA3</i>  |
| YHXW6                      | YJA10  | <i>sur7Δ</i> strain YJA10, except <i>FMP45-GFPγ::URA3</i>  |
| YHXW7                      | YHXW2  | <i>fmp45Δ</i> strain YHXW2, except <i>SUR7-GFPγ::URA3</i>  |

YHXW8 YHXW2 *fmp45Δ::NAT/fmp45Δ::HIS1 ura3Δ::λimm434/ ura3Δ::λimm434*  
*his1::hisG/his1::hisG arg4::hisG/arg4::hisG*

YHXW9 YHXW8 *fmp45Δ::NAT/fmp45Δ::HIS1 sur7Δ::UAU/SUR7 ura3Δ::λimm434/*  
*ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*

YHXW10- YHXW9 *fmp45Δ::NAT/fmp45Δ::HIS1 sur7Δ::UAU/sur7Δ::URA3*  
 UAU1 *ura3Δ::λimm434/ ura3Δ::λimm434 his1::hisG/his1::hisG*  
*arg4::hisG/arg4::hisG*

YHXW10- YHXW9 *fmp45Δ::NAT/fmp45Δ::HIS1 sur7Δ::UAU/sur7Δ::URA3*  
 UAU2 *ura3Δ::λimm434/ ura3Δ::λimm434 his1::hisG/his1::hisG*  
*arg4::hisG/arg4::hisG*

YHXW10- YHXW9 *fmp45Δ::NAT/fmp45Δ::HIS1 sur7Δ::UAU/sur7Δ::URA3*  
 UAU3 *ura3Δ::λimm434/ ura3Δ::λimm434 his1::hisG/his1::hisG*  
*arg4::hisG/arg4::hisG*

1 FIGURE LEGENDS

2

3 Figure 1. Punctate plasma membrane localization of Sur7-GFP and Fmp45-GFP.

4 (A) *C. albicans* strain BWP17 engineered to express *SUR7-GFP* or *FMP45-GFP* fusion genes.

5 Upper panels show budding cells and lower panels show cells that were induced with 10%

6 bovine calf serum to form hyphae. In each series of images, the panel on the left is a light

7 microscope image to show cell morphology. The two fluorescence microscope images on the

8 right demonstrate the localization of the Sur7-GFP and Fmp45-GFP in punctate patches in focal

9 planes that correspond to the top of the cell and to the medial portion. Arrows indicate bud  
10 localization of Fmp45-GFP.

11 (B) Localization of Sur7-GFP in *fmp45Δ* cells and Fmp45-GFP in *sur7Δ* cells. Microscope

12 images are as described in panel A. All of the fusions genes were constructed with GFP $\gamma$ , a more

13 photostable version of GFP (45). Strains used in these experiments included YHXW4 (*SUR7-*

14 *GFP*), YHXW5 (*FMP45-GFP*), YHXW6 (*sur7Δ FMP45-GFP*) and YHXW7 (*fmp45Δ SUR7-*

15 *GFP*).

16

17 Figure 2. Analysis of the cell wall and morphogenesis phenotypes of the *sur7Δ* and *fmp45Δ*

18 mutants.

19 (A) Morphology of live cells of the indicated genotype is shown in the upper images. The lower

20 images show a different set of cells viewed by fluorescence microscopy that were permeabilized

21 and stained with Calcofluor White to detect cell wall structures.

22 (B) Invasive growth assay of the indicated *C. albicans* strains. Equal numbers of cells were

23 spotted onto an agar plate containing 4% serum and then incubated at 37°C for 7 days.

1 (C) Comparison of cell wall structures in older and newer cells. Cells of the indicated genotype  
2 were crosslinked to fluorescein-maleimide and then grown for 2 or 6 hrs at 30°C in YPD. The  
3 cells were then permeabilized and stained with Calcofluor White. Fluorescent microscope  
4 images of cells stained with Calcofluor White are shown in the upper panel; the older cells that  
5 were crosslinked with fluorescein are shown in the lower panel.

