PP2A-Like Protein Phosphatase (Sit4) Regulatory Subunits, Sap155 and Sap190, Regulate Candida albicans’ Cell Growth, Morphogenesis, and Virulence

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INTRODUCTION

Candida albicans (Ca) is a commensal organism of the oral cavity, gastrointestinal tract, and vagina (Arendrup, 2013; Hebecker et al., 2014). When the host immune system is compromised, such as under conditions of long-term antibiotic treatment, immunodeficiency, or chemotherapy, C. albicans can cause mucocutaneous and life-threatening disseminated infections (Romani, 2011; Goulart et al., 2018). According to statistics, C. albicans is the fourth most common cause of hospital-acquired systemic infections with a crude mortality rate of more than 50% in the United States (Lai et al., 2008; Pfäffer and Diekema, 2010). C. albicans can grow as several cell types, including yeast, pseudohyphae, and true hyphae (Sudbery et al., 2004). Yeast form helps its spread, while hyphae have strong ability of tissue adhesion and invasion (Berman and Sudbery, 2002; Zhu and Filler, 2010). Furthermore, hyphae can avoid recognition and phagocytosis by host...
macrophages and neutrophils, thus enabling it to escape from the killing of the host immune system (Erwig and Gow, 2016). The transformation between different cell types is closely related to *C. albicans* pathogenicity (Lo et al., 1997; Saville et al., 2003), suggesting that the identification of proteins involved in morphogenesis may provide new targets for developing antifungal agents.

Reversible protein phosphorylation plays a crucial role in the control of nearly all cellular processes, and dephosphorylation is equally important to phosphorylation. Most phosphorylation events in eukaryotes involve the transfer of phosphate to serine (Ser) or threonine (Thr) residues. Removal of the phosphate is catalyzed by Ser/Thr protein phosphatases. According to the enzymological criteria, Ser/Thr protein phosphatases can be classified into two groups: type 1 (PP1) and type 2 (PP2); PP2 phosphatases can be further classified into several groups based on the requirement for metal ions: PP2A and PP2A-like enzymes do not require metal ions, PP2B is activated by calcium, and 2C is Mg²⁺ dependent (Arino et al., 2011; Albataineh and Kadosh, 2016). There are three PP2A-like phosphatases in fungi: *Sit4*, Pph3, and Ppg1 (Albataineh and Kadosh, 2016). In *Saccharomyces cerevisiae*, *Sit4* plays a critical role in cell growth, proliferation, and the regulation of the Pck1-MAPK and Tor signaling pathways (Ronne et al., 1991; Sutton et al., 1991; Angeles et al., 2002; Rohde et al., 2004). Four regulatory subunits of *Sit4* has been identified, and they are named *Sit4* association proteins (SAPs) and divided into two groups based on sequence similarity, the SAP4/SAP155 group and the SAP185/SAP190 group (Luke et al., 1996). Studies have shown that the SAPs have diverse functions, such as the regulation of cell growth, K⁺ efflux, and drug resistance (Luke et al., 1996; Manlandro et al., 2005; Miranda et al., 2010). In *C. albicans*, *Sit4* has been identified as the catalytic subunit of PP2A-like protein phosphatase, and deletion of *SIT4* causes a significant reduction in growth rate, morphogenesis, and virulence in mice (Lee et al., 2005; Miranda et al., 2010). In this study, we constructed *sit4Δ/Δ*, *sap155Δ/Δ*, *sap190Δ/Δ*, and *sap155Δ/Δ sap190Δ/Δ* mutants in *C. albicans* SC5314 background and conducted comprehensive phenotypic characterizations and comparisons. We found that *Sap190* is the main regulatory subunit of *Sit4* that plays critical roles in cell growth, cell wall integrity, hyphal morphogenesis, and virulence. *Sap155* is a redundant regulatory subunit, but it is functional and can partially compensate for the absence of *Sap190*.

