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Profilin Pfy1 is critical for cell wall integrity and virulence in *Candida albicans*

Xun Sun, Yueqing Wang, Xiaomin Yang, Xi Xiang, Lili ZOU, Xiaowen Liu, Gang Luo, and Qi Han

Corresponding Author(s): Qi Han, Beijing University of Chinese Medicine

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Re: Spectrum02593-24 (Profilin Pfy1 is critical for cell wall integrity and virulence in *Candida albicans*)

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Reviewer #1 (Comments for the Author):

The study investigates the role of profilin, an actin-binding protein, in Candida albicans, a major fungal pathogen. The authors present evidence suggesting that profilin plays a role in the morphogenesis, a process that is closely linked to the regulation of the actin cytoskeleton. Additionally, the pfy1 mutant strain was found to be hypersensitive to cell wall stress and exhibited a thicker cell wall than wild-type cells, suggesting that profilin is crucial for maintaining cell wall integrity. The authors further demonstrate that profilin is important for the virulence of C. albicans in a murine model of systemic infection.

Overall, the findings provide insights into the cellular functions of profilin in C. albicans. My main questions and suggestions for the authors are:

1. The method used to determine actin filament distribution appears to lack rigor. In Figure 1D, where actin cables are stated to be absent, some patches are detected in the mother cells of pfy1 mutant strain. The mislocalization of actin patches to the mother cell may hinder the observation of the less distinct actin cables present within the cell. Hence the statement that actin cables are absent (Lines 210) should be rephrased. Also, the authors should state the number of cells analyzed in this study.

2. It is recommended to investigate the agar invasion of the pfy1 mutant to support the role of PFY1 in hyphal formation on solid medium (Spider agar plate). Additionally, Figure 2C is not referenced in the manuscript.

3. Statistical analysis is required to determine whether the differences observed in Figure 7A are statistically significant.

Reviewer #2 (Comments for the Author):

The manuscript "Profilin Pfy1 is critical for cell wall integrity and virulence in Candida albicans" is well written and supported by the facts and the data. However, the authors need some minor corrections in the manuscript as shown below:
Line 130: MMS is abbreviated but Line 230 shows the full form. check this. Same goes for H2O2
Line 343: "that overexpreesion SC" check this
Line 351: "one of the first-line antifungals used to treat systemic candidiasis, in vitro assays" check this. I couldn't understand this.

Check for the reference page as at some place et al. mentioned.

It's good to show a figurative representation of mutant created and add that in a figure.

Did the authors do any RNA studies on the mutant just to see the genes getting upregulated or downregulated? It would be good to include that.

Dear editor and reviewers,

We feel great thanks for your professional reviews work on our manuscript. As you are concerned, there are some flaws that need to be addressed. According to your suggestions, we have made extensive corrections to our previous draft (highlighted in red), the detailed corrections are listed now.

Best!

Qi Han

Reviewer #1 (Comments for the Author):

The study investigates the role of profilin, an actin-binding protein, in Candida albicans, a major fungal pathogen. The authors present evidence suggesting that profilin plays a role in the morphogenesis, a process that is closely linked to the regulation of the actin cytoskeleton. Additionally, the pfy1 mutant strain was found to be hypersensitive to cell wall stress and exhibited a thicker cell wall than wild-type cells, suggesting that profilin is crucial for maintaining cell wall integrity. The authors further demonstrate that profilin is important for the virulence of C. albicans in a murine model of systemic infection.

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Thanks for your suggestions. We apologize for the inappropriate conclusion. The statement is now changed to “actin cables were not readily detected and patches were dispersed throughout the mother cells in pfy1Δ/Δ cells” in revision (line 219-220). The number of cells analyzed in this study is indicated in the legend for Figure 1.

2. It is recommended to investigate the agar invasion of the pfy1 mutant to support the role of PFY1 in hyphal formation on solid medium (Spider agar plate). Additionally, Figure 2C is not referenced in the manuscript.

Thanks for your suggestions. As shown in Figure 2D, the pfy1 mutant exhibited a defect in agar invasion, consistent with the conclusion that PFY1 is required for hyphal growth. In the revision, Figure 2C has been referenced (lines 230-232).

