Infection and Drug Resistance Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

The Endocytosis Adaptor Sla1 Facilitates Drug Susceptibility and Fungal Pathogenesis Through Sla1-Efg1 Regulating System in *Candida albicans*

Chenhao Suo[1,*], Yiru Gao[2,*], Sheng Yang[2], Wanli Zhang[2], Chao Li[3], Lanjing Ma[2], Yingchun Xu[4,5], Jianjun Lei[1], Chen Ding[2], Hailong Li[6], He Zhang[1], Tianshu Sun[4,7]

[1]Laboratory Animal Department, Northern Theater General Hospital, Shenyang, Liaoning, 110000, People's Republic of China; [2]College of Life and Health Science, Northeastern University, Shenyang, Liaoning, 110000, People's Republic of China; [3]Department of Emergency Medicine, the Second Affiliated Hospital of Army Medical University, Chongqing, 400037, People's Republic of China; [4]Beijing Key Laboratory for Mechanisms Research and Precision Diagnosis of Invasive Fungal Diseases, Beijing, 100730, People's Republic of China; [5]Medical Research Centre, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science, Beijing, 100730, People's Republic of China; [6]Pulmonary and Critical Care Medicine, University of Michigan, Ann Arbor, MI, 48109, USA; [7]Clinical Biobank, Medical Research Center, National Science and Technology Key Infrastructure on Translational Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, People's Republic of China

*These authors contributed equally to this work

Correspondence: Tianshu Sun; He Zhang, Email sun_tianshu@163.com; alwayszhh@163.com

Introduction: The role of endocytosis in *Candida albicans* drug-resistance and pathogenicity remains poorly understood, despite its importance as a fundamental component of intracellular trafficking.

Objective: In order to understand the role of endocytosis in *Candida albicans* cell wall integrity, drug resistance, and virulence.

Methods: Detection of intracellular endocytosis by FM4-64 staining; Scanning electron microscopy is used to detect cell wall components; Spot assay for detecting drug sensitivity; Co-ip is used to detect protein interactions.

Results: In this study, we found the functions of Sla1 in regulating endocytosis is conserved among pathogenic fungi. Our results also revealed that the deletion of the *SLA1* gene altered cell wall properties, composition, and gene expression. In addition, we showed that *C. albicans* Sla1 was responsible for hyphal development in vitro and for fungal pathogenicity in a murine infection model. Intriguingly, *sla1*Δ/Δ mutant demonstrated enhanced drug resistance, and Sla1 was found to interact with the transcription factor Efg1; the relationship between Sla1 and Efg1 impacts the expression of genes encoding components of the ergosterol biosynthesis pathway, including *ERG1, EGR11*, and *ERG25*.

Discussion: These findings have expanded our knowledge of the capabilities of Sla1 beyond its role as an endocytosis adapter and provided insights into a potential new therapeutic target for the treatment of fungal infections.

Keywords: endocytosis, Sla1, cell wall integrity, drug susceptibility, fungal pathogenicity

Introduction

Candida albicans is the most important fungal opportunistic pathogen that cause of invasive infections in hospitalized patients with considerable mortality.[1,2] It usually causes about 150 million mucosal infections and 200,000 deaths per year due to invasive and disseminated disease in susceptible populations.[3,4] The endocytosis is critical for various physiological functions in eukaryotic cells, including nutrient absorption, intercellular communication, and maintenance of the integrity of the plasma membrane and cell wall.[5] Reports have suggested that endocytosis contributes to the drug susceptibility in *C. albicans*. The uptake of caspofungin is facilitated by endocytosis and inhibitors of endocytosis reduced its uptake in vacuoles.[6] Deletion of endocytosis genes *ENT2* or *END3* causing resistance to azoles in *C. albicans*.[7] So, we focused on the endocytic process in pathogenic fungi to provide ideas for developing new therapeutic approaches.

Received: 14 August 2024
Accepted: 16 October 2024
Published: 22 October 2024

Clathrin-mediated endocytosis (CME), the most prevalent and extensively studied endocytic mechanism in fungi, enables energy-dependent and receptor-mediated entry of cargo into the cell.[8,9] Over the past decade, more than sixty CME adaptors have been identified in yeast. These adaptors are recruited, activated and disassembled at distinct locations of the plasma membrane in a specific order.[10,11] The complex of the coat module proteins Pan1, End3, and Sla1 and another complex of Sla2 and Ent1/2 convened the transition between the early and late phases of CME.[12] Sla1 is considered to be a particularly important adaptor to examine due to the fact that it connects vital components of the endocytic machinery, cargo and clathrin, and induces actin assembly.[13] However, the role of endocytosis adaptor Sla1 in *Candida albicans* drug-resistance and pathogenicity are still largely unknown.

In this study, to investigate function of endocytic adaptor Sla1 in pathogenic fungi, we characterized the Sla1 homolog genes in *C. albicans* and *C. neoformans*. Defects in endocytosis and cell wall integrity were detected in *SLA1* deletion strains. Roles of Sla1 on virulence-related characteristics in vitro and in vivo were also identified in *C. albicans*, along with changes in drug resistance. Particular attention was paid to Sla1 was found to interact with the transcription factor Efg1; the relationship between Sla1 and Efg1 impacts the expression of *ERG* family genes, due to the known roles of ergosterol biosynthesis in mediating azole resistance. Our results provide new insights into Sla1 functions that are particularly relevant to drug-resistance, pathogenesis and virulence of *C. albicans*.

Materials and Methods

Strains, Media, Stress Response and Drug Susceptibility Assay

The strains used in this work are listed in Table S1. Fungal cells were routinely cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Cells were cultured overnight and then diluted to a density of 10^7 cells/mL (OD_{600} = 1). The cell suspension was diluted serially to produce suspensions containing 10^6, 10^5, 10^4 and 10^3 cells/mL. Identical volumes (5 μL) of each of these suspensions were spotted onto plates containing YPD medium supplemented with one of the following stressors: 140 μg/mL (*C. albicans*) or 0.8% (*C. neoformans* and *S. cerevisiae*) Congo red, 1 M NaCl, 2 ng/mL fluconazole, 4 ng/mL fluconazole, 0.15 ng/mL ketoconazole, 0.2 ng/mL ketoconazole or 0.3 ng/mL ketoconazole. The plates were incubated for 48 h prior to imaging.

Generation of Fungal Mutants

The primers used are described in Table S2. The *sla1*Δ/Δ *C. albicans* mutant was constructed by PCR-mediated homologous recombination using *HIS1* and *ARG4* selection cassettes as described previously.[14] Specifically, the *SLA1* ORF was knocked out by replacement with *C. dubliniensis ARG4* or *C. dubliniensis HIS1*. First, a region upstream in the *SLA1* ORF (primer pair 220/221), a region downstream in the *SLA1* ORF (primer pair 222/223) and a selection marker, either *ARG4* or *HIS1* (primer pair 189/190), were fusion amplified, and the products were purified and transformed into *SN152* receptor cells using electroporation. Transformants were selected on SC-His or SC-Arg medium and then verified using PCR and real-time PCR. The *efg1*Δ/Δ mutant strain was constructed using the same strategy. An *SLA1* haploid rescue strain (*sla1*Δ/Δ/*SLA1*) was constructed by replacing the haploid knockout cassette with a fusion fragment containing an upstream region from the *SLA1* ORF (primer pair 220/681), the *NAT* selection marker (primer pair 682/683) and a downstream region from the *SLA1* ORF (primer pair 684/223).

The *TDH3* promoter was employed to sequentially drive gene expression in order to create a FLAG-Sla1 over-expression strain. The selection marker *ARG4* (primer pair 459/190) and *TDH3* promoter (primer pair 467/470) were amplified and inserted between an upstream site (primer pair 220/221) and a site within the ORF of *SLA1* (primer pair 490/491) in the genomic DNA of strain *SN152*. Transformants were selected on SC-Arg medium, and candidates were verified using PCR and real-time PCR.