### MATERIALS AND METHODS

#### Strains and Growth Conditions

The *Candida albicans* strains used in this study are listed in Table 1. *C. albicans* was routinely grown at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). For growth on plates, 2% agar was added to the medium. To select for nourseothricin-resistant transformants, 200 µg/mL of nourseothricin (Werner Bioagents, Jena, Germany) was added to the YPD agar plates (YPD-Nou plates). To obtain nourseothricin-sensitive derivatives in which the SAT1-flipper was excised by FLP-mediated recombination, transformants were grown overnight in YCB–BSA medium (2.34% w/v yeast carbon base, 0.4% w/v bovine serum albumin, pH 4.0) to induce the SAT2 promoter controlling CaFLP expression, and then streak-inoculated onto YPD plates containing 25 µg/mL nourseothricin and incubated at 30°C at least 2 days. Hyphal growth was induced by supplementing YPD medium with 10% fetal calf serum or DMEM, and incubating at 37°C with shaking at 200 rpm, or shaking yeast cells onto Spider agar plates (1% w/v beef extract, 1% w/v mannitol, 0.2% w/v K₂HPO₄, and 2% w/v agar, pH 7.2) to incubate at 30°C for 7 days. Pseudohyphal growth was induced by supplementing YPD medium with 15 mM hydroxyurea or 0.02% methyl methanesulfonate (MMS), and incubating at 30°C with shaking at 200 rpm.

### Strain Construction

Gene deletion was done in *C. albicans* SC5314 using the SAT-flipper method as described previously (Reuss et al., 2004). Briefly, the SAT1-flipper cassette flanked by 60 bp of upstream and downstream sequences of the target gene was amplified by PCR. Then, the PCR products were transformed into SC5314 cells using the lithium acetate protocol. After transformation, cells were recovered by culturing in fresh YPD medium at 30°C for 4 h with shaking at 200 rpm before spreading onto YPD-Nou plates. Two round of the transformation were required to obtained homzygous deletion mutants. Genomic DNA and total RNA were isolated from selected transformants to verify the mutations by PCR and RT-PCR analysis.

| Strain        | Relevant genotype       | Source            |
|---------------|-------------------------|-------------------|
| SC5314        | Wild type               | Fonzi and Irwin, 1993 |
| SIT4-Flag     | SIT4/SIT4-Flag-FRT      | This study        |
| Sap155-GFP    | SAP155/SAP155-GFP-FRT  | This study        |
| Sap155-Flag   | SAP155/SAP155-Flag-FRT | This study        |
| Sap190-GFP    | SAP190/SAP190-GFP-FRT  | This study        |
| SIT4-Flag Sap155-GFP | SIT4/SIT4-Flag-FRT | This study        |
| SIT4-Flag Sap190-GFP | SIT4/SIT4-Flag-FRT | This study        |
| Sap155-Flag   | SAP155/SAP155-Flag-FRT | This study        |
| Sap190-Flag   | SAP190/SAP190-Flag-FRT | This study        |
| Sap155-GFP    | SAP190/SAP190-GFP-FRT  | This study        |
| Sap190-GFP    | SAP190/SAP190-GFP-FRT  | This study        |
| sap155Δ/Δ     | sap155Δ/Δ              | This study        |
| sap190Δ/Δ     | sap190Δ/Δ              | This study        |
| sap190Δ/Δ     | sap190Δ/Δ              | This study        |
| sap190Δ/Δ     | sap190Δ/Δ              | This study        |
| SAP190/SAT1   | SAP190/SAP190-Δ        | This study        |
| sap190Δ/Δ + sap190Δ | SAP190/SAP190-Δ | This study        |
| sap190Δ/Δ + SAP190 | sap190/SAP190-Δ | This study        |
| sap190Δ/Δ + FRT SAT1 | SAP190/FRT | This study        |
| sap190Δ/Δ + FRT SAT1 | SAP190/FRT | This study        |
| sap190Δ/Δ + SAT1 | SAP190/FRT | This study        |
The plasmid pAG6 was constructed by Vylkova and Lorenz (2014), which is a SATI-marked version of CIP10 and used to integrate a gene into the RP10 locus by linearizing with StuI. We cloned SAP190 into the KpnI–XhoI sites of pAG6, then linearized the plasmid with StuI, and transformed it into the sap190Δ/Δ mutant to obtain SAP190 complemented strain.