3. Statistical analysis is required to determine whether the differences observed in Figure 7A are statistically significant.

Thanks for your suggestions. We have conducted a statistical analysis of Figure 7A, which demonstrated that the survival rate of mice infected with the pfy1 mutant was significantly higher compared to mice infected with the wild-type strains.

Reviewer #2 (Comments for the Author):

The manuscript "Profilin Pfy1 is critical for cell wall integrity and virulence in Candida albicans" is well written and supported by the facts and the data. However, the authors need some minor corrections in the manuscript as shown below:
Line 130: MMS is abbreviated but Line 230 shows the full form. check this. Same goes for H2O2

Thanks for your suggestions. We have corrected these issues in revision (line 132 236 237).

Line 343: "that overexpreesion SC" check this

Thanks for your suggestions. We have corrected it (line 349-350).

Line 351: "one of the first-line antifungals used to treat systemic candidiasis, in vitro assays" check this. I couldn't understand this.

Thanks for your kindly help. We have removed “in vitro assays” in revision (line 356-357).

Check for the reference page as at some place et al. mentioned.

Thanks for pointing this out. We have corrected this issue in revision.

It's good to show a figurative representation of mutant created and add that in a figure.

Thanks for your suggestions. We have added a schematic diagram of the construction of pfy1 mutant in Figure S1.

Did the authors do any RNA studies on the mutant just to see the genes getting upregulated or downregulated? It would be good to include that.

Thanks for your suggestions. qRT-PCR analysis indicated that the expression of chitin synthases was elevated in *pfy1Δ/Δ*, which is consistent with the results obtained from CFW staining. And this data was shown in Figure S4.

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Sincerely,
Chengshu Wang
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

The authors have addressed my primary concerns with the manuscript.

Reviewer #2 (Comments for the Author):

Dear Authors,
Thank you for addressing and working on the comments. I am glad about your final revised version, however there are some minor corrections to be done that I have mentioned in the main text and supplementary files. Please address those.

Profilin Pfy1 is critical for cell wall integrity and virulence in *Candida albicans*

Xun Sun[#,2,3,4], Yueqing Wang[#,3,4], Xiaomin Yang[1], Xi Xiang[3,4], Lili Zou[3,4], Xiaowen Liu[3,4], Gang Luo[5], Qi Han[*,1]

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Abstract Profilin is a small actin-binding protein that plays an important role in actin polymerization. However, its functions in *Candida albicans*, the most prevalent fungal pathogen, remain unclear. Here, we report that profilin plays a crucial role in *C. albicans* morphogenesis and virulence. Deletion of profilin results in abnormal morphogenesis and impaired hyphal development. Furthermore, *pfy1Δ/Δ* is hypersensitive to cell wall stress and displays thicker cell wall than wild type cells, indicative of a critical function of Pfy1 in cell wall integrity. In addition, our findings demonstrate that profilin is required for the virulence of *C. albicans* in a murine model of systemic infection. In conclusion, our work provides a promising target for developing antifungal drugs.

Key Words Profilin, *Candida albicans*, Cell wall, Virulence

Importance

Our research revealed Pfy1 is not only involved in hyphal development but also essential for pseudohyphal formation in response to DNA damage agents MMS and

H_2O_2. The disruption of *PFY1* resulted in striking morphological defects in both yeast and hyphal forms. Further investigation suggested that profilin plays a role in polarized growth of *C. albicans* via binding with Act1, and contributes to cell wall remodeling. Both hyphal growth and cell wall integrity are the important virulence factors of *C. albicans*. Thus, *pfy1Δ/Δ* strains significantly reduced mortality rates in mice. These findings suggested that profilin could serve as a target for developing new antifungal drugs possibly for use in combination therapies with caspofungin for treating invasive candidiasis.