In order to create an *EFG1-GFP* over-expression strain, the *EFG1* ORF (primer pair 388/389) was amplified and cloned into the *SalI* site of the plasmid pNIM1 using the in-fusion method. The intended fragment was amplified (primer pair 2625/2626) and transformed into the *SN152* and *TDH3-SLA1* strains. Correct integration was verified using PCR and real-time PCR.

C. neoformans SLA1 mutant strains were generated by transforming the fusion-amplified upstream region of the *SLA1* ORF (primer pair 992/993), a neomycin resistance marker (primer pair 23/24) and the downstream region of the *SLA1* ORF (primer pair 994/995) into the wildtype strain H99.[15] Transformants were verified by PCR. The *S. cerevisiae SLA1* mutant strains were constructed by PCR-mediated homologous recombination as described previously.[16] The upstream and downstream sequences of *SLA1* and the selective marker sequence were fusion-amplified with primer pair 1000/1001. The PCR fragments were purified, concentrated and transformed into the BY4741 strain using the LiAc/ssDNA/PEG transformation method. Correct integration was verified using PCR.

FM4-64 Staining-Based Endocytosis Assay

The lipophilic membrane dye FM4-64 was used to assess the process of endocytosis. Briefly, wildtype and mutant cells were incubated in YPD medium overnight. The cultures were diluted to an OD_{600} of 0.2 in 10 mL of YPD medium and incubated at 30°C for an additional 4 h. FM4-64 was added to a final concentration of 10 μM. The mixture was incubated on ice for 10 min and then incubated at room temperature for 1 min. Membrane transport was halted by the addition of phosphate buffer, pH 7.4, containing 10 mm NaN_3 and 10 mm NaF. After two washes with 1 mL PBS, cells were imaged using a Nikon Eclipse Ni-E microscope with a 100× objective lens (Nikon; DS-Ri1).

CSH Assay

The CSH assay was performed as previously described.[17] Briefly, cells were collected by centrifugation and washed twice using PBS, and the optical density at OD600 was measured (A0). Then, 200 μL of xylene was mixed with the cell suspension and held at 30°C for 30 min to allow phase separation. The aqueous layer was transferred to a new tube, and its OD600 was measured (A1). The percentage of CSH was calculated as [(A0 - A1)/A0] ×100.

Transmission Electron Microscopy (TEM)

Samples for transmission electron microscopy were prepared according to the protocol described previously.[18] Briefly, wildtype and mutant cells were collected after 24 h of growth in liquid YPD medium and fixed by 2.5% paraformaldehyde. The samples were then washed with PBS and postfixed for 90 min with 1% Osmium tetroxide. The fixed cells were dehydrated through a graded series of ethanol and embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were stained with uranyl acetate and lead citrate and then imaged with a JEM-1400FLASH. Multiple cells of each strain were imaged.

In vitro Hyphal Assay

Fungi were incubated in YPD Medium at 30°C overnight. Cells were collected by centrifugation and washed twice with 1 mL PBS. The cell suspension was diluted to an OD_{600} of 0.4, and aliquots (100 μL) were added into wells of a 6-well plate, and DMEM supplemented with 10% FBS (2 mL) was added. The cells were incubated at 37°C in a 5% CO_2 atmosphere for 2, 4 or 6 h and then analyzed by light microscopy.

RNA Isolation

Analyses of gene expression were performed in biological triplicate. Wildtype and mutant strains were inoculated into 10 mL YPD medium and incubated overnight at 30°C. The samples were diluted to an OD_{600} of 0.2 with YPD medium and then incubated at 30°C for 4 h. The cells were collected by centrifugation and washed twice with 10 mL PBS to remove impurities. Cells were lysed using a bead beater, and total RNA was extracted using the E.Z.N.A. Total RNA Kit I (Omega Bio-tek). Synthesis of cDNA was performed using 0.5 μg of total RNA with the MonScript™ RTIII Super Mix (Monad). Real-time PCR was performed using a CFX Connect Thermocycler (Bio-Rad). Primer sequences are listed in Table S2.

Immunoprecipitation and Immunoblotting Analyses

Cultures of wildtype and mutant cells were diluted into 50 mL of YPD medium at a OD_{600} of 0.2 and incubated at 30°C for 6 h. Proteins were extracted using lysis buffer (50 mm Tris-HCl pH 7.5, 150 mm NaCl, 0.1% NP-40), supplemented

with 1× protease inhibitor cocktail and 1 mm PMSF. Protein samples were incubated with anti-FLAG magnetic beads (MCE) at 4°C overnight. The beads were then washed three times with TBS buffer (50 mm Tris-HCl, 150 mm NaCl, pH 7.4). Proteins were eluted from the beads by heating at 95°C for 5 min in sample buffer (125 mm Tris-HCl pH 7.5, 4% SDS, 30% glycerol). The proteins were separated using 8% SDS–polyacrylamide gel, transferred to nitrocellulose membranes and detected using anti-FLAG (Sangon Biotech; catalog # D191041) and anti-GFP (Sigma; catalog # G1544) primary antibodies.

Animal Fungal Burden and Survival Experiments

Four- to six-week-old female C57BL/6 mice were purchased from Changsheng Biotech (China). Mice were housed at 22°C with a 12 h light-dark cycle. *C. albicans* cells (5×10^5 in PBS) were injected intravenously. The mice were monitored daily and sacrificed when moribund (defined as hunched posture, minimal motor activity and weight loss of 15% of starting body weight). For fungal burden analyses, infected mice were sacrificed by exposure to CO_2 at 7 d post-infection. Livers, spleens, brains, and kidneys were homogenized, and samples of the lysates were plated onto YPD agar. Fungal colony-forming units (CFU) were counted after incubation at 30°C for 2 d.

Fungal Competitive Survival Assay

Mice were administered 2 mg/mL of streptomycin, 1500 U/mL of penicillin G, and 0.2 mg/mL of gentamicin in sterile drinking water for 3 days. Treatment with streptomycin and penicillin G continued for one more day to deplete the natural gastrointestinal flora. Equal numbers of wildtype and mutant fungal cells were mixed, and 1×10^8 cells of the resulting combination were administered orally into BALB/c mice. The feces of mice were collected and homogenized in 1 mL PBS. The suspensions were diluted in PBS and plated onto SC agar to count both strains or SC-His agar supplemented with ampicillin (100 μg/mL) and gentamicin (15 μg/mL) to count mutant strains. The plates were analyzed following incubation at 30°C for 2 d.

Quantification and Statistical Analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad). The statistical test Log-rank (Mantel–Cox) was used for the animal survival tests. Statistically significant differences between the two groups were determined using an unpaired two-tailed Student's *t*-test. Statistical analyses across two or more groups were performed using one-way ANOVA or two-way ANOVA. Significant changes were defined as $p < 0.05$. *p* values are indicated in the plots or represented as $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. All experiments were performed using three biological replicates to ensure the reproducibility.

