Eisosomes promote the ability of Sur7 to regulate plasma membrane organization in *Candida albicans*

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ABSTRACT The plasma membrane of the fungal pathogen *Candida albicans* forms a protective barrier that also mediates many processes needed for virulence, including cell wall synthesis, invasive hyphal morphogenesis, and nutrient uptake. Because compartmentalization of the plasma membrane is believed to coordinate these diverse activities, we examined plasma membrane microdomains termed eisosomes or membrane compartment of Can1 (MCC), which correspond to ~200-nm-long furrows in the plasma membrane. A *pil1Δ lsp1Δ* mutant failed to form eisosomes and displayed strong defects in plasma membrane organization and morphogenesis, including extensive cell wall invaginations. Mutation of eisosome proteins Slm2, Pkh2, and Pkh3 did not cause similar cell wall defects, although *pkh2Δ* cells formed chains of furrows and *pkh3Δ* cells formed wider furrows, identifying novel roles for the Pkh protein kinases in regulating furrows. In contrast, the *sur7Δ* mutant formed cell wall invaginations similar to those for the *pil1Δ lsp1Δ* mutant even though it could form eisosomes and furrows. A PH-domain probe revealed that the regulatory lipid phosphatidylinositol 4,5-bisphosphate was enriched at sites of cell wall invaginations in both the *sur7Δ* and *pil1Δ lsp1Δ* cells, indicating that this contributes to the defects. The *sur7Δ* and *pil1Δ lsp1Δ* mutants displayed differential susceptibility to various types of stress, indicating that they affect overlapping but distinct functions. In support of this, many mutant phenotypes of the *pil1Δ lsp1Δ* cells were rescued by overexpressing *SUR7*. These results demonstrate that *C. albicans* eisosomes promote the ability of Sur7 to regulate plasma membrane organization.

INTRODUCTION

The plasma membrane is critically important as a protective barrier that also mediates a wide range of dynamic processes, including en-

docytosis, secretion, nutrient uptake, signal transduction, morphogenesis, and cell wall or extracellular matrix synthesis. Consistent with its complex function, the plasma membrane is composed of a diverse array of proteins and lipids. These various constituents are organized into specialized domains that bring together subsets of lipids and proteins by spontaneous segregation of membrane lipids, protein-mediated diffusion restrictions, and other mechanisms (Simons and Gerl, 2010; Ziolkowska *et al.*, 2012). These features are believed to contribute to proper plasma membrane function and the homeostasis of the different lipids and proteins (Malinsky *et al.*, 2013). However, the roles of plasma membrane organization are poorly understood due to the difficulties of studying hydrophobic membranes.

Recent studies with fungi have identified plasma membrane microdomains known as membrane compartment of Can1 (MCC), which are stable, protein-organized domains that can be visualized

by microscopy (Malinsky *et al.*, 2010, 2013; Douglas and Konopka, 2014). The MCC domains correspond to inward furrows in the plasma membrane (Stradalova *et al.*, 2009), which are stabilized by a complex of cytosolic proteins termed the eisosome (Walther *et al.*, 2006). In *Saccharomyces cerevisiae*, there are 50 of these punctate domains per cell, each of which is 300 nm long and 50 nm deep (Malinska *et al.*, 2003; Walther *et al.*, 2006). Outside the MCC, the plasma membrane is more dynamic—lateral diffusion of membrane proteins takes place in this region, and it also contains transient sites of endocytosis, secretion, and contact with the cortical ER (Malinska *et al.*, 2004; Stradalova *et al.*, 2012).

MCC/eisosome formation in *S. cerevisiae* is promoted by two homologous proteins, Pil1 and Lsp1, which assemble into long filaments on the cytoplasmic side of the plasma membrane (Karotki *et al.*, 2011; Olivera-Couto *et al.*, 2011; Ziolkowska *et al.*, 2011). Pil1 and Lsp1 contain BAR domains, which are believed to bend the membrane to form the furrows (Olivera-Couto *et al.*, 2011; Ziolkowska *et al.*, 2011). At least 10 other proteins assemble onto the cytoplasmic surface of the eisosomes, including Seg1, a protein that facilitates eisosome formation (Moreira *et al.*, 2012), the Pkh1/2 kinases, which regulate cell wall synthesis and stress responses (Walther *et al.*, 2007), the Slm1/2 proteins, which regulate TORC2 and sphingolipid homeostasis (Berchtold *et al.*, 2012), and quinone reductases, which were shown in *C. albicans* to promote resistance to oxidative stress (Li *et al.*, 2015). Integral membrane proteins recruited into these domains include two distinct families of tetraspanner proteins related to Sur7 and Nce102 and several nutrient transporters, including the arginine transporter Can1 (Malinsky *et al.*, 2010; Douglas and Konopka, 2014). Although the plasma membrane portion of these domains is known as the MCC and the cytoplasmic proteins that associate on the intracellular side are known as the eisosome, for simplicity we will often refer to both of these domains as eisosomes since most of our experiments focus on eisosome proteins.

Membrane furrows have been found in a wide range of fungi, lichens, and algae, but their function is not well understood (Lee *et al.*, 2015). Therefore eisosomes in the human fungal pathogen *Candida albicans* were examined in this study because of the key role that the plasma membrane plays in virulence. Previous studies showed that deletion of *SUR7* from *C. albicans* caused dramatic defects in plasma membrane organization and cell wall morphogenesis (Alvarez *et al.*, 2008; Bernardo and Lee, 2010; Wang *et al.*, 2011; Douglas *et al.*, 2012). Although eisosomes still formed in the *sur7Δ* cells, there were extensive defects outside of the eisosomes, including mislocalization of actin and septins. The *sur7Δ* cells formed deep invaginations of cell wall growth and were more susceptible to compounds that perturb the cell wall (Alvarez *et al.*, 2008; Wang *et al.*, 2011; Douglas *et al.*, 2012). To better understand the broad role of Sur7, we examined the effects of deleting the *PIL1* and *LSP1* genes, which are needed for eisosome formation. Similar but not identical defects were observed for *sur7Δ* and *pil1Δ lsp1Δ* mutants, indicating that they have overlapping but distinct functions. Consistent with this, some phenotypes of the *pil1Δ lsp1Δ* mutant could be partially rescued by overexpressing *SUR7*. These results provide new insights into membrane organization and identify a major role for *C. albicans* eisosomes in promoting Sur7 function.

RESULTS

Pil1 and Lsp1 play equal roles in eisosome formation

A previous study demonstrated that *C. albicans* Pil1 and Lsp1 localize to punctate eisosome domains (Reijnst *et al.*, 2011). To elucidate their roles in *C. albicans*, we created *PIL1* and *LSP1* deletion mutant strains. Although *C. albicans* is diploid, for simplicity the homozygous dele-
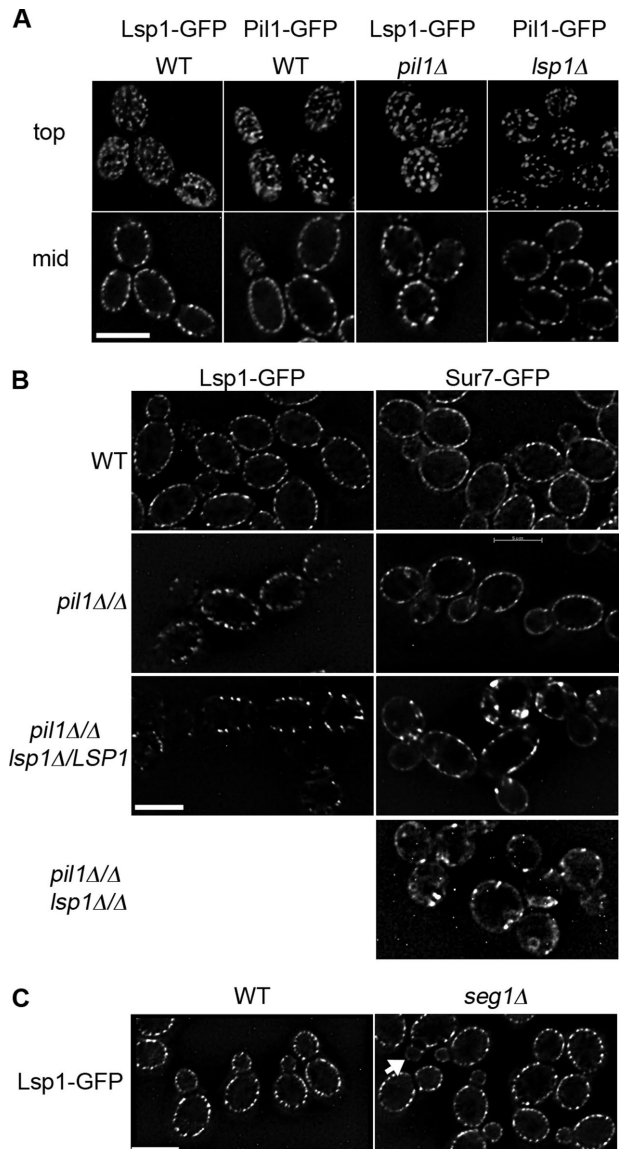

FIGURE 1: Both Pil1 and Lsp1 promote eisosome formation in *C. albicans*. Lsp1, Pil1, and Sur7 tagged with GFP at their C-termini were visualized by fluorescence microscopy. (A) Lsp1-GFP showed typical punctate localization in both the wild-type and *pil1Δ* strains. Similar results were observed for Pil1-GFP in the wild-type and *lsp1Δ* strains. This indicates that both Lsp1 and Pil1 can promote eisosome formation. Top, the punctate eisosome patches on the top of the cell; bottom, eisosomes around the periphery in a midsection of the cell. (B) Localization of Lsp1-GFP and Sur7-GFP in the indicated strains, in which the different copies of *PIL1* and *LSP1* were progressively deleted. Sur7-GFP in the *pil1Δ lsp1Δ* strain, which lacked eisosomes, gave a lower signal. Therefore the Sur7-GFP signal was increased relative to the other images to visualize this sample. (C) Lsp1-GFP localization in wild-type and *seg1Δ* strains. Strains used were *Pil1-GFP* (YLD70-1), *LSP1-GFP* (YLD76-1), *PIL1-GFP lsp1Δ* (YHXW24-1), *LSP1-GFP pil1Δ* (YLD85-1), *LSP1-GFP pil1Δ lsp1ΔLSP1* (YHXW25-1), *SUR7-GFP* (YHXW4), *SUR7-GFP pil1Δ* (YHXW26-1), *SUR7-GFP pil1Δ lsp1Δ/LSP1* (YHXW27-1), *SUR7-GFP pil1Δ lsp1Δ* (YHXW28-1), and *LSP1-GFP seg1Δ* (YLD177-1). Bars, 5 μm.