To construct SATI-marked version of GFP or Flag-tagging vectors, GFP or Flag gene sequence followed by the URA3 terminator was inserted into the Apal–XhoI sites of pSFS1. To tag protein with GFP or Flag at the C-terminus, the GFP or Flag-SATI-flipper cassette flanked by 60 bp of the coding sequence 5′ to the stop codon (without the stop codon) and 60 bp of the non-coding sequence 3′ to the stop codon was amplified by PCR. The PCR products were transformed into appropriate strains. Correct tagging was verified by PCR and Western blotting analysis. The oligonucleotide primers used to construct deletion cassette and fusion protein are shown in Table 2.

**Growth Curves**

Late-log phase **C. albicans** yeast cells were diluted to OD<sub>600</sub> = 0.01 in 10 mL of YPD medium and were cultured at 30°C with shaking at 200 rpm. 100 µL of the culture was collected every 2 h, and OD<sub>600</sub> was measured using a microplate reader. The experiment was performed in triplicate.

**Susceptibility Tests**

*Candida albicans* cells grown to the late-log phase in YPD medium were harvested and washed twice with sterile water. The cell suspensions were 10-fold serially diluted to generate suspensions containing 10<sup>6</sup> to 10<sup>9</sup> cells/mL, and 5 µL of each dilution was spotted onto YPD plates containing the indicated concentrations of chemicals or drugs. Growth was assessed by incubating the plates at 30°C for the indicated time. All experiments were performed at least thrice.

**Fluorescence Microscopy**

Log-phase **C. albicans** yeast cells were stained with 10 µg/mL DAPI to visualize nuclei. Cells were examined by differential interference contrast (DIC) and fluorescence microscopy.

**Co-immunoprecipitation (Co-IP) and Western Blotting (WB)**

Co-IP and WB was performed as described previously by Han et al. (2019).

**Macrophage Cytotoxicity Assay**

*Candida albicans* toxicity on macrophages was assessed by using a Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies, Inc). RAW264.7 macrophages were seeded at 2.5 × 10<sup>5</sup> cells per well of a 96-well tissue culture plate in phenol red-free DMEM and maintained for 6 h in a humidified incubator in 5% CO<sub>2</sub> at 37°C. *C. albicans* cells were grown to the mid-log phase in YPD medium and washed twice with sterile PBS, and these cell suspensions were co-cultured with macrophages at a 3:1 ratio for 5 h. Supernatants were transferred into new plates and the absorbance at 490 nm was measured by a microplate reader. Cytotoxicity was calculated according to the average absorbance from each triplicate set of infected host cells relative to the maximum LDH release from lysed host cells following the manufacturer’s protocol. The experiment was performed in triplicates.

**Murine Model of Disseminated Candidiasis**

Mid-log phase *C. albicans* yeast cells were washed twice and diluted to 5 × 10<sup>5</sup> cells/mL with PBS. Ten female BALB/c mice per strain were injected via the tail vein with 200 µL of the cell suspension. The mice were monitored twice daily for survival for...
RESULTS

CaSap155 or CaSap190 Interacts With CaSit4 in Co-IP Experiments

In the C. albicans genome database, orf19.642 and orf19.5160 are designated as the regulatory subunits of Sit4, and their amino-acid sequence homologies with ScSap4/cSap155/ScSap185/ScSap190 are 23.3%/26.5%/23.8%/24.9% and 26.2%/26.5%/30.9%/35.0%, respectively (Supplementary Figure S1). Thus, we named them CaSap155 (orf19.642) and CaSap190 (orf19.5160) in this study.