Results

Sla1 is Required for Endocytosis in the Fungal Kingdom

Previous studies revealed that Sla1 performs a critical role in fungi by integrating components of the endocytic machinery.[19] Homologs of Sla1 are present in multiple yeast species.[20] Thus, we hypothesized that the functions of Sla1 in modulating endocytic processes may be conserved among pathogenic fungi. Utilizing a reciprocal BLAST analysis (https://fungidb.org/), protein homologous to Sla1 in several other fungal species were identified (Figure 1A). Specifically, according to multiple sequence alignments, *S. cerevisiae* Sla1 was found to have homologs in two prevalent causes of human fungal infections-*C. albicans* and *C. neoformans, S. cerevisiae* and *C. albicans* Sla1 have three SH3 domains, but *C. neoformans* have only two (Figure 1B). Strains of *S. cerevisiae, C. neoformans* and *C. albicans* in which the Sla1 homolog was genetically deleted were prepared, and FM-64 staining was used to characterize endocytosis in these strains and in an *SLA1*-rescue strain of *C. albicans*. In this assay, FM4-64 initially binds to the plasma membrane before being absorbed via CME and then is transported to the vacuole by endocytic intermediaries.[21] Mutations that cause defects in endocytosis lead to a temporal delay in the movement of the FM4-64 dye to the vesicle. Here, in contrast to wildtype and *SLA1* rescue strains, *sla1*Δ/Δ cells of *C. albicans* exhibited a weak intracellular FM4-64 signal

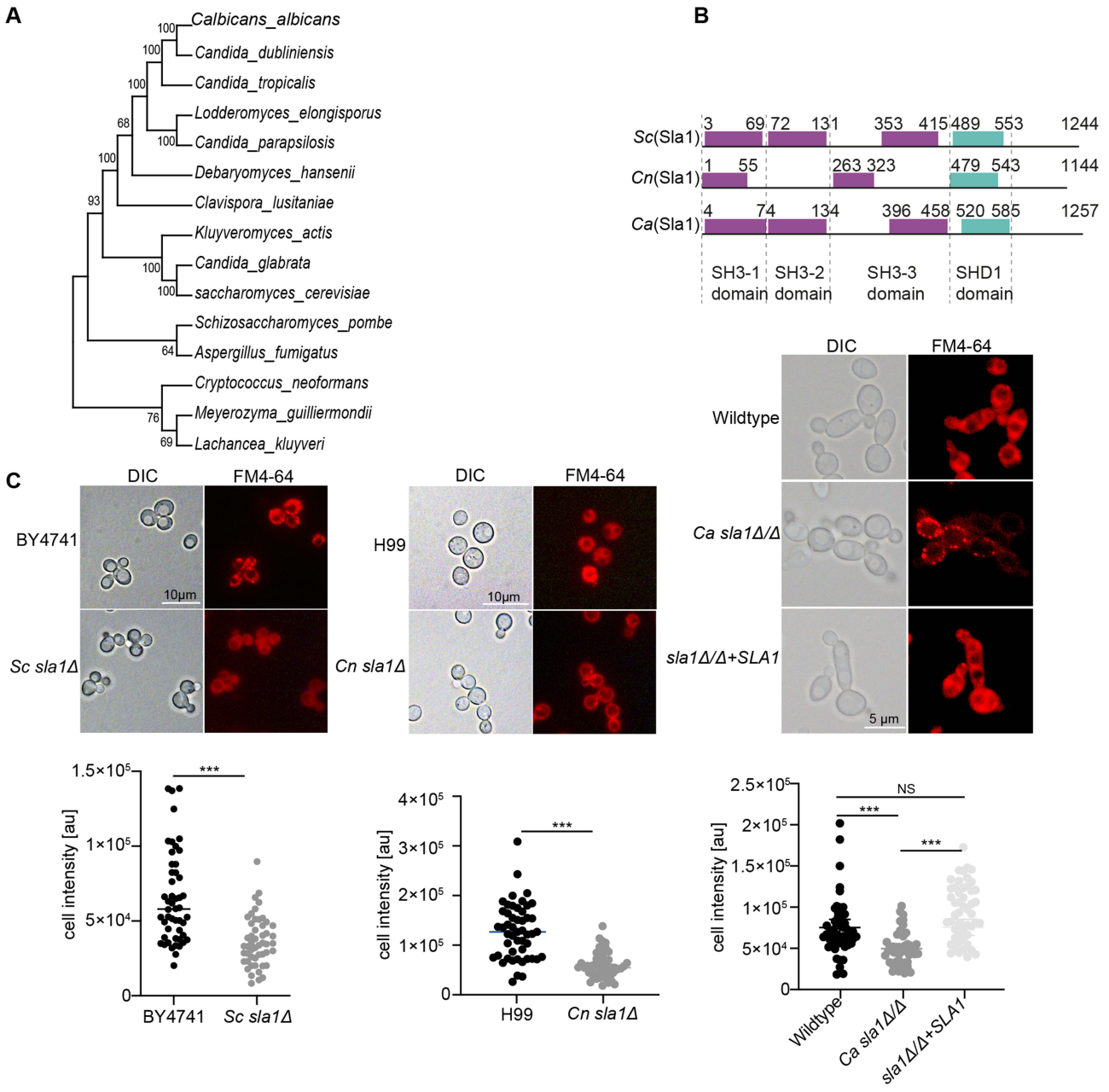

Figure 1 Sla1 is required for endocytosis in fungi. (**A**) Phylogenetic analysis of Sla1 in fungi. A phylogenetic analysis was performed using MEGA 11 (https://megasoftware.net). (**B**) Scheme of Sla1 domains. The domains were predicted using motifscan software (https://myhits.sib.Swiss/cgi-bin/motif_scan/). (**C**) Endocytosis staining analysis. Wildtype and mutants cells were stained with FM4-64, a marker of endocytosis, and imaged using DIC and fluorescence microscopy (left). Fluorescence intensity were analyzed using ImageJ toolset (n = 50). The data are presented as mean ± SD; ***p < 0.001; NS: no significance; Student's *t*-test.

(Figure 1C). These data indicate that Sla1 is conserved in multiple pathogenic fungi and *sla1*Δ/Δ affects intracellular endocytosis.

Deletion of SLA1 Leads to Defects of Cell Wall Synthesis in C. Albicans

To determine the role of *sla1*Δ/Δ in yeast cell physiology, the proliferation of *sla1*Δ/Δ strains in response to osmotic stress was then examined. Defects in cell wall integrity in the mutant strains exhibited increased susceptibility to cell wall and osmotic stressors (Figure 2A). Interestingly, we further found that the *sla1*Δ/Δ mutant, but not the wildtype, aggregated and formed flocs during cell growth in microplates (Figure 2B). Moreover, because cell surface hydrophobicity (CSH) contributes to *C. albicans* aggregation and adhesion, the CSH levels were also compared between the *sla1*Δ/Δ mutant and the wildtype.