tion strains will be referred to as *pil1Δ* and *lsp1Δ*. Analysis of Lsp1-GFP in the *pil1Δ* mutant and Pil1-GFP in the *lsp1Δ* mutant revealed that both proteins formed punctate eisosome patches in the plasma membrane similar to the wild-type control strain (Figure 1A). The

observation that Pil1 and Lsp1 have equal ability to form eisosomes was unexpected, since Lsp1 is not capable of forming eisosomes in the absence of Pil1 in *S. cerevisiae* or *Ashbya gossypii* (Walther *et al.*, 2006; Seger *et al.*, 2011), even though Pil1 and Lsp1 have very similar amino acid sequences (70% identical). Comparison of the amino acid sequences suggested that the functional differences may be due to six sites in Lsp1 from *S. cerevisiae* and *A. gossypii* that differed from residues that are conserved in the Pil1 proteins and in *C. albicans* Lsp1 (Supplemental Figure S1). These sites occur in the BAR-domain region (Ziolkowska *et al.*, 2011), where they could affect the ability to interact with the plasma membrane. The amino acid comparison also suggested that *C. albicans* Lsp1 might be more closely related to *S. cerevisiae* Pil1. However, to avoid confusion, we will use the assignments made in the *Candida* Genome Database (Pil1 orf19.778/ C1_04680W and Lsp1 orf19.3149/C2_06730W)

Although Lsp1–green fluorescent protein (GFP) formed punctate patches in the *pil1Δ* mutant, there was ~24% reduction in the number of patches (Figure 1B; 15 cells; *p* < 0.001). This was further exacerbated in a *pil1Δ* strain that contained only one copy of *LSP1* (*pil1Δ/Δ LSP1-GFP/lsp1Δ*), which displayed ~57% reduction in eisosome patches (15 cells, *p* < 0.001). Sur7-GFP showed expected localization to punctate domains in wild type, but slight abnormalities in its localization were detected in the *pil1Δ* strain (Figure 1B). Further deletion of one copy of *LSP1* from the *pil1Δ* strain resulted in atypical Sur7-GFP patches that were much larger than the punctate eisosome patches. Of interest, Sur7-GFP could still be detected at the plasma membrane in the *pil1Δ lsp1Δ* strain, which lacks eisosomes, although the size, distribution, and intensities of the Sur7-GFP patches varied (Figure 1B). Similar results have been reported for *S. cerevisiae* Sur7-GFP in a *pil1Δ* strain that lacks eisosomes (Walther *et al.*, 2006; Stradalova *et al.*, 2012). The fact that Sur7-GFP was still detectable at the plasma membrane has significance for conclusions to be made later regarding its role in the absence of Pil1 and Lsp1 (see *Discussion*).

A *C. albicans seg1Δ* mutant was also analyzed because the *S. cerevisiae* and *A. gossypii* Seg1 localizes to eisosomes and plays a role in promoting eisosome formation (Seger *et al.*, 2011; Moreira *et al.*, 2012). In contrast, a *C. albicans seg1Δ* mutant showed at most a slight delay in forming eisosomes in small buds (see arrow in Figure 1C). Seg1 may be less critical for eisosome formation in *C. albicans,* since both Pil1 and Lsp1 appear to be fully functional.

Similar but not identical cell wall invaginations are present in *sur7Δ* and *pil1Δ lsp1Δ* mutants

The *C. albicans sur7Δ* mutant forms extensive invaginations of the cell wall that accumulated with the increased age of the cell (Alvarez *et al.*, 2008; Wang *et al.*, 2011). Similar invaginations were seen for the *pil1Δ lsp1Δ* mutant using calcofluor white to stain the cell wall (Figure 2A). The *sur7Δ* and *pil1Δ lsp1Δ* mutants were also similar, in that the cells were often present in clumps, indicating a defect in cytokinesis (Figure 2A). These defects were not detected in the *pil1Δ* or *lsp1Δ* single mutants or in a *pil1Δ lsp1Δ* strain that was complemented with either *LSP1* or *PIL1*. Higher-resolution images obtained with transmission electron microscopy (TEM) revealed that, as seen previously for *sur7Δ* cells (Alvarez *et al.*, 2008), the *pil1Δ lsp1Δ* cells contained torus-shaped structures in the middle of the cell that represent a cross section of tubes of cell wall material (marked with white arrows in Figure 2B). However, the mutants differed in the types of invaginations that were present at the periphery of the cells (marked with black arrows on the outside of the cell in Figure 2B). The *sur7Δ* mutant predominantly contained pointed invaginations of cell wall at the periphery, whereas the *pil1Δ lsp1Δ*

mutant had fewer spiky invaginations and instead contained large, rounded invaginations similar to those observed in a *S. cerevisiae pil1Δ* mutant (Walther *et al.*, 2006). Thus the *sur7Δ* and *pil1Δ lsp1Δ* mutants display similar but not identical cell wall defects.

The *pil1Δ lsp1Δ* mutant displays defects in actin organization and morphogenesis

The abnormal cell morphology suggested that the *pil1Δ lsp1Δ* mutant cells have a defect in actin polarization. To examine this, we stained cells with the actin-binding agent rhodamine-phalloidin (Figure 3A). Wild-type cells showed the expected localization of actin patches at the cortical regions of the bud and actin filaments leading back into the mother cell. In contrast, actin was depolarized in the *pil1Δ lsp1Δ* mutant, as actin patches could be detected in both the bud and the mother cell, and there were fewer detectable actin filaments. This phenotype is similar to the *sur7Δ* mutant (Figure 3A). Introduction of one copy of *LSP1* partially rescued the phenotype of the *pil1Δ lsp1Δ* cells, as there were still some abnormalities.

As expected for cells with an actin defect, the *pil1Δ lsp1Δ* mutant did not efficiently form highly polarized hyphal cells after induction with serum (Figure 3B). Some cells could form elongated pseudohyphae, whereas other cells failed to initiate filamentous growth. A similar phenotype was detected for *sur7Δ* cells. This variable ability of different cells to undergo filamentous growth may be due to varying degrees of abnormal cell wall morphogenesis present in the cells at the time they were induced (Figure 2).

Although some of the *pil1Δ lsp1Δ* cells formed elongated pseudohyphae in liquid culture, the mutant cells were strongly defective in invasive growth into 1% agar–containing serum (Figure 3C). This defect was stronger than that observed for the *sur7Δ* mutant (Figure 3C). The ability of the *pil1Δ lsp1Δ* mutant to undergo invasive growth was slightly improved when assayed for ability to invade into a more rigid matrix of 4% agar. This was interesting because a mutant that lacks the MCC/eisosome domain protein Nce102 displays a similar phenotype. An *nce102Δ* mutant is defective in invading 1% agar but invades well into denser, 4% agar (Douglas *et al.*, 2013), indicating that a separate pathway activated by the surrounding matrix can compensate for the *nce102Δ* defect. Analysis of Nce102-GFP in the *pil1Δ lsp1Δ* mutant showed that it localized efficiently at the plasma membrane, although it was evenly distributed and was not present in the punctate patches seen for the wild type or the *sur7Δ* mutant (Figure 3D). This raises the possibility that the abnormal Nce102 localization contributes to the *pil1Δ lsp1Δ* phenotype. The failure of the Nce102-GFP to localize to punctate clusters in the *pil1Δ lsp1Δ* mutant is also significant because it confirms that the eisosomes are defective in this *pil1Δ lsp1Δ* mutant.