To further investigate whether Sap155 and Sap190 are regulatory subunits of Sit4 in C. albicans, we tested whether Sap155 and Sap190 physically interact with Sit4. We tagged Sap155 and Sap190 with GFP and Sit4 with Flag all at the C-terminus. We then performed co-IP experiments using the anti-GFP-antibody conjugated beads to pull down Sap155-GFP and Sap190-GFP and then detected Sit4-Flag in western blotting analysis. The results showed that both Sap155 and Sap190 physically associate with Sit4 (Figure 1A). Furthermore, we tagged Sap155 C-terminus with Flag and Sap190 C-terminus with GFP, pulled down Sap190-GFP, and then detected Sap155-Flag in western blotting analysis. We did not detect physical interaction between Sap155 and Sap190 (Figure 1B). Thus, like S. cerevisiae, Sap155 and Sap190 independently associate with Sit4 in separate complexes in C. albicans (Luke et al., 1996).

Characterization and Comparison of sit4Δ/Δ, sap155Δ/Δ, sap190Δ/Δ, and sap155Δ/Δ sap190Δ/Δ Mutants During Yeast Growth

To avoid the undesirable effects of having auxotrophic markers, we used the wild-type strain SC5314 as the parent and the SAT1-flipper method to delete the two copies of SIT4, SAP155, or SAP190, yielding the sit4Δ/Δ, sap155Δ/Δ, and sap190Δ/Δ mutants.

To investigate the functions of the regulatory subunits of Sit4 during yeast growth, wild-type (WT; SC5314), sit4Δ/Δ, sap155Δ/Δ, and sap190Δ/Δ cells were cultured in YPD liquid medium at 30°C and the growth was monitored by measuring OD600 at timed intervals (Figure 2A). Furthermore, yeast cultures of the same strains were serially diluted and spotted onto YPD plates (Figure 3). The results showed that the sap155Δ/Δ mutant exhibited normal growth, while the sap190Δ/Δ mutant grew much more slowly than WT cells. Introducing one copy of WT SAP190 at the RP10 locus of the sap190Δ/Δ mutant (sap190Δ/Δ + SAP190) fully restored the growth whereas introducing the empty vector pAG6 (sap190Δ/Δ + pAG6) had no effect, indicating that the slower growth of the sap190Δ/Δ mutant was due to the deletion of SAP190 (Figure 2B). Also, the growth of the sit4Δ/Δ mutant was slightly slower than the sap190Δ/Δ mutant (Figures 2A, 3). To further determine the roles of SAP155, we deleted SAP155 from the sap190Δ/Δ mutant to obtain the sap155Δ/Δ sap190Δ/Δ mutant. We found that the double mutant also grew little more slowly than the sap190Δ/Δ mutant while it grew at a similar rate to the sit4Δ/Δ mutant (Figures 2A, 3). These results suggest that Sap190 is the main regulatory subunit of Sit4 and is required for normal yeast growth of C. albicans, while SAP155 can partially maintain cell growth in the absence of SAP190.

Next, we stained the nucleus with DAPI and found normal nuclear localization in sit4Δ/Δ, sap155Δ/Δ, sap190Δ/Δ, and sap155Δ/Δ sap190Δ/Δ cells (Supplementary Figure S2), and these mutants had normal cytoplasmic division, suggesting that the slow growth of cells lacking SIT4 or its regulatory subunits is not due to abnormal cell division.
The antifungal drug caspofungin (CAS) is a non-competitive inhibitor of β-1,3-glucan synthase and commonly used clinically to treat a variety of fungal infections, including *C. albicans* infections (Walker et al., 2010). We next determined whether lacking Sit4 or its regulatory subunits also alters the sensitivity to CAS. We found that the sensitivity of *sap190ΔΔ* mutant gradually increased with increasing concentrations of CAS in a range between 0.064 and 0.256 µg/ml and the deletion of *SAP155* in the *sap190ΔΔ* mutant increased the sensitivity further, particularly at the concentration of 0.128 µg/ml (Figure 3). *sit4ΔΔ* and *sap155ΔΔ* *sap190ΔΔ* mutants showed similar sensitivities to CAS under these conditions. These results indicate that *sit4ΔΔ* and *sap190ΔΔ* mutants are hypersensitive to CAS, consistent with their sensitivity to osmotic and cell wall stress. The results also show that *SAP155* is functional and can partially compensate for the absence of *SAP190*.