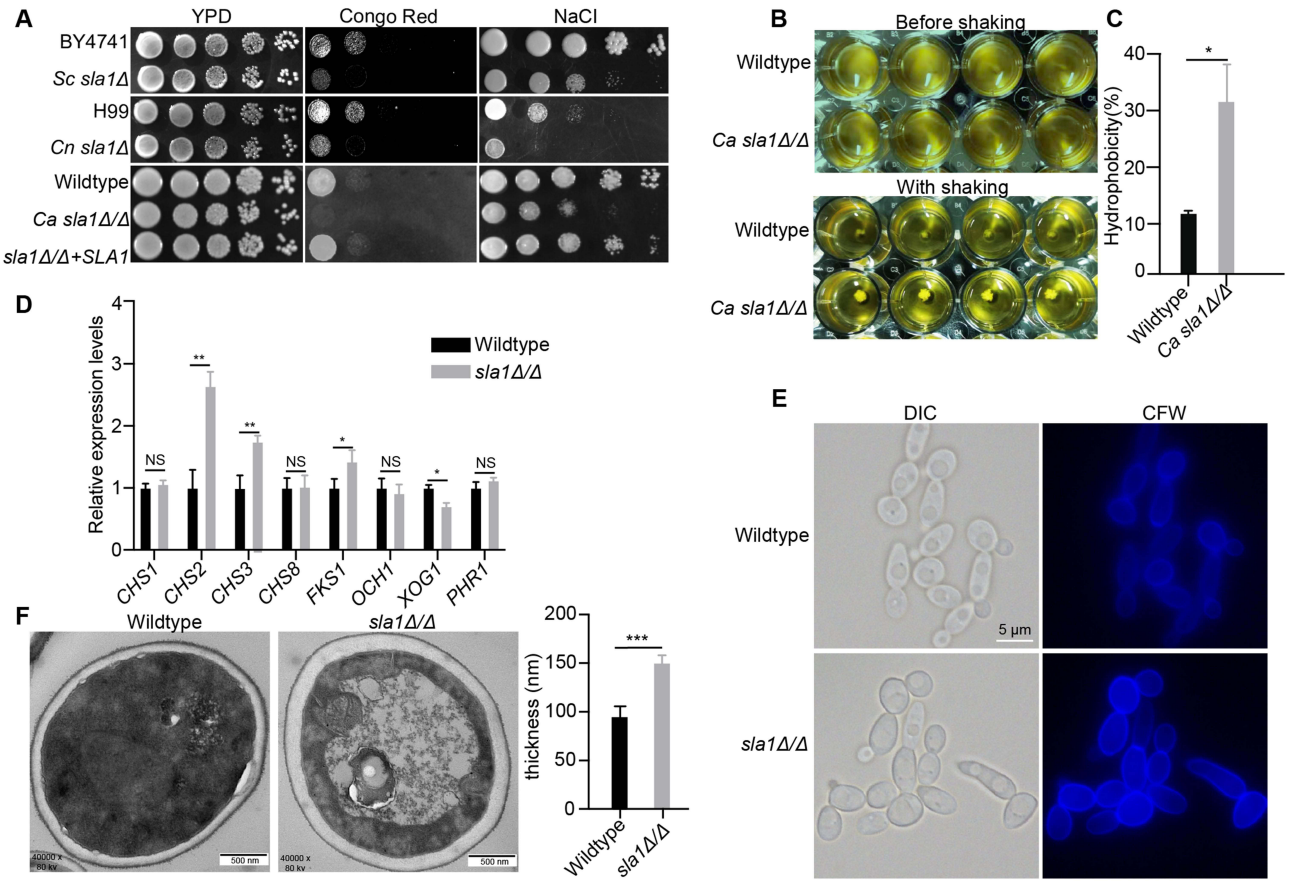

Figure 2 Depletion of sla1 leads to defects in cell wall. (**A**) Stress response of wildtype and mutants. The indicated strains were spotted on YPD agar supplemented with Congo red and NaCl. Then incubated at 30 °C for two days prior to imaging. (**B**) Cell aggregation. *C. albicans* cells were cultured overnight and transferred to a 12-well microplates and photographed immediately (top panel). Then, cells were subcultured (100 rpm) at 30°C for 30 min and photographed (bottom panel). (**C**) CSH assay. Cells were cultured overnight, and CSH was determined. The results are displayed as the mean 3 standard deviation from three independent experiments. *, P <0.05; (**D**) Expression level of cell wall biosynthesis and remodeling genes in *C. albicans SLA1* mutant strain. Real-time qPCR was performed. *, P <0.05; **, P <0.01; NS: no significance. (**E**) CFW staining. *C. albicans* cells were grown at 30 °C for 12 h and subsequently stained with CFW, Bar = 5 μm. (**F**) TEM images of *C. albicans sla1*Δ/Δ mutants and wildtype. The cell wall ultrastructure of wildtype and *sla1*Δ/Δ cells as observed by transmission electron microscopy (left), Bar = 500 nm. The thickness of the cell wall was measured (right), The cell walls of at least 5 cells were measured in each experiment. ***p < 0.001; Student's *t*-test.

Indeed, the CSH of the *sla1*Δ/Δ mutant was increased compared to the wildtype (Figure 2C). CSH is closely associated with cell wall compositions, we suspected that Sla1 may act as a transcription regulator to control the expression of cell wall-related genes. *CHS1, CHS2, CHS3* and *CHS8* are involved in chitin synthesis. *FKS1, OCH1, XOG1* and *PHR1* are involved in glucan and glycan synthesis. We found that *sla1*Δ/Δ mutant mainly affects the synthesis of chitin, as its deletion upregulates the expression of chitin synthase *CHS2* (Figure 2D). Chitin content influences the cell wall rigidity. To determine if the *sla1*Δ/Δ mutants strengthen the cell wall through increased chitin deposition, we analyzed the chitin deposition by CFW staining and observed it with fluorescence microscopy, the *sla1*Δ/Δ showed higher fluorescence than control, indicating a higher chitin content in the cell wall (Figure 2E). In addition, Chitin content also influences the cell wall structure, to confirm our observation, we analyzed the ultrastructure of cell wall by transmission electron microscopy (TEM). Both the wildtype and *sla1*Δ/Δ mutant strains exhibited characteristic bilayered cell walls with a translucent carbohydrate region sandwiched between two electron-dense mannoprotein layers (Figure 2F). We measured the cell wall thickness and our results revealed that the *sla1*Δ/Δ strain showed a thicker cell wall than that of the wildtype strain (Figure 2F), which may be due to the accumulation of chitin in *sla1*Δ/Δ cells. These observations suggest that deletion of *SLA1* impacts *C. albicans* cell wall properties.

Sla1 is a Virulence Gene Involved with Hypha Formation and Fungal Virulence

Because the cell wall plays a critical role in the interaction of *C. albicans* with the host immune system. In addition, the mycelium of *C. albicans* is pathogenic,[22] hyphal production was found to be delayed in *sla1*Δ/Δ *C. albicans* strains, and

this production was recovered in *sla1*Δ/Δ strains upon complementation with *SLA1* (Figure 3A) and the regulation of mycelium development by Sla1 thus may have an impact on virulence. To investigate the influence of Sla1 on virulence in vivo, the C57BL/6 mouse were challenged with the wildtype and *sla1*Δ/Δ strains, and their survival rates over time were recorded. The survival curve of C57BL/6 mice infected with *sla1*Δ/Δ cells was substantially delayed relative to that of animals infected with the wildtype strain, indicating that the *sla1*Δ/Δ mutant is severely attenuated in its pathogenicity (Figure 3B). CFU analyses of the organs of infected mice were performed to quantify the effects of the loss of Sla1 on fungal growth in vivo. Significant decreases in fungal growth in the kidney, brain, spleen and liver were identified in C57BL/6 mice infected with *sla1*Δ/Δ fungal cells (Figure 3C). The effect of the mutation strain on growth was further investigated through a competitive commensal colonization experiment in which mice were initially infected with equal numbers of wildtype and *sla1*Δ/Δ mutant strains. When the feces of these mice were collected and analyzed for fungal colonization at 24, 48 and 72 h post-infection, colonization by *sla1*Δ/Δ mutant strains were found to be significantly reduced relative to colonization by the wildtype strain (Figure 3D). Taken together, these data demonstrate that Sla1 is an indispensable mediator of bloodstream infection and commensal colonization.