Differential effects of inhibitors on *pil1Δ lsp1Δ* and *sur7Δ* mutants

The unusual cell wall invaginations prompted us to examine whether the *pil1Δ lsp1Δ* mutant was sensitive to agents that exacerbate cell wall defects. The *pil1Δ lsp1Δ* mutant was similar to *sur7Δ* cells in being more sensitive than wild-type cells to calcofluor white, Congo red, and SDS (Figure 4A) and the ergosterol synthesis inhibitor fluconazole (Figure 4B). However, it was significant that the two mutants displayed differential sensitivity to these inhibitors. Of the two mutants, *sur7Δ* was more sensitive to calcofluor white and Congo red, which bind to the cell wall. In contrast, the *pil1Δ lsp1Δ* mutant was more sensitive to the detergent SDS, and it showed a clearer zone of growth inhibition surrounding an Etest strip containing a gradient of fluconazole, an antifungal drug that blocks ergosterol synthesis (Figure 4B). Control studies confirmed that these phenotypes were
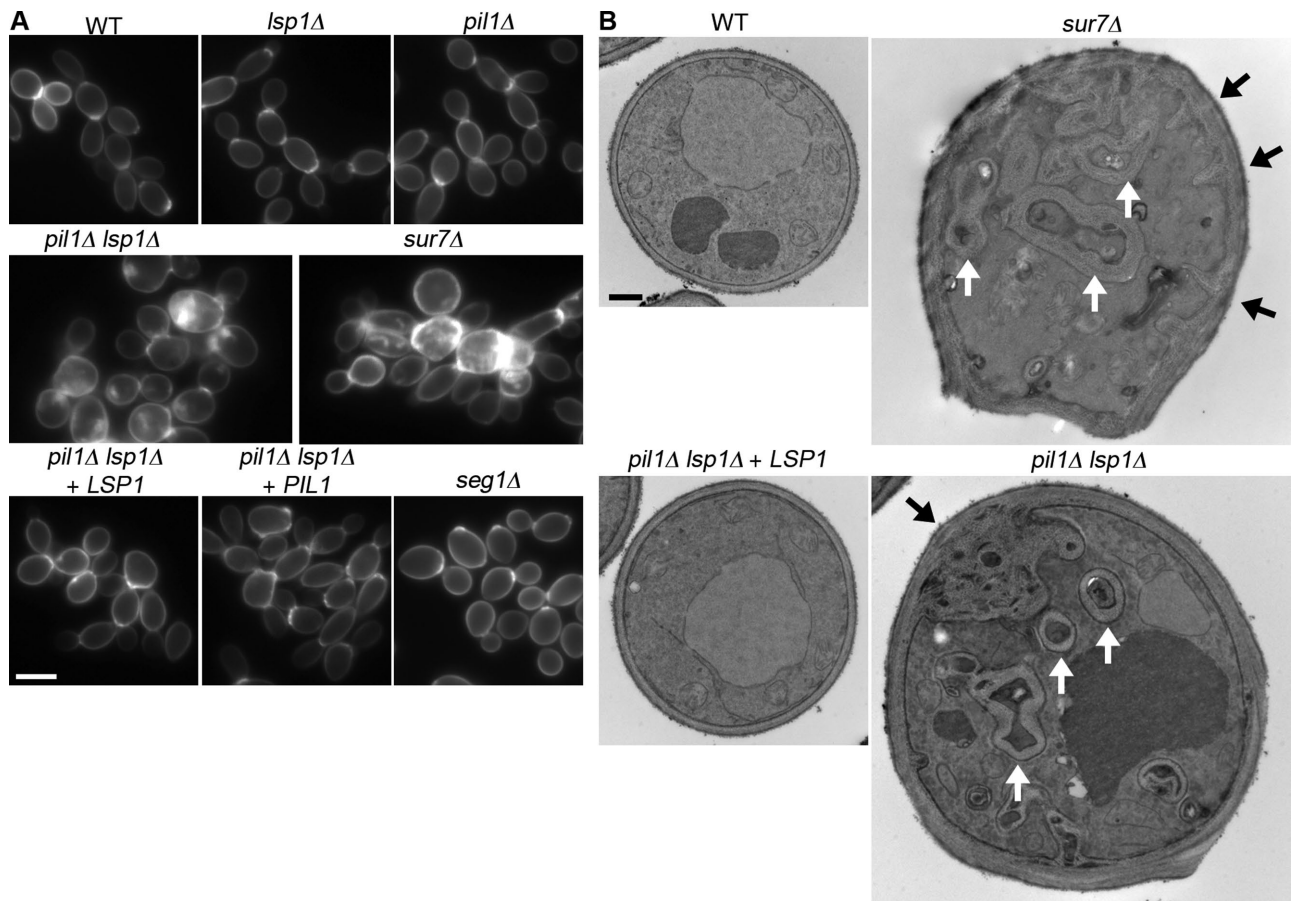
FIGURE 2: The *pil1Δ lsp1Δ* mutant forms large invaginations of the cell wall. (A) The cells were stained with calcofluor white, and then cell wall structures were photographed by fluorescence microscopy. Note that the *pil1Δ* and *lsp1Δ* mutants displayed very little altered cell wall, whereas the *pil1Δ lsp1Δ* double mutant displayed cell wall invaginations comparable to those of the *sur7Δ* mutant. White bar, 5 μm. (B) Cell sections detected by transmission electron microscopy. Note the presence of thicker cell walls and invaginations of cell wall in both the *pil1Δ lsp1Δ* double mutant and the *sur7Δ* mutant. Black arrows on the outer edges of the cells point to regions where there are spiky invaginations in the *sur7Δ* mutant and the large, round cell wall invagination in the *pil1Δ lsp1Δ* mutant. The white arrows point to the cell wall tubes. Black bar, 1 μm. The strains used were wild type (DIC185), *lsp1Δ* (YLD77-11-24-1), *pil1Δ* (YLD73-9-2-1), *pil1Δ lsp1Δ* (YHXW21-1), *sur7Δ* (YJA11), *pil1Δ lsp1Δ* with a copy of *LSP1* reintegrated (YHXW23-1), *pil1Δ lsp1Δ* with a copy of *PIL1* integrated (YHXW22-1), and *seg1Δ* (YLD182-40-14-3).

complemented by reintroduction of a copy of *LSP1* into the *pil1Δ lsp1Δ* mutant cells. The greater sensitivity of the *pil1Δ lsp1Δ* mutant to fluconazole is consistent with studies showing that certain cell wall mutants, such as those affecting the Pkc1 pathway, are more susceptible to fluconazole (LaFayette *et al.*, 2010). However, the *pil1Δ lsp1Δ* mutant was not more sensitive to cercosporamide, an inhibitor of fungal Pkc1 (Sussman *et al.*, 2004), whereas the *sur7Δ* mutant was fivefold more sensitive to cercosporamide (Figure 4C). Taken together, these data demonstrate that *pil1Δ lsp1Δ* and *sur7Δ* have similar but not identical defects in cell wall function.

Slm2 does not play an important role in *C. albicans* cell wall morphogenesis

To investigate the basis for the cell wall defects of the *pil1Δ lsp1Δ* and *sur7Δ* mutants, we analyzed the *C. albicans* orthologues of the *S. cerevisiae* Slm1 and Slm2 proteins, which localize to eisosomes and influence morphogenesis (Berchtold *et al.*, 2012). Two *C. albicans* proteins showed significant homology to the Slm proteins. The Orf19.3505 protein (C6_02060W) had greater similarity to Slm1/2 from *S. cerevisiae* (~40% identity over 425 amino acids) than

Orf19.4043 (C5_05440C; ~30% identity over 300 amino acids). When fused to GFP, Orf19.3035 colocalized with Lsp1–red fluorescent protein (RFP) in punctate eisosome patches in the plasma membrane (Figure 5A), as expected for the Slm proteins (Kamble *et al.*, 2011; Berchtold *et al.*, 2012). We will refer to Orf19.3505 as Slm2, since it shows higher amino acid similarity to Slm2 than to Slm1 from *S. cerevisiae*. In contrast, Orf19.4043 showed better similarity to Ask10 and Rgc1 from *S. cerevisiae*, and Orf19.4043-GFP localized to the cytoplasm (Figure 5A), as do Ask10 and Rgc1 under these conditions (Huh *et al.*, 2003; Beese *et al.*, 2009). Thus, consistent with *C. albicans* not having undergone the ancient genome duplication that *S. cerevisiae* did, *C. albicans* appears to have just one orthologue of each.

In *pil1Δ lsp1Δ* cells, Slm2-GFP was not in punctate patches and instead was usually present in large patches near the ends of the cells (Figure 5B). A small fraction of cells showed a variety of different localization patterns that are likely due to Slm2-GFP associated with the plasma membrane surrounding the abnormal cell wall invaginations. In contrast, *sur7Δ* cells contained Slm2-GFP in the expected punctate eisosome patches, although there were fewer patches than in wild-type cells.
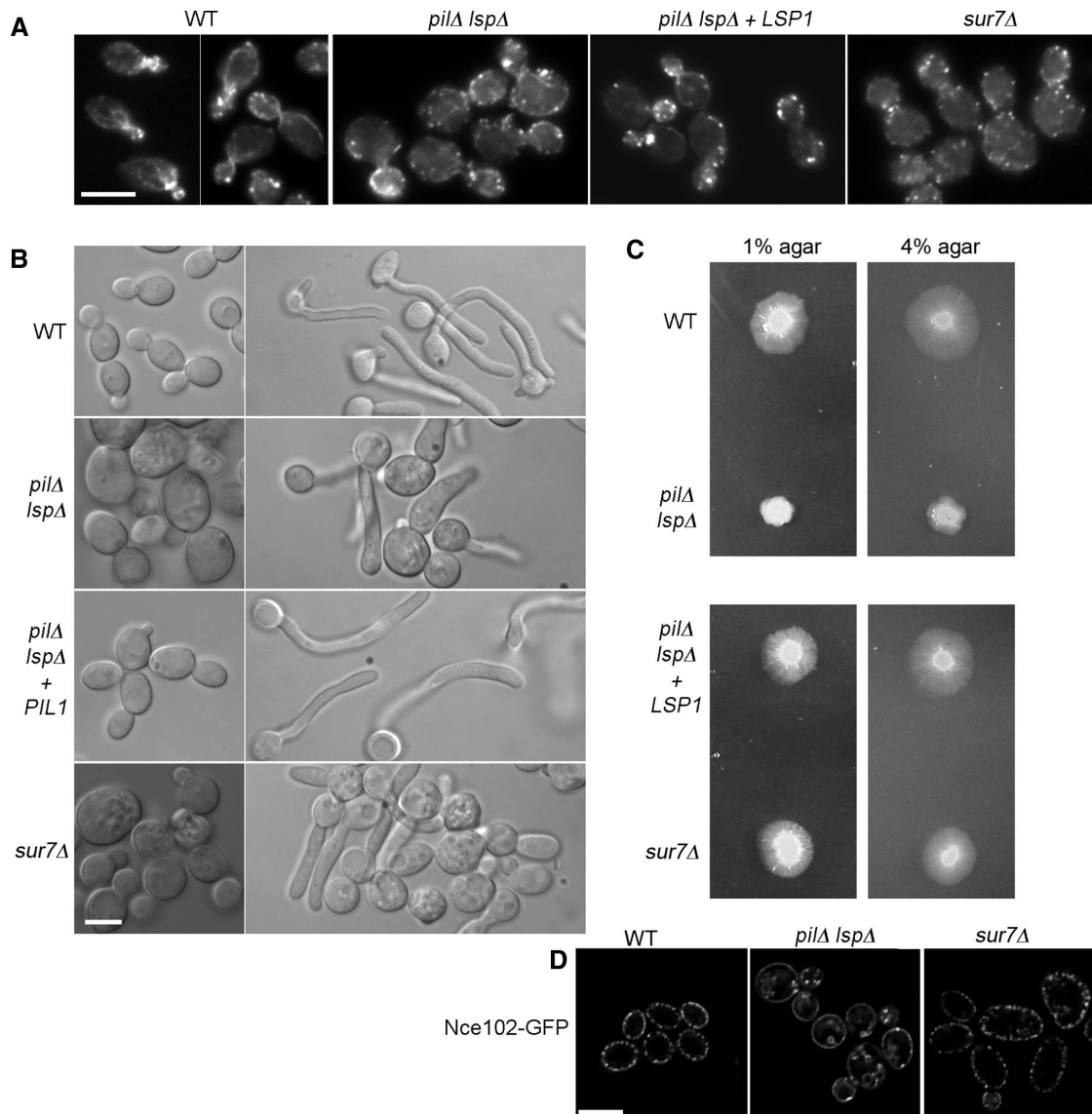
FIGURE 3: The *pil1Δ lsp1Δ* mutant displays defects in actin localization and hyphal morphogenesis. (A) The indicated cells types were stained with rhodamine-phalloidin to detect actin. Note the lack of polarized actin patches and fewer actin cables in the *pil1Δ lsp1Δ* cells and the *sur7Δ* cells. (B) Left, budding cells. Right, cells in synthetic medium that were treated with 15% serum for 1.5 h at 37°C to induce hyphal formation. Although the *pil1Δ lsp1Δ* mutant could be induced to form elongated cells, they did not form typical hyphae with long, parallel walls. (C) Cells were spotted onto solid-medium plates containing 4% bovine serum and then either 1% agar (left) or 4% agar (right). The higher agar concentration weakly rescued the invasive growth defect of the *pil1Δ lsp1Δ* mutant. (D) Nce102-GFP localized to punctate patches in the wild-type and *sur7Δ* strains, which form eisosomes. In contrast, Nce102-GFP localized evenly in the plasma membrane in the *pil1Δ lsp1Δ* strain, consistent with the absence of eisosomes. The Nce102-GFP signal seen in the center of the cells is due to the plasma membrane that is associated with the cell wall invaginations and does not represent a punctate localization of Nce102-GFP in the *pil1Δ lsp1Δ* or *sur7Δ* cells. Strains used were wild type (DIC185), *pil1Δ lsp1Δ* (YHXW21-1) *sur7Δ* (YJA11), and *pil1Δ lsp1Δ* with a copy of *LSP1* reintegrated (YHXW23-1), *NCE102-GFP* (YHXW15), *NCE102-GFP pil1Δ lsp1Δ* (YHXW29-1), and *NCE102-GFP sur7Δ* (YHXW30-1). Bars, 5 μm.