### The Functions of *SAP155* and *SAP190* in Filamentous Growth Caused by Different Inducing Factors

We next examined the hyphal growth of the mutants of *Sit4* and its regulatory subunits under various inducing conditions. We found that although *sit4ΔΔ*, *sap190ΔΔ*, and *sap155ΔΔ* *sap190ΔΔ* cells could grow hyphae, their hyphae were much shorter compared with those of WT cells in YPD medium containing 10% serum at 37°C (Figure 4A). Interestingly, the hyphal length of *sap190ΔΔ* cells was longer than that of *sit4ΔΔ* and *sap155ΔΔ* *sap190ΔΔ* mutants. The same phenotype was also observed when hyphal growth was induced in DMEM medium (Supplementary Figure S3). *sap155ΔΔ* *sap190ΔΔ* cells did not exhibit significant defects in hyphal growth under these tested conditions. On Spider plates, WT and *sap155ΔΔ* *sap190ΔΔ* cells formed colonies with long filaments radiating from the colony periphery, while the edge of *sit4ΔΔ*, *sap190ΔΔ*, and *sap155ΔΔ* *sap190ΔΔ* colonies were smooth (Figure 4A). Furthermore, it is interesting that both *sit4ΔΔ* and *sap155ΔΔ* *sap190ΔΔ* colonies show wrinkling at the colony center, which is very different from the morphology of the other strains. These results indicate that *SAP155* is required for hyphal growth and that *SAP155* can partially compensate for the loss of *SAP190* under some inducing conditions.

Genotoxic stress, such as DNA replication inhibition and DNA damage, can cause *C. albicans* cell cycle arrest, leading to filamentous growth and the formation of pseudohyphae (Gow et al., 2011). Hydroxyurea (HU) is an inhibitor of DNA replication, and MMS causes DNA methylation, leading to DNA damage. To investigate the roles of *Sit4* and its regulatory subunits in response to the genotoxic stress, we grew *C. albicans* in the presence of 15 mM HU and 0.02% MMS. We observed that, under HU treatment, while nearly all WT cells grew into long filaments, the majority of *sit4ΔΔ*, *sap190ΔΔ*, and *sap155ΔΔ* *sap190ΔΔ* cells remained in the yeast form with a small number of cells showing slight elongation (Figure 4B). The phenotypes of *sit4ΔΔ* and *sap155ΔΔ* *sap190ΔΔ* cells were similar, both exhibiting more severe defects than *sap190ΔΔ* cells. Under MMS treatment, *sit4ΔΔ*,

### Deletion of *SAP155* Renders the *sap190ΔΔ* Mutant More Sensitive to Cell Wall Stress

A previous study has shown that the deletion of *Sit4* led to hypersensitivity to osmotic stress (Lee et al., 2004). We found that the *sap155ΔΔ* *sap190ΔΔ* and *sit4ΔΔ* *mutants grew at similar rates but both significantly more slowly than SC5314 (WT) on YPD plates containing 1.5 M NaCl (Figure 3). To investigate the functions of the regulatory subunits *SAP155* and *SAP190* in maintaining cell wall integrity, WT, *sit4ΔΔ*, *sap155ΔΔ*, and *sap190ΔΔ* cells were spotted onto YPD plates containing 80 µg/ml Calcofluor White (CFW) or 100 µg/ml Congo Red (CR). We found that the growth of the *sap190ΔΔ* mutant was slower than WT and the *sap155ΔΔ* mutant, while the *sap155ΔΔ* *sap190ΔΔ* and *sit4ΔΔ* *mutants grew much more slowly than the *sap190ΔΔ* mutant, and *sit4ΔΔ* and *sap155ΔΔ* *sap190ΔΔ* mutants grew at similar rates (Figure 3). These results suggest that the deletion of *SAP155* further sensitizes the *sap190ΔΔ* mutant to osmotic and cell wall stress.
sap190Δ/Δ, and sap155Δ/Δ sap190Δ/Δ cells did not undergo filamentous growth (Figure 4B). After a 9-h MMS treatment, a small number of sap190Δ/Δ cells exhibited a slightly elongated yeast morphology, while all sit4Δ/Δ and sap155Δ/Δ sap190Δ/Δ cells remained in the typical yeast form. In spite of the defects in the genotoxic stress-induced filamentous growth, none of the mutants of SIT4 and its regulatory subunits showed altered sensitivity to either HU or MMS (Figure 4C). These results indicate that, firstly, Sit4 with its regulatory subunits plays an important role in regulating DNA-replication-inhibition and DNA-damage-induced filamentous growth. Secondly, Sit4 and its regulatory subunits have different roles in filamentous growth in response to different types of genotoxic stress. Thirdly, Sap190 plays a critical role in the filamentous growth induced by genotoxic stress, while Sap155 partially compensates for the loss of SAP190.