Deletion of SLA1 Leads to Antifungal Drug Resistance

Modifications in the cell wall can impact drug tolerance, numerous studies have demonstrated that CSH is linked to cellular drug resistance.[23] We next investigated the susceptibility to drug treatments by culturing cells on YPD media containing fluconazole or ketoconazole. The *sla1*Δ/Δ strain showed resistance to the two azole drugs (Figure 4A). The

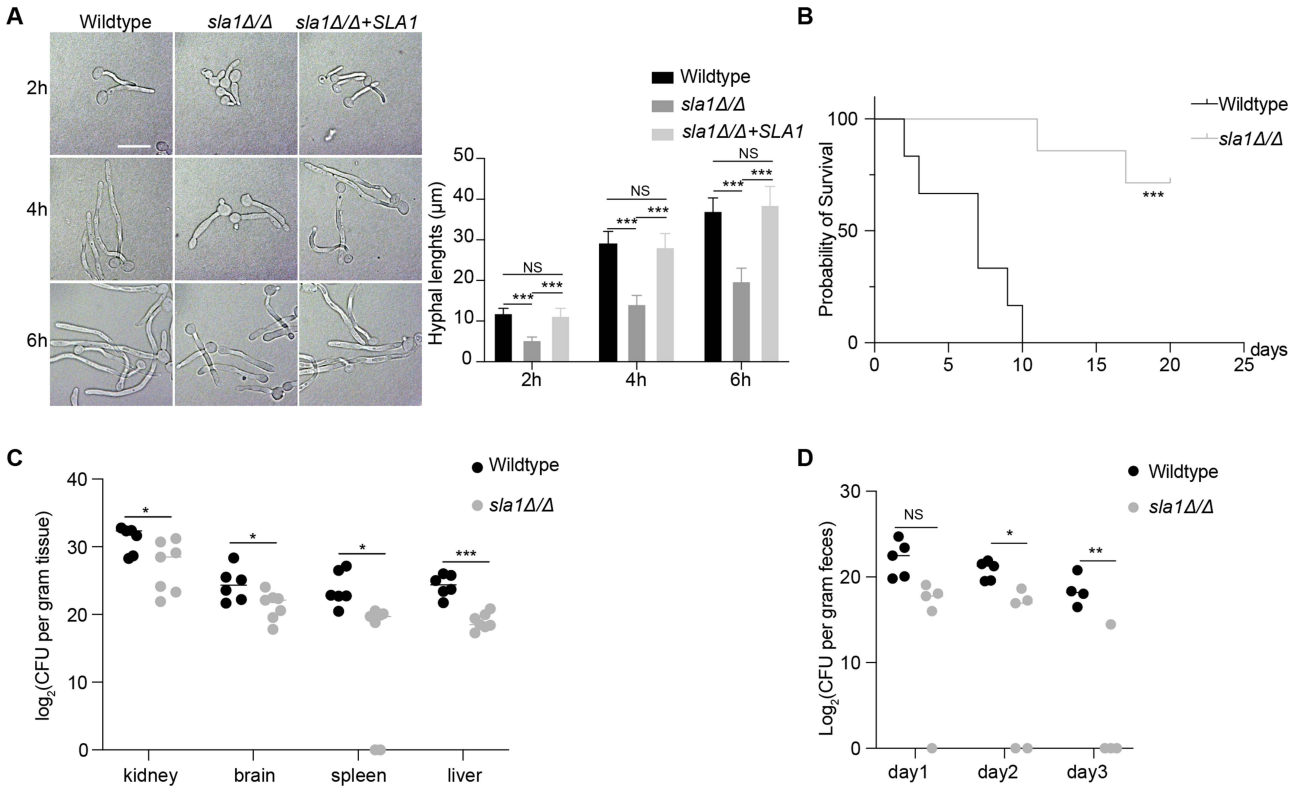

Figure 3 Depletion of *sla1* decreases fungal virulence. (**A**) Hyphal structures of *SLA1* mutant strain. Wildtype, *sla1*Δ/Δ, and *sla1*Δ/Δ+*SLA1* cells were incubated in DMEM supplemented with 10% fetal bovine serum at 37 °C for 2, 4, or 6 hours, and the formation of hyphae was observed. scale bars = 10 μm, and representative images were shown (left). Hyphal length quantification(right), Hyphal structures described in (**A**) were measured and plotted using at least 20 hyphal cells. The data are presented as the mean ± SD. ***p < 0.001; NS: no significance; Student's *t*-test. (**B**) Animal survival assays. C57BL/6 mice ($n = 10$) were infected with wildtype or mutant *C. albicans*, and survival rates were plotted as Kaplan–Meier survival curves. (**C**) CFU analysis of mutation strains. Fungal burdens were evaluated at day 7 post infections. *p < 0.05, ***p < 0.001; Student's *t*-test ($n = 7$). (**D**). Analysis of commensal colonization. Prior to infections, mice were administered sterile water supplemented with 2 mg/mL streptomycin and 1500 U/mL of penicillin G and 0.2 mg/mL gentamicin to deplete the native GI bacteria. The cells of the two strains were combined in equal proportions, and 1×10^8 cells of the mixture were administered by gavage. The feces were collected, homogenized in PBS, and plated on SC agar or SC-His agar supplemented with ampicillin (100 μg/mL) and gentamicin (15 μg/mL). *p < 0.05; **p < 0.01; NS: no significance; Student's *t*-test ($n = 5$).

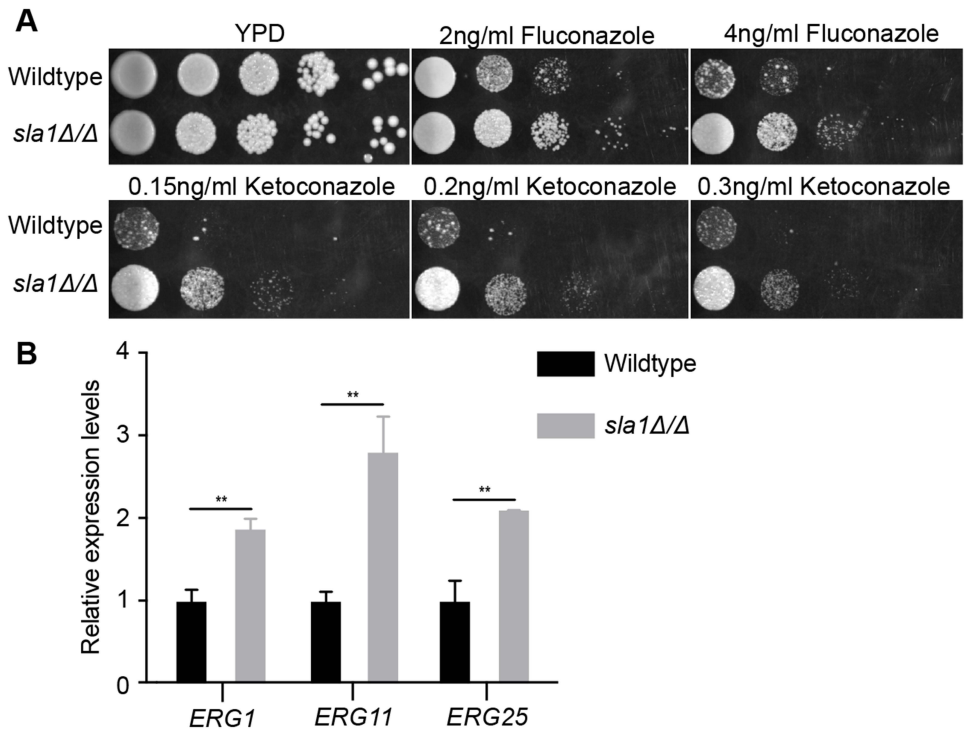

Figure 4 Deletion of *SLA1* leads to anti-fungal drug resistance. (**A**) Anti-fungal drug sensitivity test. Wildtype and *sla1*Δ/Δ cells were spotted on YPD agar supplemented with fluconazole or ketoconazole. Plates were incubated at 30 °C for 2 days prior to imaging. (**B**) RT-qPCR analyses of *ERG* gene expression level in the *sal1*Δ/Δ strain. Expressions of *ERG1, ERG11*, and *ERG25* were quantified using real-time qPCR. The data are presented as the mean ± SD. **p < 0.01; Student's *t*-test(n=3).

products of the *ERG* genes, which are involved in the production of ergosterol, have been shown to mediate the resistance to azole compounds in *C. albicans*.[24] Conversely, the lanosterol demethylase Erg11 is recognized as the primary target of azole drugs.[24] Accordingly, the increased expression of *ERG11* and other *ERG* genes has been shown to compensate for azole-mediated inhibition of Erg11 and to confer azole resistance in *C. albicans*.[25] Interestingly, we revealed that the levels of expression of *ERG1, ERG11* and *ERG25* genes were highly up-regulated in the *sla1*Δ/Δ mutant (Figure 4B). These data indicate that the resistance to fluconazole and ketoconazole conferred by the loss of Sla1 may arise from the up-regulation of *ERG* family genes.