Of interest, a *C. albicans slm2Δ* mutant grew well, which contrasts with the fact that an *S. cerevisiae* mutant lacking both of the redundant *SLM1* and *SLM2* genes is not viable (Berchtold *et al.*, 2012). Furthermore, there were no detectable abnormalities in budding for the *C. albicans slm2Δ* cells or in the ability to form hyphae when induced with serum (Figure 5C). Overexpression of *SLM2* by introduction of an extra copy under control of the strong *ADH1* promoter also did not have obvious effects on morphogenesis and did not cause formation of cell wall invaginations. Thus, although Slm2-GFP localizes to eisosomes, it does not appear to play a significant role in morphogenesis and therefore seems unlikely to contribute to the defects observed for the *pil1Δ lsp1Δ* and *sur7Δ* mutants.

pkh mutants form larger eisosomes but not cell wall invaginations

The Pkh kinases were next investigated for a role in the *pil1Δ lsp1Δ* phenotype since in *S. cerevisiae*, the Pkh1 and Pkh2 paralogues localize to eisosomes and regulate Pkc1 and other stress responses
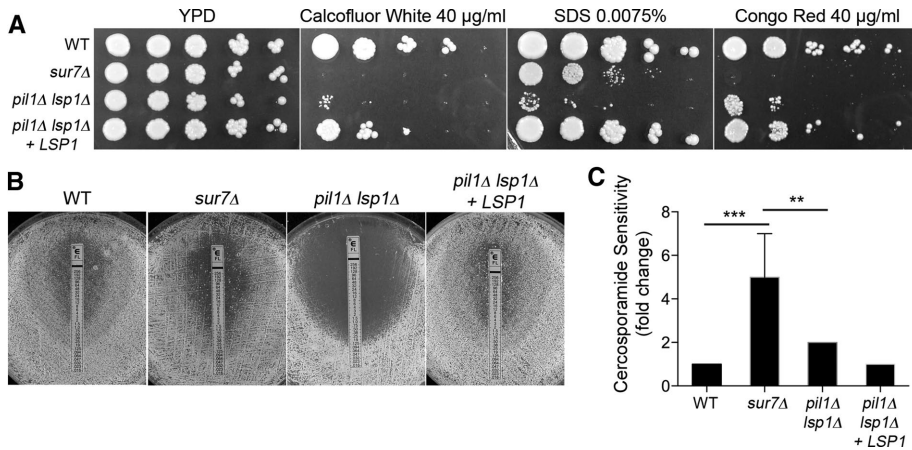
FIGURE 4: The *pil1Δ lsp1Δ* and *sur7Δ* mutants show differential sensitivity to cell wall–perturbing agents. (A) Dilutions of cells were spotted onto solid-medium agar plates containing the indicated perturbing agent. The plates were incubated for 2 d at 37°C and then photographed. (B) The sensitivity to fluconazole was assayed by plating a lawn of cells and then placing an E strip containing a gradient of fluconazole on top. Plates were incubated for 2 d at 37°C and then photographed. (C) Sensitivity to cercosporamide was determined by growing cells in the presence of different concentrations of this Pkc1 inhibitor at 37°C for 2 d. Strains used were wild type (DIC185), *pil1Δ lsp1Δ* (YHXW21-1), *sur7Δ* (YJA11), and *pil1Δ lsp1Δ* with a copy of *LSP1* reintegrated (YHXW23-1). **$p < 0.01$, ***$p < 0.001$.

(Roelants *et al.*, 2002). The more distantly related Pkh3 kinase has not been studied well but appears to carry out similar functions (Inagaki *et al.*, 1999). *C. albicans* contains only one gene related to *PKH1* and *PKH2* (C1_12410C or orf19.5224). We previously referred to this gene as *PKH1* (Douglas *et al.*, 2013), but will now refer to it as *PKH2* to conform to the *Candida* Genome Database. The *C. albicans pkh2Δ* mutant displayed increased sensitivity to Congo red and SDS, and these phenotypes were exacerbated by further deletion of one copy of *PKH3* (*pkh2Δ/Δ pkh3Δ/PKH3*; Douglas *et al.*, 2013). We were unable to isolate a *pkh2Δ pkh3Δ* double mutant, perhaps because the combined defect makes cells sick or is lethal.

The role of the Pkh kinases in *S. cerevisiae* eisosome formation is controversial; some studies indicated they promote eisosome formation, and other studies reported that they promote eisosome disassembly (Douglas and Konopka, 2014). Of interest, the *C. albicans pkh2Δ/Δ pkh3Δ/PKH3* mutant showed abundant eisosomes (Figure 6A). In addition, filaments of Lsp1-GFP were detected in Z-stack images from the middle of the cell away from the plasma membrane, indicating a role for the Pkh kinases in preventing polymerization of Pil1 and Lsp1 in the cytoplasm. Analysis of the top view of the cells showed that the *pkh2Δ/Δ pkh3Δ/PKH3* mutant cells displayed an extended meshwork of Lsp1-GFP rather than the isolated punctate patches seen in wild-type cells (Figure 6B). The *pkh2Δ* mutant and to a lesser degree the *pkh3Δ* mutant also showed enlarged eisosome structures. This indicates that the Pkh kinases are not required for eisosome assembly in *C. albicans* and are instead needed to prevent assembly or promote disassembly.

The effects of mutating the Pkh kinases on membrane furrow formation were analyzed in cells processed by freeze-etching and imaged by TEM (samples shown in Figure 6E, and whole cells shown in Supplement Figures S2–S5). As expected, wild-type cells formed typical membrane furrows, which were absent in *pil1Δ lsp1Δ* cells. Both the *sur7Δ* and *nce102Δ* cells contained furrows, although on average they were slightly longer than in the wild-type cells (Supplemental Figure S6). The *pkh3Δ* mutant cells formed unusual furrows that were wider. In contrast, the *pkh2Δ* and the *pkh2Δ/Δ pkh3Δ/PKH3* mutants formed long chains of furrows (Figure 6E). This sug-

gests that extensive filament formation of Lsp1-GFP seen in *pkh2Δ/Δ pkh3Δ/PKH3* cells (Figure 6A) induces chains of furrows rather than longer furrows that have been seen in other species (Kabeche *et al.*, 2011; Lee *et al.*, 2015).

In spite of the altered eisosomes, the cell wall morphology of the *pkh2Δ/Δ pkh3Δ/PKH3* mutant cells was normal, as we did not detect invaginations after staining with calcofluor white (Figure 6C). To determine whether a defect in Pkh kinase activity might contribute to the other phenotypes in the *sur7Δ* or *pil1Δ lsp1Δ* mutants, we tested cells for sensitivity to agents that exacerbate cell wall defects. The results showed that there was no direct correlation between phenotypes of the *pkh2Δ/Δ pkh3Δ/PKH3* mutant and *sur7Δ* or *pil1Δ lsp1Δ* cells. The *pkh2Δ/Δ pkh3Δ/PKH3* mutant was less sensitive to either calcofluor white or SDS than are the *sur7Δ* and *pil1Δ lsp1Δ* cells (Figure 6D). Conversely, the *pkh2Δ/Δ pkh3Δ/PKH3* cells were more sensitive to caffeine than were the *sur7Δ* or *pil1Δ lsp1Δ* cells. The response to rapamycin distinguished all three mutants (Figure 6D). The *pkh2Δ/Δ pkh3Δ/PKH3* cells were slightly sensitive to rapamycin, the *sur7Δ* cells were very sensitive to rapamycin, and the *pil1Δ lsp1Δ* cells were slightly resistant (note the better growth of *pil1Δ lsp1Δ* cells in columns 3 and 4 of Figure 6D). These differences indicate that the defects caused by the absence of the Pkh kinases do not correlate with the phenotypes of the *sur7Δ* and *pil1Δ lsp1Δ* mutants. These results also further demonstrate that the *sur7Δ* and *pil1Δ lsp1Δ* mutants have distinct phenotypes.

PI$_{4,5}$P$_2$ clusters in the plasma membrane at sites of cell wall invaginations

Studies on the role of the Inp51, a phosphatidylinositol 4,5-bisphosphate (PI$_{4,5}$P$_2$) phosphatase, showed that an *inp51Δ* mutant that has elevated levels of the lipid PI$_{4,5}$P$_2$ formed invaginations of cell wall growth that are similar to those seen in the *sur7Δ* and the *pil1Δ lsp1Δ* mutant cells (Badrane *et al.*, 2012). PI$_{4,5}$P$_2$ is an important regulatory lipid that binds to many different signaling and morphogenesis proteins (Sun *et al.*, 2013). Therefore we examined the localization of PI$_{4,5}$P$_2$ in the plasma membrane using a previously described fusion between the human PLCδ1 PH domain and RFP (Badrane *et al.*, 2012). As expected, the PH-RFP probe localized to the plasma membrane in wild-type cells (Figure 7A). In contrast, both the *sur7Δ* and the *pil1Δ lsp1Δ* cells showed abnormal clusters of PH-RFP at sites of invaginations of the plasma membrane. These results indicate that eisosomes regulate the distribution of PI$_{4,5}$P$_2$ in the plasma membrane. Eisosomes in *S. cerevisiae* and *Schizosaccharomyces pombe* have been implicated in regulating PI$_{4,5}$P$_2$ levels and distribution into patches in response to stress (Murphy *et al.*, 2011; Frohlich *et al.*, 2014; Kabeche *et al.*, 2014, 2015).