**Characterization and Comparison of the Virulence of sit4Δ/Δ, sap155Δ/Δ, sap190Δ/Δ, and sap155Δ/Δ sap190Δ/Δ Mutants in vitro and in vivo**

Macrophages are the first line of host defense against *C. albicans* infection, but *C. albicans* can escape through its hyphal growth, which can penetrate and cause the lysis of macrophages (Erwig and Gow, 2016). Next, we co-cultured *C. albicans* with RAW264.7 macrophages and then measured the activity of lactate dehydrogenase (LDH) released by macrophages into the supernatant to determine the macrophage cytotoxicity of *C. albicans*. We found that cells lacking SAP155 did not alter the macrophage cytotoxicity, while cells lacking SAP190 resulted in less damage to macrophages (Figure 5). Furthermore, sap155Δ/Δ sap190Δ/Δ and sit4Δ/Δ mutants exhibited similar macrophage cytotoxicity, while both caused less damage to macrophages than the sap190Δ/Δ mutant.

We next investigated the role of Sit4 and its regulatory subunits in virulence using a mouse model of systemic infection. Mice were injected with the WT (SC5314), sit4Δ/Δ, sap155Δ/Δ, sap190Δ/Δ, or sap155Δ/Δ sap190Δ/Δ strains via the tail vein, and their survival was monitored for 20 days. The results showed that all mice injected with WT or sap155Δ/Δ strains died within 9 days, and their survival median was 4–6 days. Mice infected with the sap190Δ/Δ mutant all died within 18 days, and the survival median was 10–11 days. However, 50% of mice injected with the sit4Δ/Δ or sap155Δ/Δ sap190Δ/Δ mutant survived for at least 20 days (Figure 6A). To assess the ability of these mutants to colonize the kidney, five mice in each group were sacrificed 48 h post-infection to quantify CFUs and perform PAS staining of kidney sections. The results showed that CFUs in the kidneys of mice infected with the
sit4Δ/Δ, sap190Δ/Δ, or sap155Δ/Δ sap190Δ/Δ mutant were much less than that in mice infected with the WT strain or the sap155Δ/Δ mutant. There was no statistically significant difference between the CFUs of sit4Δ/Δ and sap155Δ/Δ sap190Δ/Δ mutants, but the CFUs of both mutants were marked more than that of the sap190Δ/Δ mutant (Figure 6B). Also, the PAS staining of kidney sections revealed many long filaments of C. albicans cells in mice infected with SC5314 or the sap155Δ/Δ strain. In contrast, a small number of C. albicans cells were found in the kidney of mice infected with the sap190Δ/Δ mutant, and none was found in the kidney of mice infected with the sit4Δ/Δ or sap155Δ/Δ sap190Δ/Δ mutant (Figure 6C). The results demonstrate that SAP190 is required for the virulence of C. albicans and that SAP155 can partially compensate for the loss of virulence in the absence of SAP190 during co-cultivation with macrophages and systemic infection of mice.