Sla1 Controls Drug Sensitivity and Pathogenicity by Activating the Transcription Factor Efg1

Sla1 in *S. cerevisiae* is known to shuttle between the nucleus and actin cortical patches, its location is unknown in *C. albicans*. We found that the Sla1 protein has an NLS sequence (Figure S1A), and fluorescence localization experiments showed that Sla1 is localized in the cytoplasm and nucleus (Figure S1B). We suspected that Sla1 may perform its wide range of functions by regulating the activity of particular transcription factors. We screened a *C. albicans* transcription factor deletion library, obtained from the Fungal Genetics Stock Center,[26] and identified six candidate transcription factors whose deletion led to drug resistance that was similar to that of the *sla1*Δ/Δ mutant (Figure S1C). Subsequently, we investigated the patterns of expression of these six transcription factors in the context of the mutant strains (Figure S1D), and we found that the transcription of *EFG1* was drastically reduced in the *sla1*Δ/Δ strain (Figure 5A). In addition, when we performed immunoprecipitation experiments from *C. albicans* strains overexpressing Sla1 and Efg1, we discovered the existence of a complex containing both Sla1 and Efg1 (Figure 5B). The resistance to antifungal drugs of *efg1*Δ/Δ strain was similar to that seen in *sla1*Δ/Δ (Figure 5C), and levels of expression of *ERG1, ERG11*, and *ERG25* were significantly increased in *efg1*Δ/Δ cells (Figure 5D). More importantly, *efg1*Δ/Δ mutant is severely attenuated in its pathogenicity (Figure 5E), which was consist with that in *sla1*Δ/Δ. These findings proposed

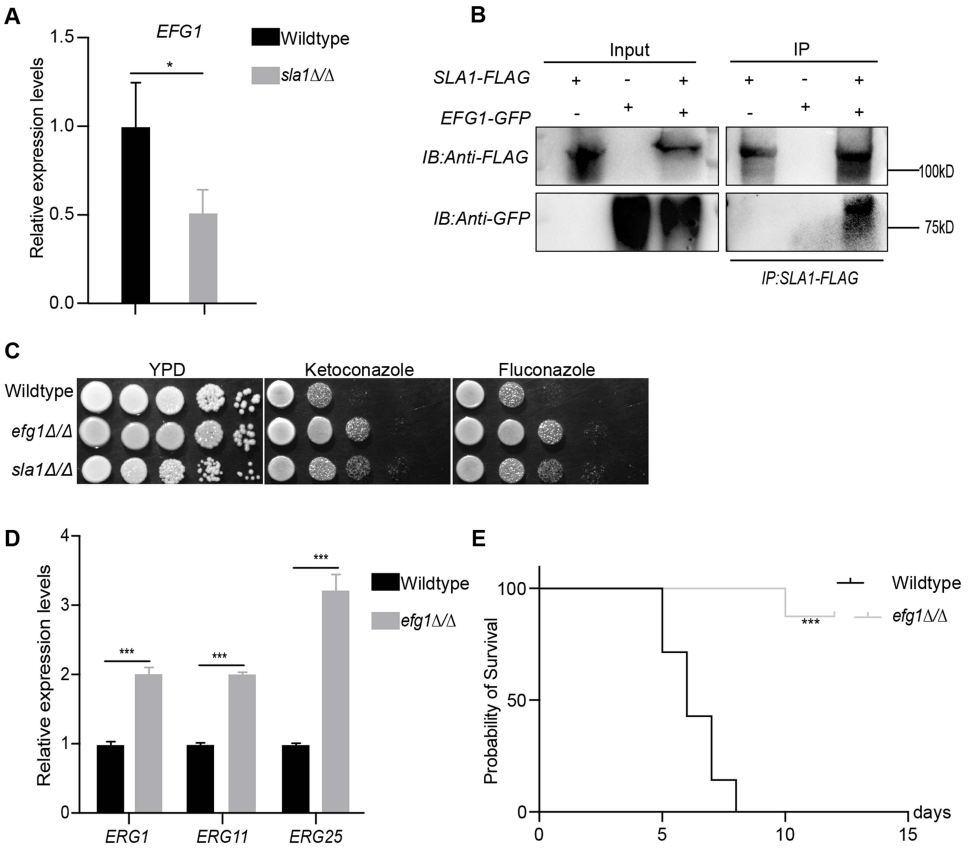

Figure 5 Sla1 regulates *EFG1* expression and interacts with Efg1. (**A**) Expression level of *EFG1* in *C. albicans sla1*Δ/Δ. The data are presented as mean ± SD. *p < 0.05; Student's *t*-test (*n* = 3). (**B**) Co-immunoprecipitation (Co-IP) of Sla1-FLAG and Efg1-GFP in *C. albicans*. Anti-FLAG magnetic beads was used to pull down complexes. (**C**) Anti-fungal sensitivity of *SLA1 and EFG1* mutant strains. Spotting assay of wildtype and mutant strains were performed on YPD agar supplemented with 4 ng/mL fluconazole or 0.3 ng/mL ketoconazole. Plates were incubated at 30 °C for 2 d prior to imaging. (**D**) Expression level of *ERG1*, *ERG11* and *ERG25*. The data are presented as mean ± SD; ***p < 0.001; Student's *t*-test (*n* = 3). (**E**) Animal survival assays. C57BL/6 mice (*n* = 7) were infected with wildtype or *efg1*Δ/Δ mutant strains, and survival rates were plotted as Kaplan–Meier survival curves.

a model in which the regulation of drug sensitivity by Sla1 is achieved through activating the regulations of *ERG* genes by the transcription factor Efg1.

Discussion

Interactions between Sla1 and clathrin are critical for coat production, regulation of endocytic progression and membrane curvature. In *S. cerevisiae*, defects in interactions between Sla1 and clathrin cause delays of multiple stages of endocytosis, as well as morphological abnormalities in the action network and alterations to the membrane lipid profile.[20] For example, an interaction between clathrin and Sla1, in complex with Pan1 and End3, is required to bring the Sla2/Ent1/2 complex to the clathrin coat prior to the execution of endocytosis. Consistent with these findings in *S. cerevisiae*, our study indicates that *C. albicans* or *C. neoformans* strains lacking *SLA1* were unable to endocytose FM4-64 into the cytoplasm (Figure 1C). These findings suggested that abnormalities in endocytosis may be prevalent in *SLA1* mutants of pathogenic fungi.