Badrane *et al.* (2012) also found that septins were mislocalized to ectopic sites in the *inp51Δ* mutant (similar to what was observed in *sur7Δ* cells; Alvarez *et al.*, 2008). We therefore examined a GFP-tagged version of the Cdc10 septin and found that it was mislocalized to sites away from the bud neck in *pil1Δ lsp1Δ* cells, similar to the *sur7Δ* cells (Figure 7A). In both of these mutants, Cdc10-GFP was detected near regions of membrane invagination that contained
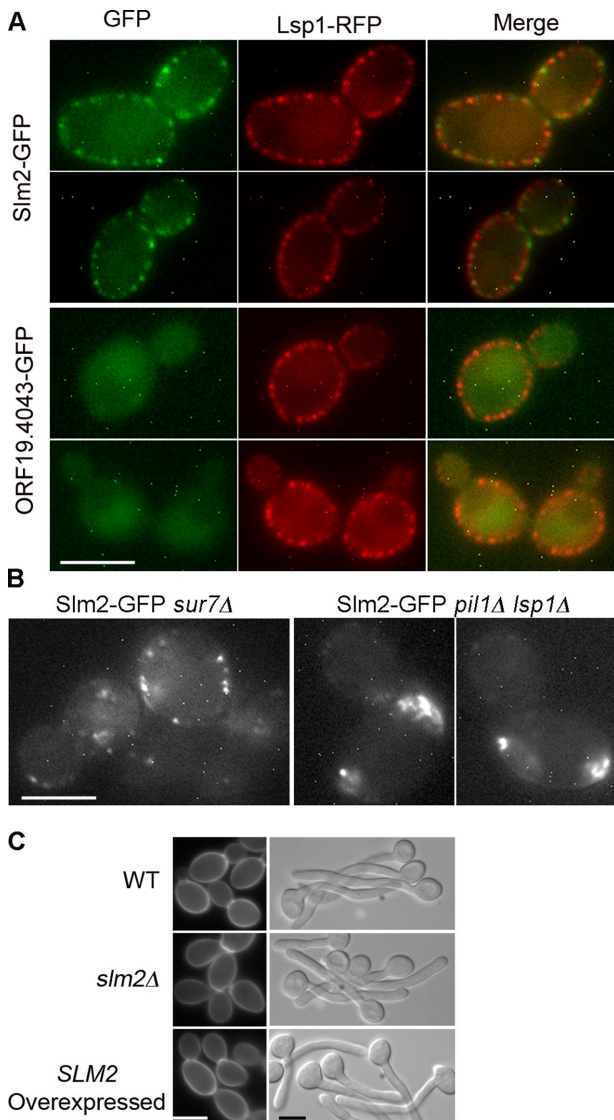
FIGURE 5: The *C. albicans* orthologue of *S. cerevisiae* Slm1/2 localizes to eisosomes but is not needed for proper cell wall morphogenesis. (A) Orf19.3505-GFP (Slm2-GFP) colocalized with Lsp1-RFP, indicating that it is present in eisosomes, consistent with it being the *C. albicans* orthologue of *S. cerevisiae* Slm1 and Slm2 proteins. In contrast, Orf19.4043-GFP displayed a diffuse cytoplasmic localization, consistent with it being the orthologue of the *S. cerevisiae* Ask10 and Rgc1 proteins. (B) In the *sur7Δ* mutant, Orf19.3035-GFP (Slm2-GFP) localized to punctate clusters, although there were fewer patches than in a wild-type strain. In contrast, Orf19.3035-GFP showed a very different abnormal localization in the eisosome mutant *pil1Δ lsp1Δ*. The predominant pattern was two major spots at opposite poles of the cells. (C) Left, cells lacking or overexpressing *ORF19.3035* (*SLM2*) do not display cell wall or morphogenesis defects. Cells were stained with calcofluor white. Right, the cells could be induced to form hyphae by treatment with 15% serum for 1.5 h. Strains used were *ORF19.3035-GFP LSP1-RFP* (YHXW32-1), *ORF19.4043-GFP LSP1-RFP* (YHXW31-1), *ORF19.3505-GFP sur7Δ* (YHXW33-1), *ORF19.3505 pil1lsp1* (YHXW34-1), *orf19.3505Δ* (YHXW36-1), and *ORF19.3505* overexpressed (YHXW37-1). Bars, 5 µm.

enriched levels of PH-RFP. However, some regions enriched in PH-RFP contained low or nondetectable levels of Cdc10-GFP. Septin proteins may be recruited to these regions because it was shown in

S. cerevisiae that the binding to $PI_{4,5}P_2$ promotes assembly of septin filaments (Bertin *et al.*, 2010).

Comparison of the cell wall defects indicated that the phenotypes of the *sur7Δ* and *pil1Δ lsp1Δ* cells are not solely due to abnormal regulation of Inp51. Calcofluor white staining revealed that the *sur7Δ* and *pil1Δ lsp1Δ* cells had more extensive cell wall invaginations than the *inp51Δ* cells (Figure 7B). Whereas only 12% of the *inp51Δ* cells showed major cell wall invaginations, >40% of the *pil1Δ lsp1Δ* cells and 60% of the *sur7Δ* cells did so. Furthermore, the *inp51Δ* mutant did not show increased sensitivity to calcofluor white under conditions in which *sur7Δ* and the *pil1Δ lsp1Δ* cells are strongly inhibited (Figure 7C). Thus the phenotypes of *sur7Δ* and the *pil1Δ lsp1Δ* cells must be due, at least in part, to effects on other pathways that regulate $PI_{4,5}P_2$ and not just Inp51.

Overexpression of *SUR7* partially rescues phenotypes of *pil1Δ lsp1Δ* mutant cells

The similar but distinct phenotypes of the *sur7Δ* and the *pil1Δ lsp1Δ* cells suggested that Sur7 and the eisosome proteins Pil1 and Lsp1 have independent functions. For example, as described earlier, *sur7Δ* cells were more sensitive to compounds that affect the cell wall (e.g., cercosporamide and calcofluor white) and to rapamycin, whereas *pil1Δ lsp1Δ* cells were more susceptible to fluconazole and had a stronger defect in invasive hyphal growth. To examine this relationship further, we tried to construct a *sur7Δ pil1Δ lsp1Δ* triple mutant but were not successful. It was rare to find a mutant with one copy of *SUR7* deleted in the *pil1Δ lsp1Δ* mutant under conditions in which they were plentiful in the wild-type background, and deletion of a second copy of *SUR7* showed that there was still a wild-type *SUR7* present. Owing to these difficulties, we instead examined the effects of overexpressing *SUR7* in the *pil1Δ lsp1Δ* mutant.

Of interest, overexpressing *SUR7* by integrating an extra copy of *SUR7* in the *pil1Δ lsp1Δ* cells caused a significant improvement in resistance to SDS, calcofluor white, and Congo red (Figure 8A). Overexpression of *SUR7* also decreased the clumps of large cells that represent the more extreme phenotype of *pil1Δ lsp1Δ* cells (Figure 8B). Overexpression of *SUR7* caused a reduction in the number of cells with obvious cell wall abnormalities from 40.4% in the *pil1Δ lsp1Δ* cells to 18.4% for *pil1Δ lsp1Δ* cells with an extra copy of *SUR7* (Figure 8C). Integration of two extra copies of *SUR7* (2xSUR7) in the *pil1Δ lsp1Δ* cells further improved the phenotypes but did not fully restore cells to a wild-type phenotype.

In contrast to the foregoing results, overexpression of *SUR7* only caused a minor change in the susceptibility of *pil1Δ lsp1Δ* cells to a low dose of fluconazole and did not cause a significant difference at higher doses (Figure 8D). This is consistent with *SUR7* playing a minor role in resistance to fluconazole compared with Pil1 and Lsp1 (Figure 4B).