6 (D) Quantitation of Calcofluor White-stained cells after labeling with fluorescein-maleimide  
7 (FM) at time zero versus new cells that were not labeled (NonFM). Outside indicates cells that  
8 showed altered Calcofluor White staining of the outer cell wall and Intracellular indicates altered  
9 Calcofluor White staining within the cell due to ectopic growth of the cell wall.

10

11 Figure 3. The *sur7Δ* mutant is more sensitive to chemicals that exacerbate the growth defects of  
12 cell wall mutants.

13 Ten-fold dilutions of the wild type control strain DIC185, YJA11 (*sur7Δ*), YJA12 (*sur7Δ* +  
14 *SUR7*), and YHXW3 (*fmp45Δ*) were spotted onto rich medium YPD plates containing the  
15 following concentration of the indicated additive: 0.06% SDS, 0.04 mg/ml Calcofluor White,  
16 0.1 mg/ml Congo Red, 0.15 mg/ml hygromycin. The plates were incubated at 37°C for two days  
17 and then photographed.

18

19

20 Figure 4. Altered sensitivity of the *sur7Δ* mutant to antifungal drugs.

21 (A) *C. albicans* strains were tested for sensitivity to nikkomycin Z, tunicamycin, and  
22 cercosporamide by assaying their ability to grow in 96 well plates containing a two-fold dilution  
23 series of each drug. Sensitivity to caspofungin was tested with Etest strips, as described



1 previously for the *sur7Δ* mutant (2). Results represent the average of at least two independent  
2 experiments.

3 (B) The *fmp45Δ* mutant displays wild type level of sensitivity to amphotericin. Cells were  
4 spread on a plate containing RPMI 1640 medium, the Etest strip was applied, and then the plates  
5 were incubated at 37°C for 48 hrs. The Etest strips release a gradient of drug causing a zone of  
6 growth inhibition. Strains used in these assays included DIC185, YJA11 (*sur7Δ*), YJA12 (*sur7Δ*  
7 + *SUR7*), and YHXW3 (*fmp45Δ*).

8

9 Figure 5. Decreased alkali insoluble fraction in *sur7Δ* cell walls.

10 The chemical composition of the cell walls was compared for the wild type (DIC185), *sur7Δ*  
11 (YJA11) and *sur7Δ* + *SUR7* complemented (YJA12) strains. (A) The relative amount of alkali  
12 soluble (AS) and alkali insoluble (AI) fractions as a percent of the total dry weight of the cell  
13 wall. (B) Ratio of AI/AS for each strain. (C) Glucose composition of the AI fractions. (D)  
14 Mannose and glucosamine composition of the AI fractions. (E) Mannose and glucose  
15 composition of the AS fractions. Cell wall fractions were analyzed as described in the Materials  
16 and Methods. The results represent the average of 4 independent experiments ± SD.

17

18 Figure 6. Increased osmolarity suppresses cell lysis defect of *sur7Δ* mutant at elevated  
19 temperature.

20 Wild type (DIC185) and *sur7Δ* strain (YJA11) were grown overnight at 42°C in the presence or  
21 absence of 1 M sorbitol to increase osmolarity. The fraction of lysed cells was determined by  
22 staining with Trypan Blue. For comparison, cells were grown in either rich YPD medium or

1 synthetic SD medium. The results represent the average of at least two independent experiments  
2  $\pm$  SD.