Cell wall integrity is also known to be dependent on many of these same proteins, highlighting the relationship between adaptor proteins involved in endocytosis and cell wall integrity. For example, the regulation of the actin cytoskeleton by the Arp2/3 complex involves an interaction with the Pan1/End3p complex,[27] and *C. albicans end3*Δ/Δ and *ent2*Δ/Δ mutants have been shown to have reduced cell wall integrity.[7] Consistent with these findings, we found that the *C. albicans sla1*Δ/Δ mutant lacked cell wall integrity, according to Congo red stress response assays (Figure 2A). Remarkably, our investigation revealed that the *sla1*Δ/Δ mutant, unlike the wildtype, exhibited aggregation and floc formation during cell growth in microplates (Figure 2B). Additionally, we find a notable increase in the CSH of the

*sla1*Δ/Δ mutant compared to the wildtype (Figure 2C). Given that CSH is intricately linked to cell wall compositions, it is noteworthy that we observed a slight increase in chitin content for the *sla1*Δ/Δ mutants (Figure 2D and 2E), Chitin content is closely related to cell wall, TEM shows that the cell wall of *sla1*Δ/Δ strain is thicker than that of the wildtype strain (Figure 2F). These findings suggest that defects in cell wall integrity may be a common feature in endocytosis mutants.

The cell wall is crucial during both *C. albicans* commensalism and infections.[17] In addition, the deletion of *C. albicans* endocytosis genes *ENT2* or *END3* causing cell wall integrity, hyphal formation and reduced virulence-related process including secretion of extracellular aspartyl proteases, cell–cell adhesions and host cell killing in vitro.[7] Previous studies demonstrated that Sla1 regulated hyphal growth in *C. albicans*,[28] and was involved in two aspects of fungal virulence: cytoskeletal polarity and mycelia development. We found that the *C. albicans sla1*Δ/Δ mutant exhibited defects in hyphal development (Figure 3A). Our results also confirmed the effect of Sla1 on pathogenicity in vivo (Figure 3B). In our analysis, the virulence of the *sla1*Δ/Δ mutant was severely diminished in a murine infection model, particularly in the colonization of host organs during blood infections and competitive survival in host feces during gastrointestinal infections (Figure 3C and 3D). Of note, components of the *C. albicans* cell wall serves as major pathogen-associated molecular patterns (PAMPs), which are recognized by various pattern recognition receptors (PRRs) to trigger host immune responses,[29,30] The *sla1Δ/Δ* mutant may affect PAMPs, thereby impacting the host's immune response, which requires further research. In aggregate, our findings clearly show that Sla1 is associated with cell wall integrity, hypha, and virulence in *C. albicans*.

Over the past decade, endocytosis has emerged as a critical factor in therapeutic drug delivery, and direct inhibition of endocytosis has been proposed as a new form of cancer therapy.[31] In particular, the endocytic pathway has been exploited in strategies to improve current cancer treatments. For example, the antibody-drug conjugate T-DM1 uses the monoclonal antibody trastuzumab to target the anti-cancer agent emtansine to tumors expressing human epidermal growth factor receptor-2; antibody-mediated attachment of the complex to this receptor triggers endocytosis, leading to the intracellular release of the drug.[32] In this strategy, the increased endocytosis efficiency is considered to be a critical factor for the initiation of T-DM1 therapy.[32] In addition, alterations to endocytosis have been implicated in the development of artemisinin resistance.[33] Although endocytosis and drug resistance are well studied in the field of anti-tumor drugs, there have been few studies on the relationship between drug resistance and endocytosis in fungi. It is well known that fungal resistance to azole drugs mainly affects the synthesis of ergosterol.[32] Our study found that *sla1*Δ/Δ mutants was sensitive to cell membrane pressure (Figure 2A) and maintained a high expression level of *ERG* genes. But, to our best knowledge, there is no reports demonstrated the regulation role of Sla1 to *ERG* family genes.

Sla1 in *S. cerevisiae* is known to shuttle between the nucleus and actin cortical patches. We found that the Sla1 protein of *C. albicans* has an NLS sequence (Figure S1A), We suspected that Sla1 may perform its wide range of functions by regulating the activity of particular transcription factors. We screened a *C. albicans* transcription factor deletion library, obtained from the Fungal Genetics Stock Center,[26] and identified six candidate transcription factors whose deletion led to drug resistance that was similar to that of the *sla1*Δ/Δ mutant (Figure S1C). Subsequently, we found that the transcription of *EFG1* was drastically reduced in the *sla1*Δ/Δ strain (Figure 5A). We also determined Efg1 to be a viable candidate for an Sla1-interacting transcription factor due to its known role as an essential modulator for hyphal development,[34,35] and demonstrated their interactions at the molecular level (Figure 5A and 5B). Accordingly, loss of both Sla1 and Efg1 led to similar phenotypes. For example, it was intriguing that both *sla1*Δ/Δ and *efg1*Δ/Δ mutants exhibited strong resistance to challenge with the antifungal agents fluconazole and ketoconazole (Figure 4A and 5C).

Moreover, in both *sla1*Δ/Δ and *efg1* Δ/Δ mutants, the expression of the *ERG* family genes, which are associated with drug resistance, was dramatically enhanced (Figure 4B and 5D). These results suggest that Sla1 regulates pathways leading to drug resistance and that Efg1 may be involved in the underlying mechanisms. Co-immunoprecipitation assays revealed further that Sla1 may regulate Efg1 through complex formation (Figure 5B), while the mechanisms underlying the effects of these interactions require further analysis. *S. cerevisiae* Sla1 has been predicted by bioinformatics analyses to have three classic nuclear localization sequences and three bipartite nuclear localization sequences.[36] We also found that the Sla1 of *C. albicans* has an NLS sequence (Figure S1A), and fluorescence localization experiments showed that Sla1 is localized in the cytoplasm and nucleus (Figure S1B). One potential model, then, involves the binding of Sla1 to

Efg1 in the nucleus of the cell, leading to the activation of expression of *EFG1*. This action would be expected to limit the ability of Efg1 to up-regulate the expression of *ERG* family genes. So far, Efg1 had not been reported to directly regulate the expression of *ERG* genes; we are the first to study possibility that Efg1 modulates fungal drug resistance by regulating *ERG* gene expression.

In conclusion, Sla1 has critical functions in connecting the early and late phases of endocytosis, including the recruitment of cargo, coupling to clathrin and stimulation of actin assembly. We demonstrated its important roles in cell wall integrity, hyphal growth and the virulence in the prevalent human fungal pathogens *C. albicans*. Additionally, we found that deletion of Sla1 affect endocytosis, plasma membrane composition and also depress *EFG1* expression, leading to up-regulation of *ERG* family gene and thus increases drug resistance. Overall, our research proposed an axis in which Sla1 regulates susceptibility to antifungal drugs by stimulating the activity of transcription factor Efg1. Our data provides theoretical support for advancing disease treatment and developing new antifungal drugs.

Ethics Statement

The animal study protocol was formulated according to the Laboratory animal Guideline for ethical review of animal welfare (GB/T 35892-2018) and approved by the Animal Welfare & Ethics Committee of Peking Union Medical College Hospital (XHDW-2022-033). All efforts were made to minimize animal suffering.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This research was funded by the Liaoning Province Science and Technology Plan Joint Project (2023JH2/101700140 to C.S), the National Natural Science Foundation of China (31870140 to C.D.), the National High Level Hospital Clinical Research Funding (2022-PUMCH-A-264 to T.S).