**DISCUSSION**

PP2A-like phosphatases share high homology with PP2A enzymes which contain a catalytic subunit and a regulatory subunit (Albataineh and Kadosh, 2016). C. albicans Sit4 has been identified as a PP2A-like catalytic subunit (Lee et al., 2004). In this study, according to the amino-acid sequence analyses, we identified two proteins in C. albicans identified as PP2A-like catalytic subunit (Lee et al., 2004). In addition, PP2A-like phosphatases have been shown to play a role in stress response and cell morphology (Luke et al., 1996). We show here that Sap190 is the main regulatory subunit of Sit4 and plays...
FIGURE 6 | The virulence of \textit{sit4}\textsuperscript{Δ/Δ}, \textit{sap155}\textsuperscript{Δ/Δ}, \textit{sap190}\textsuperscript{Δ/Δ}, and \textit{sap155}\textsuperscript{Δ/Δ} \textit{sap190}\textsuperscript{Δ/Δ} mutants in a murine model of disseminated candidiasis BALB/c mice were injected via the tail vein with 10\textsuperscript{6} yeast cells of SC5314, \textit{sit4}\textsuperscript{Δ/Δ}, \textit{sap190}\textsuperscript{Δ/Δ}, or \textit{sap155}\textsuperscript{Δ/Δ} \textit{sap190}\textsuperscript{Δ/Δ} strains (15 mice per \textit{C. albicans} strain) and monitored for (A) survival over a period of 20 days. The results of survival curves were analyzed using Kaplan-Meier test. The statistical analysis showed that the survival curves of \textit{sap190}\textsuperscript{Δ/Δ} strain are significantly different compared to SC5314 or \textit{sap155}\textsuperscript{Δ/Δ} strain, and the survival curves of \textit{sit4}\textsuperscript{Δ/Δ} or \textit{sap155}\textsuperscript{Δ/Δ} \textit{sap190}\textsuperscript{Δ/Δ} strain are significantly different compared to SC5314, \textit{sap155}\textsuperscript{Δ/Δ}, or \textit{sap190}\textsuperscript{Δ/Δ} strain. Five mice were sacrificed after 48 h of the injection to determine the fungal load in the kidney (the results of fungal burdens were analyzed with Mann-Whitney test. *p < 0.05 compared between the indicated two groups. n.s: no significance) and (C) conduct histological examinations of kidney sections. Size bars = 0.13 mm. Arrows indicate \textit{C. albicans} cells in the renal tissues.

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critical roles in cell growth, cell wall integrity, morphogenesis, and virulence in mice. In the SC5314 background, deleting \textit{SAP155} does not produce any apparent defects, but deleting it in the \textit{sap190}\textsuperscript{Δ/Δ} background leads to more severe defects, indicating that \textit{Sap155} is functionally redundant and can partially compensate for the absence of \textit{Sap190}. These findings also indicate that \textit{C. albicans} retains redundant regulatory subunits of \textit{Sit4}, which may enhance its adaptability to some adverse environmental factors.

Cytokinesis and nuclear localization in \textit{sit4}\textsuperscript{Δ/Δ}, \textit{sap155}\textsuperscript{Δ/Δ}, \textit{sap190}\textsuperscript{Δ/Δ}, and \textit{sap155}\textsuperscript{Δ/Δ} \textit{sap190}\textsuperscript{Δ/Δ} mutants do not exhibit any discernable defects. In \textit{S. cerevisiae}, \textit{SIT4} is required for the G1/S transition (Sutton et al., 1991), and in \textit{Debaryomyces hansenii}, deletion of \textit{SIT4} causes an increased number of G1 phase cells (Chawla et al., 2017). Therefore, like in other fungal species, the regulatory subunits of \textit{Sit4} may affect cell growth though the G1/S transition in \textit{C. albicans}.