3

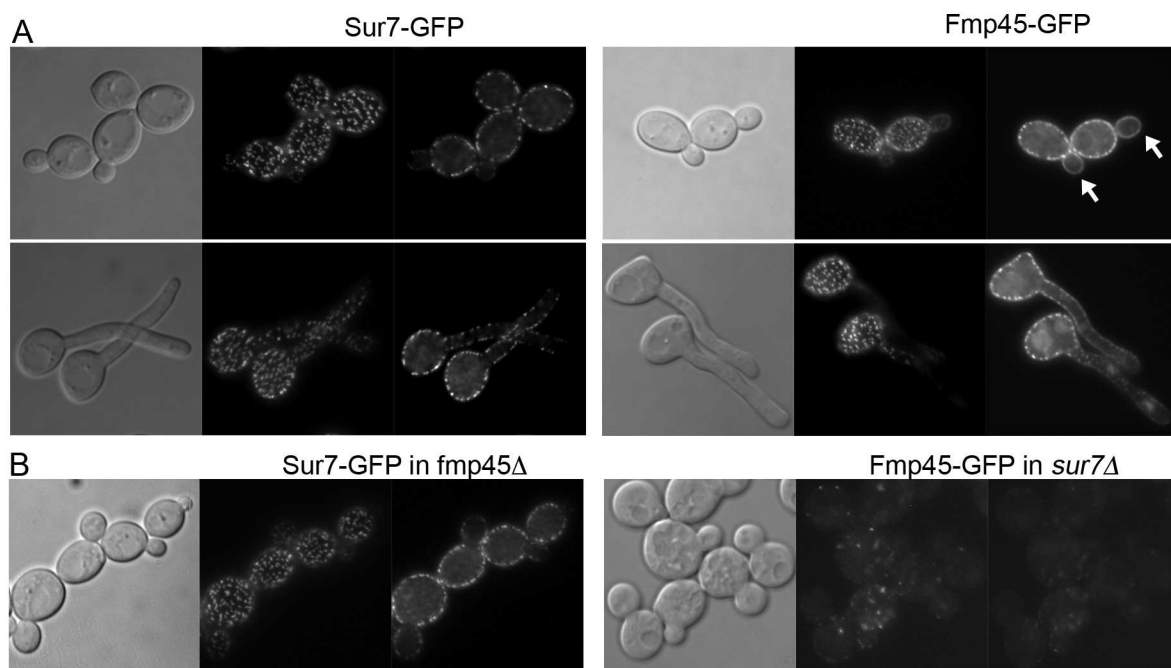
4 Figure 7. Spontaneous lysis of both large and small *sur7* $\Delta$  cells.

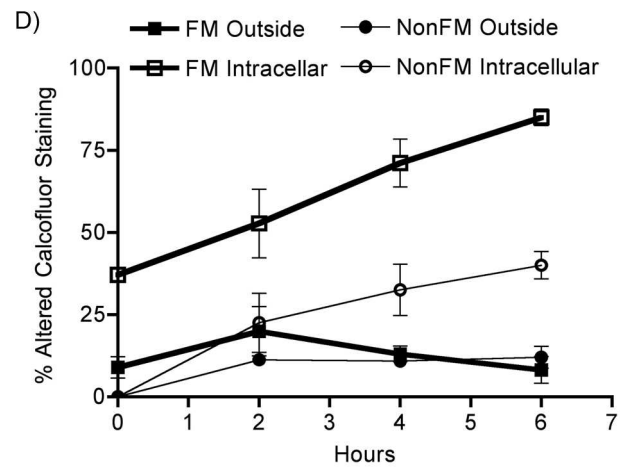
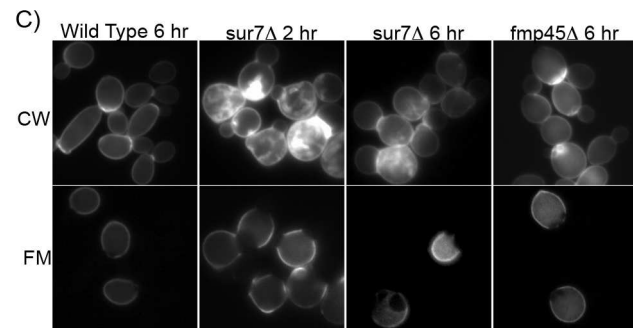
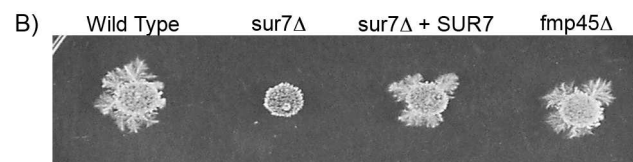
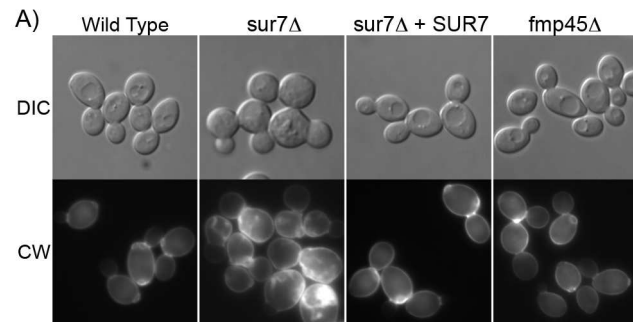
5 The *sur7* $\Delta$  strain YJA11 was grown at 30°C in either (A) rich YPD or (B) synthetic SD medium  
6 and then stained with Trypan Blue to detect lysed cells.