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Richardson JP. Candida albicans: a Major Fungal Pathogen of Humans. *Pathogens*. 2022;11(4):459.
2. Spampinato C, LEONARDI D. Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. *Biomed Res Int*. 2013;2013:204237. doi:10.1155/2013/204237
3. Lopes JP, Lionakis MS. Pathogenesis and virulence of Candida albicans. *Virulence*. 2022;13(1):89–121. doi:10.1080/21505594.2021.2019950
4. Henriques M, Williams D. Pathogenesis and Virulence of Candida albicans and Candida glabrata. *Pathogens*. 2020;9(9):752. doi:10.3390/pathogens9090752
5. Tolsma TO, Febvre HP, Olson DM, et al. Cargo-mediated recruitment of the endocytic adaptor protein Sla1 in S. cerevisiae. *J Cell Sci*. 2020;133(19):247684.
6. Jaber QZ, Bibi M, Ksiezopolska E, et al. Elevated Vacuolar Uptake of Fluorescently Labeled Antifungal Drug Caspofungin Predicts Echinocandin Resistance in Pathogenic Yeast. *ACS Cent Sci*. 2020;6(10):1698–1712. doi:10.1021/acscentsci.0c00813
7. Rollenhagen C, Agyeman H, Eszterhas S, et al. Candida albicans END3 Mediates Endocytosis and Has Subsequent Roles in Cell Wall Integrity, Morphological Switching, and Tissue Invasion. *Microbiol Spectr*. 2022;10(2):e0188021. doi:10.1128/spectrum.01880-21
8. Woodard TK, Rioux DJ, Prosser DC. Actin- and microtubule-based motors contribute to clathrin-independent endocytosis in yeast. *Mol Biol Cell*. 2023;34(12):ar117. doi:10.1091/mbc.E23-05-0164
9. Prischich D, Camarero N, Encinar Del DEDO, et al. Light-dependent inhibition of clathrin-mediated endocytosis in yeast unveils conserved functions of the AP2 complex. *iScience*. 2023;26(10):107899. doi:10.1016/j.isci.2023.107899
10. Traub LM, Bonifacino JS. Cargo recognition in clathrin-mediated endocytosis. *Cold Spring Harb Perspect Biol*. 2013;5(11):a016790. doi:10.1101/cshperspect.a016790
11. Sun Y, Schoneberg J, Chen X, et al. Direct comparison of clathrin-mediated endocytosis in budding and fission yeast reveals conserved and evolvable features. *Elife*. 2019;8:e50749.

12. Sun Y, Leong NT, Wong T, et al. A Pan1/End3/Sla1 complex links Arp2/3-mediated actin assembly to sites of clathrin-mediated endocytosis. *Mol Biol Cell*. 2015;26(21):3841–3856. doi:10.1091/mbc.E15-04-0252
13. SKRUZNY M, BRACH T, Ciuffa R, et al. Molecular basis for coupling the plasma membrane to the actin cytoskeleton during clathrin-mediated endocytosis. *Proc Natl Acad Sci U S A*. 2012;109(38):E2533–42. doi:10.1073/pnas.1207011109
14. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen Candida albicans. *Eukaryot Cell*. 2005;4(2):298–309. doi:10.1128/EC.4.2.298-309.2005
15. TOFFALETTI DL, RUDE TH, JOHNSTON SA, et al. Gene transfer in Cryptococcus neoformans by use of biolistic delivery of DNA. *J Bacteriol*. 1993;175(5):1405–1411. doi:10.1128/jb.175.5.1405-1411.1993
16. Brachmann CB, DAVIES A, COST GJ, et al. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. 1998;14(2):115–132. doi:10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2
17. WANG WH, LAI TX, Wu YC, et al. Associations of Rap1 with Cell Wall Integrity, Biofilm Formation, and Virulence in Candida albicans. *Microbiol Spectr*. 2022;10(6):e0328522. doi:10.1128/spectrum.03285-22
18. BACKHAUS K, RIPPERT D, Heilmann CJ, et al. Mutations in SNF1 complex genes affect yeast cell wall strength. *Eur J Cell Biol*. 2013;92(12):383–395. doi:10.1016/j.ejcb.2014.01.001
19. TANG HY, Xu J, CAI M. Pan1p, End3p, and S1a1p, three yeast proteins required for normal cortical actin cytoskeleton organization, associate with each other and play essential roles in cell wall morphogenesis. *Mol Cell Biol*. 2000;20(1):12–25. doi:10.1128/MCB.20.1.12-25.2000
20. Tolsma TO, CUEVAS LM, Di Pietro SM. The Sla1 adaptor-clathrin interaction regulates coat formation and progression of endocytosis. *Traffic*. 2018;19(6):446–462. doi:10.1111/tra.12563
21. LETTNER T, ZEIDLER U, GIMONA M, et al. Candida albicans AGE3, the ortholog of the S. cerevisiae ARF-GAP-encoding gene GCS1, is required for hyphal growth and drug resistance. *PLoS One*. 2010;5(8):e11993. doi:10.1371/journal.pone.0011993
22. ARKOWITZ RA, BASSILANA M. Recent advances in understanding Candida albicans hyphal growth. *F1000Res*. 2019;8:1.
23. DANCHIK C, CASADEVALL A. Role of Cell Surface Hydrophobicity in the Pathogenesis of Medically-Significant Fungi. *Front Cell Infect Microbiol*. 2020;10:594973. doi:10.3389/fcimb.2020.594973
24. HENRY KW, NICKELS JT, EDLIND TD. Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors J]. *Antimicrob Agents Chemother*. 2000;44(10):2693–2700. doi:10.1128/AAC.44.10.2693-2700.2000
25. Song JL, HARRY JB, Eastman RT, et al. The Candida albicans lanosterol 14-alpha-demethylase (ERG11) gene promoter is maximally induced after prolonged growth with antifungal drugs. *Antimicrob Agents Chemother*. 2004;48(4):1136–1144. doi:10.1128/AAC.48.4.1136-1144.2004
26. Homann OR, Dea J, Noble SM, et al. A phenotypic profile of the Candida albicans regulatory network. *PLoS Genet*. 2009;5(12):e1000783. doi:10.1371/journal.pgen.1000783
27. LEE A, Robbins N, XIE JL, et al. Functional Genomic Analysis of Candida albicans Adherence Reveals a Key Role for the Arp2/3 Complex in Cell Wall Remodelling and Biofilm Formation. *PLoS Genet*. 2016;12(11):e1006452. doi:10.1371/journal.pgen.1006452
28. Reijnst P, Jorde S, Wendland J. Candida albicans SH3-domain proteins involved in hyphal growth, cytokinesis, and vacuolar morphology. *Curr Genet*. 2010;56(4):309–319. doi:10.1007/s00294-010-0301-7
29. GARCIA-RUBIO R, DE OLIVEIRA HC, RIVERA J, et al. The Fungal Cell Wall: candida, Cryptococcus, and Aspergillus Species. *Front Microbiol*. 2019;10:2993. doi:10.3389/fmicb.2019.02993
30. Bojang E, GHUMAN H, Kumwenda P, et al. Immune Sensing of Candida albicans. *J Fungi*. 2021;7(2):119.
31. Banushi B, Joseph SR, Lum B, et al. EndocytosiSs in cancer and cancer therapy. *Nat Rev Cancer*. 2023;23(7):450–473.
32. Liang K, Mei S, Gao X, et al. Dynamics of Endocytosis and Degradation of Antibody-Drug Conjugate T-DM1 in HER2 Positive Cancer Cells. *Drug Des Devel Ther*. 2021;15:5135–5150. doi:10.2147/DDDT.S344052
33. Behrens HM, Schmidt S, Spielmann T. The newly discovered role of endocytosis in artemisinin resistance. *Med Res Rev*. 2021;41(6):2998–3022. doi:10.1002/med.21848
34. Park YN, Conway K, Pujol C, et al. EFG1 Mutations, Phenotypic Switching, and Colonization by Clinical a/alpha Strains of Candida albicans. *mSphere*. 2020;5(1):10–128.
35. Glazier VE. EFG1, Everyone's Favorite Gene in Candida albicans: a Comprehensive Literature Review. *Front Cell Infect Microbiol*. 2022;12:855229. doi:10.3389/fcimb.2022.855229
36. Gardiner FC, Costa R, Ayscough KR. Nucleocytoplasmic trafficking is required for functioning of the adaptor protein Sla1p in endocytosis. *Traffic*. 2007;8(4):347–358. doi:10.1111/j.1600-0854.2007.00534.x