Introduction

Fungal diseases are a global public health threat, causing more than one billion human infections, and nearly two million deaths annually [1]. In October 2022, the World Health Organization (WHO) published the first-ever fungal priority pathogen list, identifying 19 fungi with the greatest public health impact and emerging antifungal resistance risk. These pathogens were categorized into three priority groups (Critical, High, and Medium), and *Candida* species were designated as the highest priority, "Critical" [2]. As listed in the Critical Priority Group, *C. albicans* is the most common opportunistic pathogen in humans, which colonizes mucosal surfaces and gastrointestinal tract of healthy individuals; however, in immunocompromised patients, *C. albicans* can invade solid organs such as kidneys, liver, and spleen, causing life-threatening infection with high mortality despite antifungal treatment [3-5]. The limited effectiveness of current antifungal drugs and the rising prevalence of antifungal resistance indicate a need to develop new therapies for invasive candidiasis [6].

Profilin is a ubiquitously expressed protein and serves as a key regulator of actin polymerization, playing a critical role in cellular function [7]. In budding yeast, the *PFY1* gene encodes profilin, which has been identified as a multifunctional protein involved not only in regulating actin dynamics but also in maintaining Ca^{2+} homeostasis, controlling the relative abundance of actin and tubulin, intracellular transport [8-12]. *Candida glabrata* Pfy1 is an ortholog of human profilin-4 [13]. Although profilin is structurally conserved among eukaryotes, there is only limited sequence identity between yeast and mammals [9], suggesting that profilin could be a potential antifungal target. Indeed, Keigo Ueno *et al* have designed novel peptides binding to the active interface of profilin against *Candida glabrata* [13], where *PFY1* is an essential gene. Unlike *Saccharomyces cerevisiae* and *Candida glabrata*, *C. albicans* does not require profilin for its survival [14]; however, PEP-IA18, an antimicrobial peptide derived from profilin of *Spodoptera frugiperda*, still displayed potent anti-fungal effects against *C. albicans* [15]. Unfortunately, our understanding of the function and related mechanisms of profilin in *C. albicans* remains limited.

In this study, we deleted *PFY1* in *C. albicans* and conducted comprehensive phenotypic characterizations. Our findings demonstrate that Pfy1 plays a pivotal role in hyphal development, cell wall integrity, and virulence of *C. albicans*. Based on these results, we propose that targeting Pfy1 could be a promising therapeutic strategy for the treatment of invasive candidiasis.

Materials and methods

Strains and growth conditions

The *C. albicans* strains used in this study are listed in **Table 1**. *C. albicans* was routinely grown at 30ºC with shaking at 200 rpm 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 YPD agar plates (YPD-Nou plates). To obtain the 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 *SAP4* promoter controlling *Ca*FLP expression, and then streak-inoculated onto YPD plates containing 25 μg/mL nourseothricin and incubated at 30ºC at least 2 days.

Hyphal growth was induced by supplementing YPD medium with 10% fetal calf serum, and incubating at 37 ºC with shaking at 200 rpm, or steaking yeast cells onto Spider agar plates (1% w/v beef extract, 1% w/v mannitol, 0.2% w/v K_2HPO_4, 2% w/v agar, pH 7.2) to incubate at 30ºC for 7 days. Invasive growth was assayed using YPD plates grown at 30°C for two days. After this period the plates were photographed before and after thoroughly washing the cells from the surface. Any cells remaining embedded in the agar were interpreted as evidence of invasive growth. Pseudohyphal growth was induced by supplementing YPD medium with 0.02% MMS or 20 mM H_2O_2 (Sigma-Aldrich), and incubating at 30ºC with shaking at 200 rpm.

Strain construction

The deletion of *PFY1* was done in *C. albicans* SC5314 using the *SAT*-flipper method as described previously [16]. Briefly, the *SAT1*-flipper cassette flanked by 60 bp of upstream and downstream sequences of the *PFY1* 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 rounds of the transformation were required to obtained homozygous deletion mutants. Genomic DNA and total RNA were isolated from selected transformants to verify the mutations by PCR and RT-PCR analysis.

We cloned one copy of wild type *PFY1* into the *Xho*I-*Hind*III sites of *pAG6* [17], then linearized the plasmid with *Stu*I, and transformed it into the *pfy1*Δ/Δ mutant to obtain *PFY1* complemented strain.