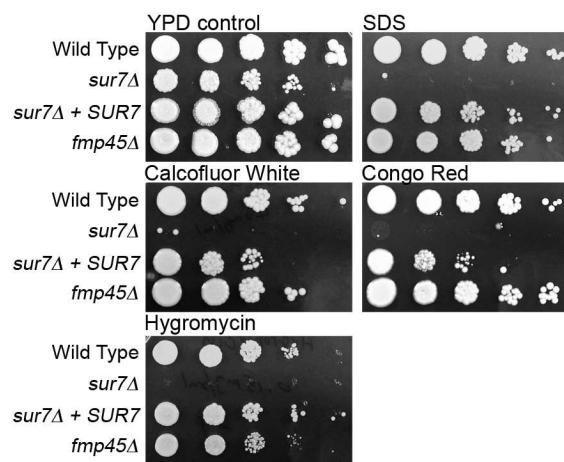
7

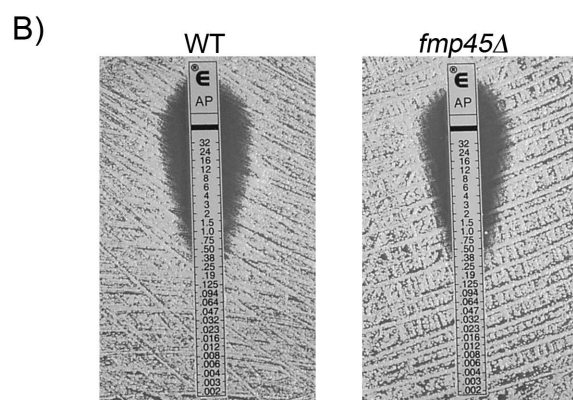
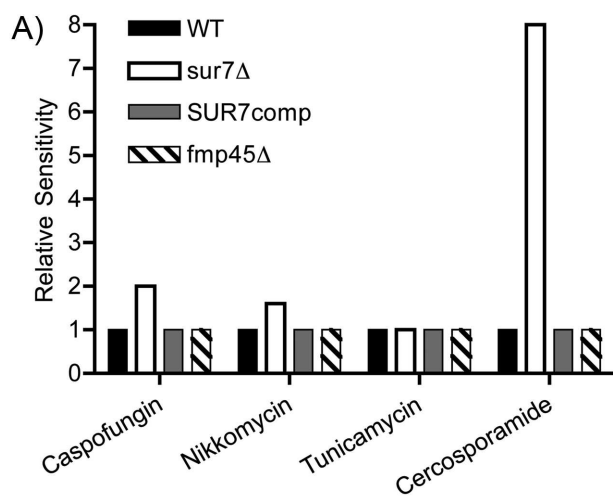
8 Figure 8. Phenotypes of *sur7* $\Delta$ *fmp45* $\Delta$  mutants.