DISCUSSION

C. albicans eisosomes were examined to better define the roles of plasma membrane compartmentalization in this important human fungal pathogen. A *pil1Δ lsp1Δ* mutant that lacks eisosomes displayed strong defects in plasma membrane organization, cell wall morphogenesis, and invasive hyphal growth that were similar to *sur7Δ* cells that do make eisosomes (Alvarez *et al.*, 2008). Further comparison of *sur7Δ* and *pil1Δ lsp1Δ* phenotypes indicated that one key function for eisosomes in *C. albicans* is to promote the ability of Sur7 to regulate the distribution of $PI_{4,5}P_2$ and cell wall morphogenesis. The *pil1Δ lsp1Δ* mutant also had distinct properties, such as stronger defects in invasive hyphal growth and increased sensitivity
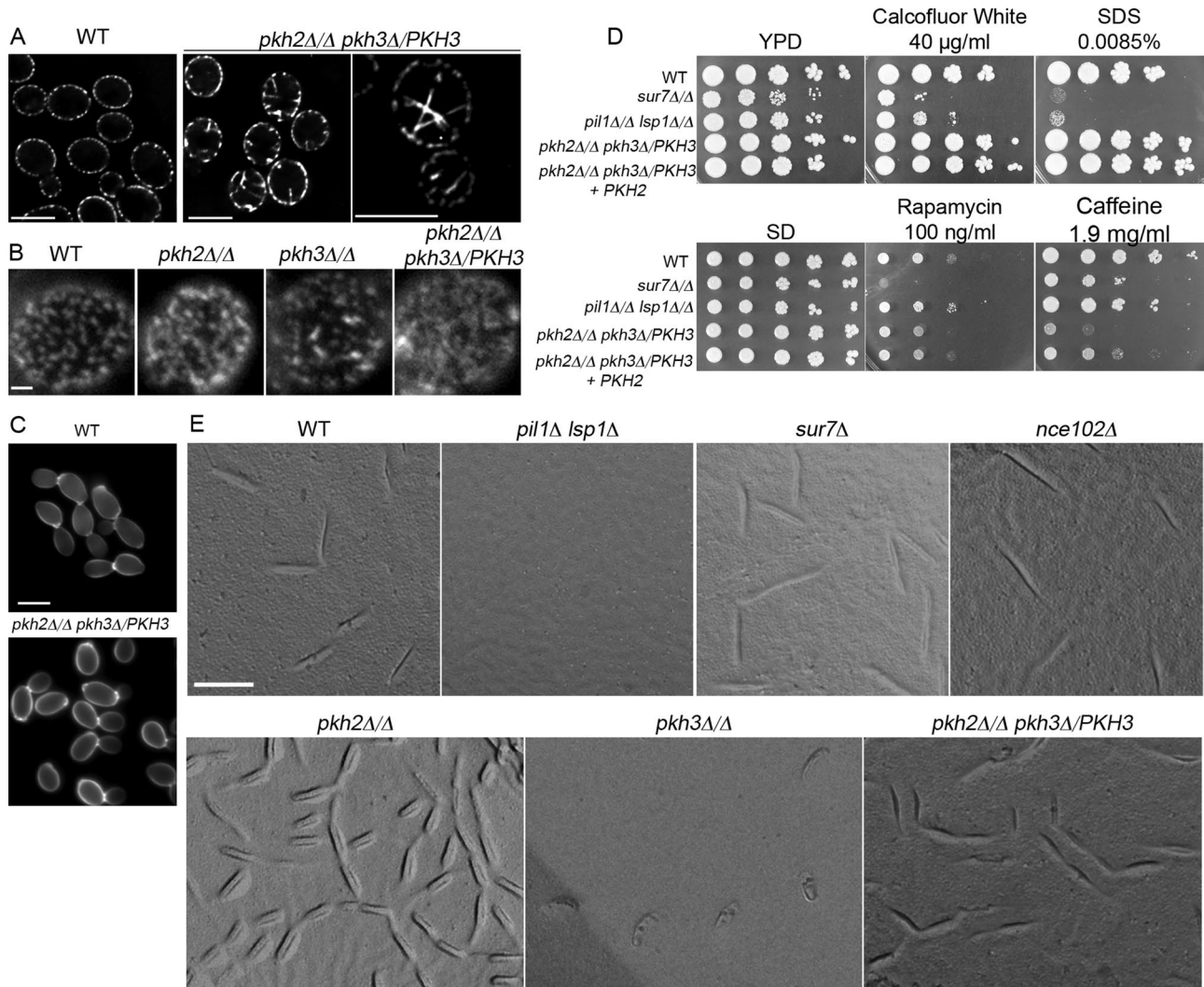
FIGURE 6: The Pkh2 and Pkh3 kinases prevent formation of elongated eisosomes. (A) Localization of Lsp1-GFP in wild-type control and *pkh2Δ/Δ pkh3Δ/PKH3* cells. The full diploid genotype is listed for this strain to designate that it lacks *PKH2* and is heterozygous for *PKH3*. Cells were analyzed by fluorescence microscopy, and representative midsections are shown. Right, an enlarged midsection for the *pkh2Δ/Δ pkh3Δ/PKH3* strain, revealing intracellular filaments of Lsp1-GFP. (B) Top view of cells, showing the pattern of Lsp1-GFP in the indicated wild-type or *pkh* mutant strain. Deletion of the *PKH* genes resulted in formation of elongated patches of Lsp1-GFP. (C) Cell walls were detected by staining with calcofluor white. (D) Dilutions of the indicated cells were spotted onto agar plates to test the effect on growth of different inhibitory compounds. These data revealed a different pattern of sensitivity to these agents for the *pil1Δ lsp1Δ* mutant and the *pkh2Δ/Δ pkh3Δ/PKH3* mutant. The top row of assays was carried out with rich YPD medium. The bottom row of spot assays was carried out with SD medium (synthetic medium with dextrose). (E) Plasma membrane furrows were analyzed in the indicated strains by freeze-etching methods and TEM. Strains used for A and B were *LSP1-GFP* (YLD76-1), *LSP1-GFP pkh2Δ* (YLD173-2), *LSP1-GFP pkh3Δ* (YLD178-1), and *LSP1-GFP pkh2Δ/Δ pkh3Δ/ PKH3* (YLD174-4). Strains used for C–E were wild-type control (DIC185), *sur7Δ* (YJA11), *pil1Δ lsp1Δ* (YHXW21-1), *pkh2Δ/Δ pkh3ΔPKH3* (YLD146-6-54-1), and *pkh2Δ/Δ pkh3ΔPKH3* plus *PKH2* (YLD203-3-3). Bars, 5 µm (A, C), 1 µm (B), 250 nm (E).

to fluconazole, indicating that eisosomes have other functions independent of Sur7.

Eisosome variation among species

Analysis of *C. albicans* mutants lacking different eisosome proteins revealed important similarities and differences with other fungi. A major difference is that Sur7 has important roles in *C. albicans*, whereas its deletion causes relatively minor phenotypes in *S. cerevisiae* (Young *et al.*, 2002; Alvarez *et al.*, 2008). In addition, Pil1 and Lsp1 have equal roles in *C. albicans*, whereas Lsp1 cannot promote eisosome formation on its own in *S. cerevisiae* or *A. gossypii* (Walther

et al., 2006; Seger *et al.*, 2011). This may help to explain why *seg1Δ* had a minor effect in *C. albicans*, since its role in promoting eisosome formation may not be as important when Lsp1 is fully functional. Although the role of the Pkh kinases in regulating *S. cerevisiae* eisosome formation is controversial (Douglas and Konopka, 2014), in *C. albicans*, the Pkh kinases were not needed for eisosome formation. Of interest, the *pkh2Δ* and *pkh3Δ* phenotypes indicated that the Pkh kinases are important for regulating the placement and width of eisosomes, which represents new roles for these kinases. It was also unexpected that a *C. albicans* strain lacking Slm2 had no obvious phenotypes (Figure 5), whereas production of either Slm1 or
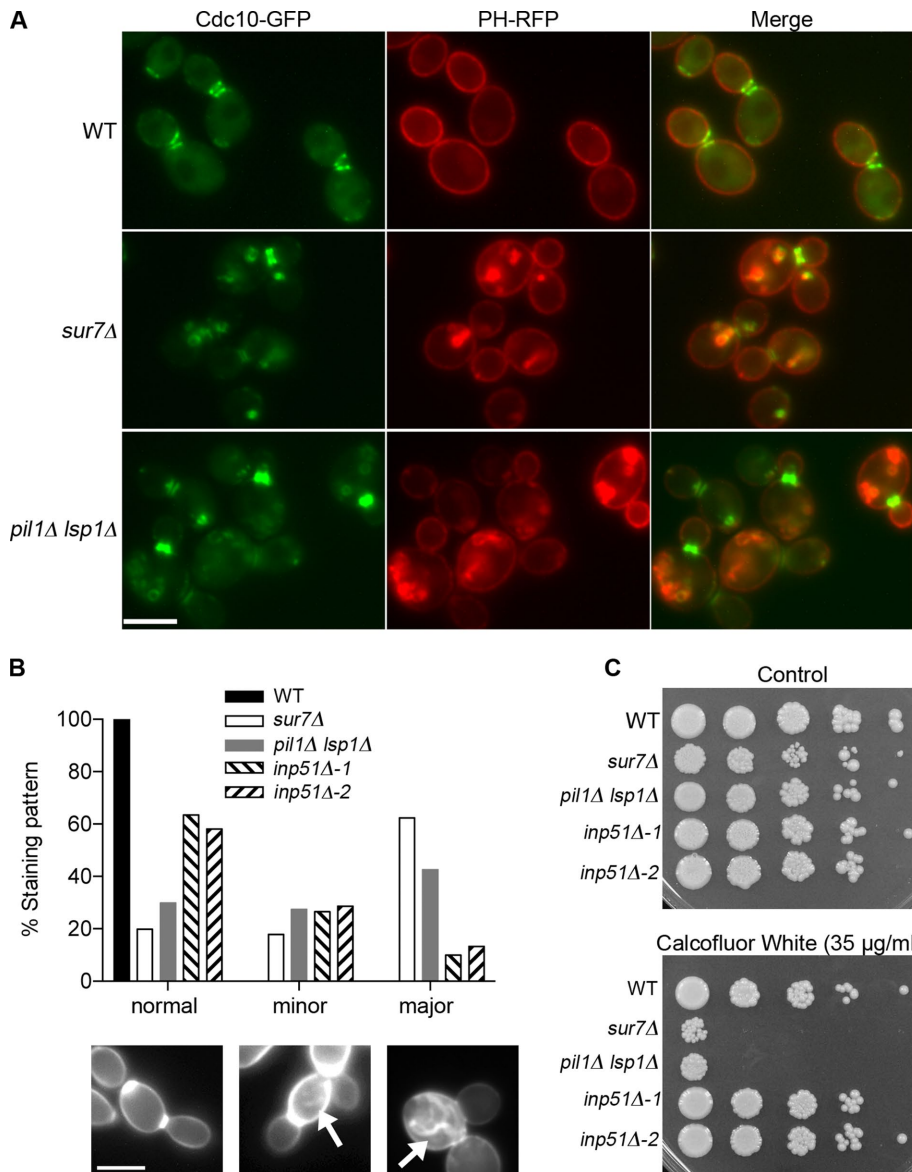
FIGURE 7: A PH-domain probe indicates that sites of cell wall invaginations correlate with increased PI$_{4,5}$P$_2$. (A) Indicated cell types carrying the Cdc10 septin fused to GFP (Cdc10-GFP) and the PLCδ1 PH domain fused to RFP (PH-RFP) were analyzed by fluorescence microscopy. Note the similar localization of these PI$_{4,5}$P$_2$-binding proteins. (B) Cell walls were stained with calcofluor white to detect abnormalities. The fraction of cells displaying strongly abnormal cell wall was higher in the *sur7Δ* and *pil1Δ lsp1Δ* mutants than in the *inp51Δ* mutant, which lacks this PI$_{4,5}$P$_2$ phosphatase. Bottom, representative samples of cells that were scored as wild type, minor defect, or major defect. The contrast was adjusted when scoring samples to make minor defects more obvious. (C) Dilutions of cells were spotted onto rich YPD medium in the absence and presence of 35 µg/ml calcofluor white to perturb the cell wall. Two independent *inp51Δ* mutants. Strains used were *CDC10-GFP PH-RFP* (YHXW47-1), *CDC10-GFP PH-RFP sur7Δ* (YHXW41-1), *CDC10-GFP PH-RFP pil1Δ lsp1Δ* (YHXW39-1), WT control (DIC185), *inp51Δ-1* (YXH42-1), *inp51Δ-2* (YXH42-2), *sur7Δ* (YJA11), and *pil1Δ lsp1Δ* (YHXW21-1). Bars, 5 µm.

might also help to explain why eisosome proteins are not as highly conserved as is the presence of plasma membrane furrows, which are detected in a broad range of fungi, algae, and lichens (Lee *et al.*, 2015).