To construct the *SAT1*-marked version of GFP or mCherry-tagging vectors, GFP or mCherry gene sequence followed by the *CaURA3* terminator was inserted into the *Apa*I–*Xho*I sites of *pSFS1*. To tag protein with GFP or mCherry at the C-terminus, the GFP or mCherry-*SAT1*-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 were 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 were shown in **Table 2**.

Growth curves

Late-log phase *C. albicans* yeast cells were diluted to OD_{600} = 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 hours, and OD_{600} was measured using a microplate reader (THERMO, Multiskan FC). The experiment was performed in triplicate.

Susceptibility tests

C. albicans cells grown to the late-log phase in YPD medium were harvested and washed twice with sterile water. The cell suspensions were ten-fold serially diluted to generate suspensions containing 10^3 to 10^6 cells/mL, and 5 μL of each dilution was

spotted onto YPD plates containing the indicated concentrations of MMS or H_2O_2 (Sigma-Aldrich). Growth was assessed by incubating the plates at 30°C for the indicated time. All experiments were performed at least three times.

Fluorescence Microscopy

Log-phase *C. albicans* yeast cells were stained with 20 μg/mL Calcofluor White (Sigma-Aldrich) to visualize cell wall chitin. Cells were examined by differential interference contrast (DIC) and fluorescence microscopy (Olympus IX73). To visualize the chitin content, the exposure time for the images of Calcofluor White fluorescence was fixed.

Log-phase *C. albicans* yeast cells were stained with 1 μL, 200 T/mL Fluorescein Phalloidin (LABLEAD, G0059) to visualize actin. Cells were examined by differential interference contrast (DIC) and fluorescence microscopy (Olympus IX73). To visualize actin organization, the exposure time for the images of Phalloidin fluorescence was fixed.

Co-immunoprecipitation (Co-IP) and Western blotting (WB)

Co-IP and WB was performed as previously described by Han *et al.* [18]. Briefly, mid-log phase C. *albicans* yeast cells were suspended in lysis buffer as previously described by Han *et al.* [18], and then mechanically lysed by five rounds of bead-beating at 5500 rpm for 60 s at 4°C by using Tomy Microsmash (Tomy-Seiko, MS-100R). Cell lysates were centrifuged and the supernatant was transferred to a new tube to mixed with 20-30 μL antibody-conjugated beads (Santa Cruz) that had been pretreated. After incubated at 4°C for 8 h, the beads were washed for three times and then boiled for 5 min with loading buffer added.

Proteins were resolved by SDS-PAGE and then transferred to a polyvinylidenedifluoride membrane (Millipore). The membrane was immersed in 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h, and then incubated in TBST with containing the primary antibody at 4°C for 8 h. After that, the membrane was washed for three times with TBST and then incubated with the secondary antibody conjugated with hydrogen peroxidase (Beyotime). Protein bands were detected by using the chemiluminescence system (Bio-Rad, ChemiDoc XRS+).

mCherry and GFP antibodies were purchased from Beyotime (China).

Quantitative RT-PCR

Total RNA was purified using the RNeasy Minikit and DNase-treated at room temperature for 15 min using the RNase-free DNase set (Qiagen). cDNA was synthesized using the Maxima H Minus cDNA synthesis master mix (Thermo Scientific), and qRT-PCR was performed using the iQ SYBR green supermix (Bio-Rad) in 96-well plates.

XTT reduction assay

This method developed by Ramage *et al.* [19] that was used to quantify biofilm formation. *C. albicans* cells were grown to mid-log phase in YPD medium, washed twice with sterile PBS, and suspended in DMEM medium at a density 10^6 cells/mL. 100 µL of cell suspensions were added into wells of 96 well plates and incubated at 37^{o}C, 5% CO_2, 100% humidity for 2 days. Then, non-adherent cells were removed by washing thrice with sterile PBS and leaving behind mature biofilm. A Colorimetric XTT Reduction Assay Kit (KeyGEN BioTECH) was used to quantify the biofilm formation. 200 µL of XTT-menadione solution was added to the prewashed biofilms and background control wells and incubated at 37 ^{o}C in the dark for 3 h. Supernatants were transferred into new plates. Average values of XTT reduction reading at 450 nm of each strain is expressed as a percentage of the value of the WT strain.