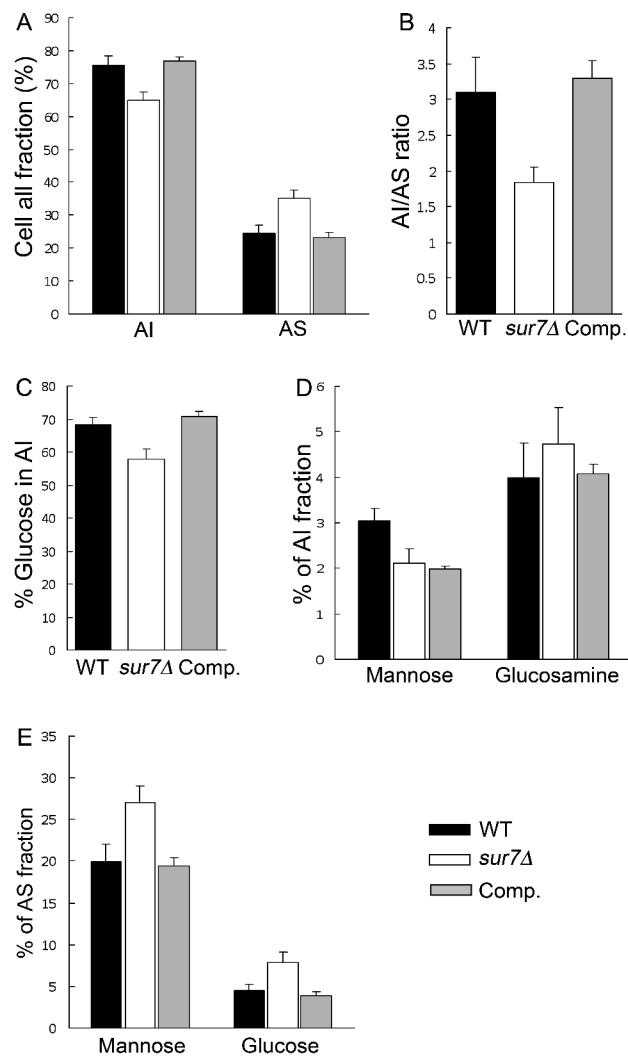
9 Three *sur7* $\Delta$ *fmp45* $\Delta$  double mutants were generated by the UAU method as described in the  
10 Materials and Methods and are labeled UAU-1, UAU-2, and UAU-3. These mutants were  
11 compared to the following strains: wild-type control (DIC185), *sur7* $\Delta$  (YJA11), and *fmp45* $\Delta$   
12 (YHXW3). (A) Permeabilized cells stained with Calcofluor White and then examined  
13 microscopically to determine the fractions of cells displaying a normal cell wall, altered cell  
14 wall, or intracellular cell wall pattern (n = >200). (B) Cells induced with 10% serum and then  
15 examined for hyphal formation (n = >200). (C) Sensitivity to cercosporamide tested by broth  
16 dilution assays in 96 well plates. (D) Cells grown overnight at 42°C and then stained with  
17 Trypan Blue to determine viability (n = >200). Error bars indicate SD.

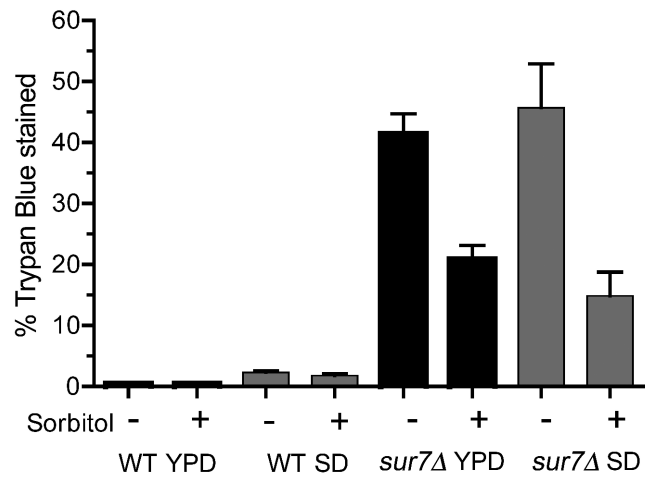






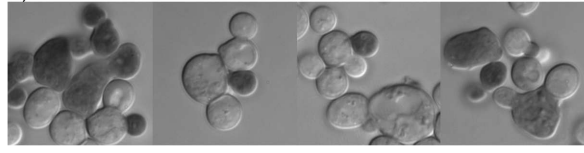








A) YPD



B) SD