An important role for *C. albicans* Pil1 and Lsp1 is to promote Sur7 function

The fact that Sur7 plays a key role in *C. albicans* makes it a good model system for determining the function of this MCC/eisosome component. Sur7 is an integral membrane protein that is predicted to span the plasma membrane four times. Of interest, the first extracellular loop of Sur7 contains an amino acid motif similar to one found at the same position in the claudin proteins, which are a family of tetraspan proteins that promote the formation of specialized membrane domains at sites of tight junctions in mammalian cells (Alvarez *et al.*, 2008). Claudin proteins mediate contacts across tight junctions that restrict the passage of chemicals or microorganisms between cells (Furuse and Tsukita, 2006). Sur7 could play a similar type of structural role in eisosomes, perhaps mediating contact across furrows or forming a lateral scaffold.

To better define Sur7 function, we compared a *sur7Δ* mutant that makes eisosomes to a *pil1Δ lsp1Δ* mutant that lacks eisosomes. Both mutants showed abnormal cell wall invaginations (Figure 2), mislocalized actin (Figure 3), and increased sensitivity to agents that affect cell wall function (Figure 4). However, there were significant differences. For example, the *sur7Δ* mutant was more sensitive than the *pil1Δ lsp1Δ* mutant to agents that affect the cell wall, including Congo red, calcofluor white, and the Pkc1 inhibitor cercosporamide (Figure 4). In contrast, the *pil1Δ lsp1Δ* mutant was more sensitive than the *sur7Δ* mutant to agents that affect the plasma membrane, such as SDS and the ergosterol synthesis inhibitor fluconazole (Figure 4). The *sur7Δ* cells were also distinct, in that they were more susceptible to caffeine and rapamycin (Figure 6), whereas the *pil1Δ lsp1Δ* mutant had a stronger defect in invasive hyphal growth (Figure 3). These differences indicate that Sur7 and Pil1/Lsp1 have overlapping but distinct functions.

The weaker signal of Sur7-GFP in the plasma membrane of *pil1Δ lsp1Δ* cells led us to examine the effects of overexpressing *SUR7*. The results showed that *SUR7* overexpression in the *pil1Δ lsp1Δ* mutant increased resistance to the cell wall inhibitors Congo red and calcofluor white and improved cell wall morphology (Figure 8). The ability of *SUR7* overexpression to suppress *pil1Δ lsp1Δ* defects indicates that Sur7 can function in the absence of Pil1 and Lsp1. This indicates that an important role of eisosomes is to promote Sur7

Slm2 is required in *S. cerevisiae* for morphogenesis and viability (Berchtold *et al.*, 2012). Similarly, Nce102 does not appear to have a significant influence on eisosome assembly or furrow formation in *C. albicans* as it does in *S. cerevisiae*, *S. pombe*, and *Aspergillus nidulans* (Frohlich *et al.*, 2009; Stradalova *et al.*, 2009; Kabeche *et al.*, 2011; Douglas *et al.*, 2013). These species-specific differences suggest that eisosome proteins have overlapping functions that enable some proteins to take on stronger roles in different species. This
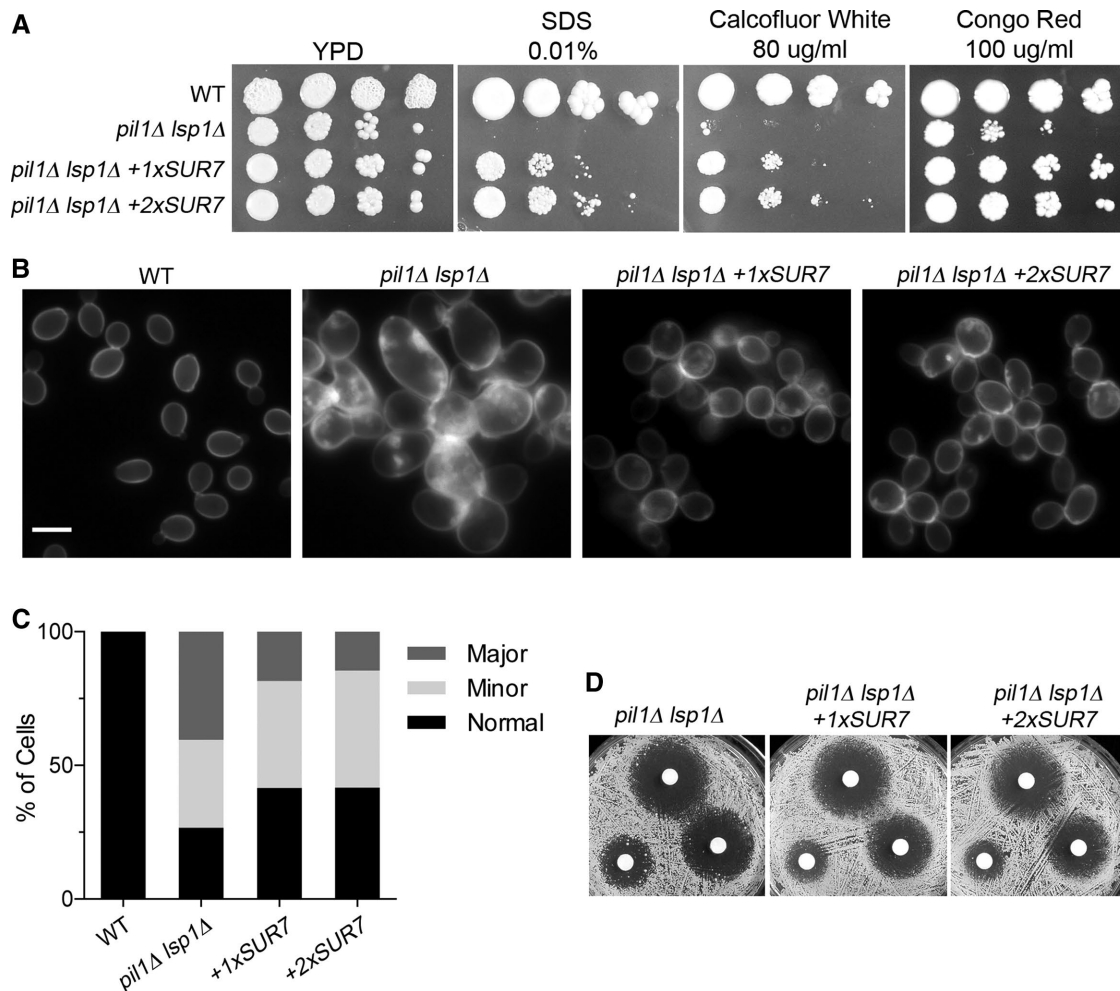
FIGURE 8: Overexpression of *SUR7* partially suppresses the cell wall phenotypes of *pil1Δ lsp1Δ* cells. Wild type, *pil1Δ lsp1Δ*, and versions of *pil1Δ lsp1Δ* carrying one extra copy of *SUR7* integrated into the genome (+1x*SUR7*) or two extra copies of *SUR7* (+2x*SUR7*) were analyzed. (A) Spot assays showing the growth of the strains indicated on the left to the compounds listed above. YPD indicates the rich nutrient medium, which was used as the base medium for all of the assays. (B) Wild-type and indicated mutant cells were stained with calcofluor white, and then cell wall structures were photographed by fluorescence microscopy. (C) Relative proportions of cells with cell wall invaginations detected by calcofluor white staining as described in Figure 7B. (D) Relative sensitivity to fluconazole for the indicated strains. Cells were spread on an agar plate, and then filter disks containing 0.25, 0.5, or 1 µg fluconazole were placed on top and the plates incubated for 2 d at 37°C. Strains used were wild-type control (DIC185), *pil1Δ lsp1Δ* (YHXW21-1), *pil1Δ lsp1Δ +1xSUR7* (YHXW45-1), and *pil1Δ lsp1Δ +2xSUR7* (YHXW46-1). Bar, 5 µm.

function, most likely by stabilizing it in the membrane. Consistent with this, Sur7 is one of the most stable proteins in *S. cerevisiae* (Thayer *et al.*, 2014). Eisosomes also likely have other roles in the cell, as *SUR7* overexpression did not rescue the fluconazole sensitivity of the *pil1Δ lsp1Δ* cells (Figure 8D).

Eisosomes regulate morphogenesis

Eisosomes regulate cell wall growth in a manner distinct from the new cell wall synthesis at the tips of buds and hyphae. Eisosomes take time to form and are not present at these sites of new morphogenesis (Moreira *et al.*, 2009; Reijnst *et al.*, 2011). Consistent with this, *sur7Δ* daughter cells look relatively normal, but the older, mother cells grow larger and form cell wall invaginations (Wang *et al.*, 2011). Similar results were observed for the *pil1Δ lsp1Δ* cells. The phenotypes of a *C. albicans inp51Δ* mutant first suggested that altered regulation of $PI_{4,5}P_2$ might be involved in forming the cell wall invaginations. Mutation of the $PI_{4,5}P_2$ phosphatase *INP51* in *C. albicans* caused cell wall invaginations and mislocalization of septins similar to what was observed for the *sur7Δ* and *pil1Δ lsp1Δ* mutants (Alvarez *et al.*, 2008; Badrane *et al.*, 2012). In *S. cerevisiae*, an *inp51Δ inp52Δ* mutant lacking two $PI_{4,5}P_2$ phosphatases also forms abnormal invaginations (Singer-Kruger *et al.*, 1998). $PI_{4,5}P_2$ plays important roles by influencing actin localization and recruiting morphogenesis proteins to the plasma membrane (Strahl and Thorner, 2007; Vernay *et al.*, 2012). A PH-RFP probe that binds $PI_{4,5}P_2$ showed that sites of altered cell wall growth were associated with elevated levels of $PI_{4,5}P_2$ in the *sur7Δ* and *pil1Δ lsp1Δ* mutants (Figure 7) similar to what was reported for the *inp51Δ* mutant (Badrane *et al.*, 2012). Consistent with this, septin proteins that bind $PI_{4,5}P_2$ (Bertin *et al.*, 2010) and cell wall morphogenesis proteins also localized at these sites. This suggests that $PI_{4,5}P_2$ inappropriately recruits morphogenesis proteins that promote cell wall invaginations.