The yeast-to-hyphae morphological transition is recognized as the most important trait for \textit{C. albicans} infection. Previous studies have shown that \textit{SIT4} is involved in the morphogenesis of \textit{C. albicans} (Lee et al., 2004; Noble et al., 2010). Our results show that \textit{sap190}\textsuperscript{Δ/Δ} cells exhibited slower hyphal formation with shorter hyphal length, and \textit{sap155}\textsuperscript{Δ/Δ} \textit{sap190}\textsuperscript{Δ/Δ} cells exhibit more severe defects, which was similar to \textit{sit4}\textsuperscript{Δ/Δ} mutant, although the \textit{sap155}\textsuperscript{Δ/Δ} mutant did not show any discernible defects. The results suggest that both regulatory subunits of \textit{Sit4} are involved in regulating morphogenesis. In \textit{C. albicans}, cell wall integrity is closely correlated with morphogenesis. For example, the deletion of cell wall protein-coding genes \textit{ECM33} and \textit{CSF4} led to abnormal hyphal growth (Alberti-Segui et al., 2004; Martinez-Lopez et al., 2004). Thus, \textit{SAP155} and \textit{SAP190} may regulate morphogenesis partially through their roles in cell growth and cell wall integrity. However, the targets of \textit{SIT4} and its regulatory subunits are remain unclear in \textit{C. albicans}, thus their roles in the morphogenesis may be also through other unknown mechanisms.

Genotoxic stress, such as DNA replication inhibition and DNA damage, can activate cell cycle checkpoints via the
phosphorylation of the checkpoint protein kinase Rad53, causing *C. albicans* to form pseudohyphae (Gow et al., 2011). Deletion of RAD53 not only led to a defect in filamentous growth, but also caused hypersensitivity to genotoxic stress (Shi et al., 2007). Interestingly, we show here that under HU treatment, only a small percentage of *sit4ΔΔ*, *sap190ΔΔ*, and *sap155ΔΔ* *sap190ΔΔ* cells could undergo filamentous growth forming short filaments, and under MMS treatment, these mutant cells remained in the yeast form. However, deletion of *SIT4* or its regulatory subunits did not alter the sensitivity to HU and MMS, suggesting that *Sit4* with its regulatory subunits are involved in DNA-replication and DNA-damage checkpoint pathways to specifically regulate the filamentous growth. In future studies, we will explore the relationship between *SIT4* phosphatase complexes and Rad53 under different genotoxic stresses.

Although the exact mechanism by which Sap155 and Sap190 regulate the functions of *Sit4* remains unclear, deleting SAP190 causes reduced macrophage cytotoxicity in *vitro* and impaired virulence in mice, and deleting SAP155 in *sap190ΔΔ* background resulted in more severe defects. Therefore, the regulatory subunits of *Sit4* could serve as targets for developing new antifungal drugs.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**ETHICS STATEMENT**

The animal experiments were carried out in accordance to National Advisory Committee for Laboratory Animal Research Guidelines, and all procedures were approved by the IACUC of the Agency for Science, Technology and Research of Singapore.

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**AUTHOR CONTRIBUTIONS**

QH, JS, and YW conceived and created the experimental design. QH, CP, and YQW conducted the experiments. QH, YW, and LZ prepared the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02943/full#supplementary-material

**FIGURE S1** | Sequence alignment of *S. cerevisiae* Sap155 and Candida albicans Sap155 (orf19.642), *S. cerevisiae* Sap190 and C. albicans Sap190 (orf19.5160) Completely conserved residues are colored cyan.

**FIGURE S2** | The location of nucleus in *sit4ΔΔ*, sap155ΔΔΔ, sap190ΔΔΔ, and sap155ΔΔΔ* sap190ΔΔΔ cells. Yeast cells of *C. albicans* strains of the indicated genotype were stained with DAPI to visualize the nucleus. Size bars = 12 μm.

**FIGURE S3** | The hyphal growth of *sit4ΔΔ*, *sap155ΔΔΔ*, *sap190ΔΔΔ*, and *sap155ΔΔΔ* *sap190ΔΔΔ* mutants in DMEM. Late-log phase yeast cells of SC5314, *sap155ΔΔΔ*, *sap190ΔΔΔ*, and *sap155ΔΔΔ* *sap190ΔΔΔ* strains were re-inoculated at 1:20 dilution into fresh DMEM and incubated at 37°C with shaking at 200 rpm. Photos were taken at 1 h and 3 h. Size bars = 16 μm.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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