Patches of $PI_{4,5}P_2$ were detected in the plasma membrane of *S. pombe* and *S. cerevisiae* in response to osmotic stress, which might be related to the $PI_{4,5}P_2$ patches seen in *C. albicans* sur7Δ and pil1Δ lsp1Δ mutants (Kabeche *et al.*, 2015). These stress-induced $PI_{4,5}P_2$ patches were not associated with cell wall invaginations, perhaps because they are transient. *S. cerevisiae* and *S. pombe* eisosomes have been linked to negative regulation of $PI_{4,5}P_2$ levels (Frohlich *et al.*, 2014; Kabeche *et al.*, 2014, 2015). An *S. cerevisiae* pil1Δ mutant that fails to recruit Inp51 to the plasma membrane displayed elevated $PI_{4,5}P_2$ levels (Frohlich *et al.*, 2014). However, *C. albicans* Sur7 does not exclusively regulate Inp51, since the phenotype of inp51Δ cells is weaker than that of sur7Δ or pil1Δ lsp1Δ cells (Figure 7). Eisosomes in *C. albicans* must either regulate additional $PI_{4,5}P_2$ phosphatases or interfere with $PI_{4,5}P_2$ levels in some other way. Of interest, since $PI_{4,5}P_2$ is important for eisosome assembly (Olivera-Couto *et al.*, 2011), there could be a feedback loop in which increased $PI_{4,5}P_2$ levels promote eisosome assembly, which in turn negatively regulates $PI_{4,5}P_2$ levels.

Eisosome proteins promote fungal virulence

The pil1Δ lsp1Δ mutant defects provide additional evidence that eisosomes are important for *C. albicans* virulence functions. Previous studies showed that a sur7Δ mutant has a very strong virulence defect in a mouse model of disseminated candidiasis (Alvarez *et al.*, 2008). An nce102Δ mutant has decreased virulence, which is likely due to its defect in invasive hyphal growth (Douglas *et al.*, 2013). A family of four quinone reductase proteins that localize to eisosomes in *C. albicans* promotes a novel antioxidant pathway that is needed for virulence (Li *et al.*, 2015). Furthermore, the pil1Δ lsp1Δ mutant shows increased susceptibility to fluconazole (Figure 4). Thus eisosome proteins represent interesting new drug targets. Further studies on eisosomes will contribute to developing new therapeutic approaches and improving the efficacy of current antifungal drugs.

MATERIALS AND METHODS
Strains and media

The *C. albicans* strains are described in Supplemental Table S1. Cells were grown in either rich yeast extract/peptone/dextrose (YPD) medium or complete synthetic medium containing yeast nitrogen base, dextrose, amino acids, and uridine (Sherman, 1991). Deletion mutants lacking *LSP1*, *PIL1*, *SEG1*, *ORF19.3505* (SLM2), *PKH2*, or *PKH3* were constructed in *C. albicans* strain BWP17 essentially as described previously (Wilson *et al.*, 1999). PCR primers containing ~70 base pairs of sequence homologous to the sequences flanking the open reading frame of the targeted gene were used to amplify either the *ARG4* or *HIS1* selectable marker genes. Proper integration of the deletion cassettes was verified by PCR using combinations of primers that flanked the deleted gene and primers that annealed within the deletion cassette, as well as primers that were used to confirm the absence of the deleted gene. The *ura3* auxotrophy was corrected by transforming cells with an *IRO1-URA3* fragment that was released from plasmid pBSK-URA by digestion with *Not*I and *Pst*I. Alternatively, *URA3* was used to select for reintegration of a copy of the corresponding wild-type gene to create complemented strains. Complementing plasmids were constructed by inserting a *C. albicans* gene containing the open reading frame plus sequences ~1 kb upstream and 300 base pairs downstream between the *Sac*I and *Sac*II sites of pDDB57. Plasmids were cut within the promoter region and transformed into the appropriate deletion mutant to create the complementing strain. The *PIL1* complementing plasmid was cut with *Sna*BI, the *LSP1* comple-

menting plasmid with *Bsa*BI, the *PKH2* complementing plasmid with *Sna*BI, and the *SUR7-URA3* and *SUR7-NAT1* complementing plasmids with *Bsr*GI. To overexpress *SUR7*, the *SUR7-URA3* plasmid carrying the open reading frame and ~1 kb upstream sequence was cut in the promoter region with *Bsr*GI and then integrated into the genome to create strain HXWY45-1. *SUR7* overexpression strain HXWY46-1 was created by integrating the *SUR7-NAT1* plasmid in a similar manner, using selection for nourseothricin resistance.

A pil1Δ lsp1Δ double mutant was constructed by deleting one copy of *PIL1* with *ARG4* and one copy of *LSP1* with *HIS1*, as described. The second copy of each allele was then deleted using the SAT flipper (Reuss *et al.*, 2004). In brief, PCR primers containing ~70 base pairs of homology to *PIL1* or *LSP1* were used to amplify the SAT Flipper cassette, which contains a nourseothricin resistance gene. After obtaining a deletion of the second allele of *PIL1*, we grew the cells on maltose medium to stimulate recombination to pop out the SAT flipper cassette and leave behind one copy of the FRT recombination site. This process was then repeated to delete the remaining copy of the *LSP1* gene and create the lsp1::HIS1/lsp1::FRT pil1::ARG4/pil1::FRT strain, which for brevity is referred to as the pil1Δ lsp1 mutant.

The GFP variant was fused to the 3′ ends of the open reading frames for *LSP1*, *PIL1*, *SEG1*, *SUR7*, *ORF19.3505* (SLM2), *ORF19.4043*, and *NCE102* as described previously (Zhang and Konopka, 2010; Douglas *et al.*, 2013). The correct integration was confirmed by PCR analysis and production of a fluorescent protein, which was detected by microscopy. The human PLCδ1 PH domain fused to RFP was introduced near the RP10 locus as described previously (Badrane *et al.*, 2012).

Fluorescence microscopy

Strains that produce fluorescent fusion proteins were grown overnight and analyzed by microscopy using a Zeiss (Jena, Germany) Axiovert 200 M microscope equipped with an AxioCam HRm camera and Zeiss AxioVision software for deconvoluting images. Actin localization was examined by phalloidin staining. Cells were grown to log phase in YPD medium at 30°C, fixed in 5% formaldehyde for 75 min at 30°C, incubated at room temperature for 30 min in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1% Triton X-100, and then washed twice in 0.1 M potassium phosphate buffer (pH 7.5). The cells were then stained by addition of 5 U of rhodamine-phalloidin (Invitrogen, Carlsbad, CA), incubated overnight at 4°C, washed, and then examined by fluorescence microscopy. Cell-wall chitin was stained by incubating cells with 40 μg/ml calcofluor white (Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO) as described previously (Pringle, 1991). Calcofluor white does not cross the plasma membrane, which prevents it from efficiently staining the invaginations of cell wall growth in the mutant cells. To better visualize the cell wall invaginations, we first permeabilized cells with methanol and acetone and then stained them with calcofluor white.

Electron microscopy

TEM was used to analyze cell wall invaginations in cells that were fixed with 3% EM-grade glutaraldehyde in 1× sodium cacodylate buffer (pH 7.4) at room temperature for 1 h. The fixed cells were resuspended in a 4% permanganate solution, washed, and then stained with uranyl acetate. The samples were then dehydrated by resuspending the cells in a graded series of acetone, washed with acetonitrile, and then embedded in Epon resin. A Reichert-Jung UltracutE ultramicrotome was used to cut ultrathin sections (80 nm), which were placed on Formvar-coated slot copper grids.

The sections were then viewed with a Tecnai12 BioTwinG^2 electron microscope (FEI, Hillsboro, OR), and an XR-60 CCD Digital Camera System (Advanced Microscopy Techniques, Woburn, MA) was used to acquire digital images. Sample preparation and TEM analyses were carried out at the Central Microscopy Imaging Center at Stony Brook University.

For analysis of membrane furrows, cells from an overnight culture were harvested by centrifugation (1 min at $1500 \times g$) and washed in 50 mM potassium phosphate buffer (pH 5.5). A 2-µl aliquot of the concentrated cell suspension was loaded onto a gold carrier and frozen rapidly in liquid nitrogen. The sample was cut with a cold knife ($\leq-185°C$), etched for 4 min ($-97°C$; pressure $\leq1.310^5$ Pa) in a CFE-50 freeze-etch unit (Cressington, Watford, United Kingdom), shadowed (1-nm Pt/C, 45°C; 10-nm C, 90°C), and cleaned in fresh 70% H_2SO_4 for 16 h (Rachel et al., 2002). Air-dried sample surface replicas were examined in an FEI Morgagni 268(D) transmission electron microscope at 80 kV. Images were captured with a Megaview II (Olympus Corp., Münster, Germany) charge-coupled device camera.

Growth inhibition assays

Spot assays were used to test the sensitivity of cells to SDS, Congo red, calcofluor white, rapamycin, and caffeine. A 10-fold dilution series of cells was prepared, and then 3 µl of each dilution was spotted on the surface of YPD plates containing the indicated concentration of chemical. The plates were incubated at 37°C for 2 d and then photographed to record the extent of growth. The sensitivity of cells to cercosporamide was assayed by testing the ability of cells to grow in a twofold dilution series of different concentrations of cercosporamide in 96-well plates at 37°C for 2 d. The extent of growth in each well was then quantified using a spectrophotometer. The sensitivity of cells to fluconazole was assayed using Etest strips (AB Biodisk North America, Piscataway, NJ). We spread 1×10^6 cells on an agar plate containing RPMI 1640 medium (Sigma-Aldrich), 0.165 M 3-(N-morpholino)propanesulfonic acid, pH 7, and 2% dextrose. An Etest strip was applied to the surface of each agar plate; the plates were incubated at 37°C for 48 h and then photographed. Similar studies were also carried out by placing filter disks carrying different amounts of fluconazole on the lawns of cells spread on an agar plate.

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