Murine model of disseminated candidiasis

The murine models were constructed as previously described by Han *et al.* [17]. Mid-log phase *C. albicans* yeast cells were washed twice and diluted to 5×10^6 cells/mL with PBS. Thirteen 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 21 days. To determine the organ fungal burden, three mice were infected with each strain as described above and sacrificed at 48 h after the injection to surgically remove the kidney. One kidney from each mouse was removed, weighed, and homogenized. The homogenate was serially diluted in PBS and spread onto YPD plates for counting colony forming units (CFUs) per gram of kidney. Another kidney was fixed with formaldehyde followed by 70% ethanol and then embedded in paraffin. Thin sections

were cut and stained with periodic acid-Schiff staining for microscopic examination.

Statistical analyses

All data shown in this study were from at least 3 independent experiments as means ± SD. The results of the *in vitro* experiments were analyzed with unpaired two tails Student's *t*-test. The results of survival curves and fungal burdens were analyzed using Kaplan-Meier test and Mann-Whitney test, respectively.

Results

Profilin has a role in polarized growth in *C. albicans*

To explore the role of profilin in *C. albicans*, we generated *pfy1* mutant (*pfy1Δ/Δ*, *orf19.5076Δ/Δ*) in the SC5314 background (Figure S1 and S2). Given that the *PFY1* homolog in *S. cerevisiae* is an essential gene, our initial assessment focused on whether *PFY1* affects the growth of *C. albicans*. In YPD liquid medium, *pfy1Δ/Δ* displayed a growth curve like that of the wild-type (WT) strain (Figure 1A). On YPD solid plate, *pfy1Δ/Δ* showed no growth defects or temperature sensitivity when grown at 30 ℃/37 ℃ (Data not shown), indicating *PFY1* was not crucial for cell growth in *C. albicans*.

However, microscopic examination revealed that the *pfy1*Δ/Δ mutant exhibited rounder and larger cell morphology compared to the WT strain (Figure 1B and C). The aberrant cell morphology of *pfy1Δ/Δ* was restored by introducing one copy of wild-type *PFY1* at *RP10* locus (*pfy1Δ/Δ+PFY1*) (Figure 1B and C). In addition, cellular polarity was investigated by staining cells with Calcofluor white (CFW) to monitor chitin distribution [21]. In contrast to the WT and *pfy1Δ/Δ+PFY1* strains, where chitin staining was primarily localized to bud sites, *pfy1Δ/Δ* cells displayed a rounded shape with delocalized CFW staining patterns (Figure 1B).

Profilin plays a critical role in actin cable assembly in *S. cerevisiae* [39]. We next employed phalloidin to visualize actin filament distribution. In the WT cells, actin cables were visible in mother cells and oriented toward the small buds, where the cortical patches were concentrated; however, actin cables were not readily detected and patches were dispersed throughout the mother cells in *pfy1Δ/Δ* cells , even when small

buds present (Figure 1D). All these results suggesting that profilin has a role in polarized growth of *C. albicans*.

The loss of *PFY1* results in defective filamentous growth in *C. albicans*

As filamentous growth represented the most extreme form of polarized growth [20-22], the hyphal development of *pfy1Δ/Δ* under various inducing conditions were investigated. After induction in YPD containing 10% serum at 37 ℃ for 1 h, both WT and *pfy1Δ/Δ* cells could initiate hyphal formation. However, after 3 h induction period, *pfy1Δ/Δ* failed to produce hyphae or develop significantly shorter hyphae compared to the WT strain (Figure 2A and B). On Spider plates, *pfy1* mutant exhibited a markedly reduced capacity to form wrinkly colonies, whereas the WT and reintegrant strains retained the ability for wrinkly colony formation (Figure 2C). Similarily, *pfy1Δ/Δ* displayed decreased invasion of agar (Figure 2D). These results indicated that *PFY1* is required for hyphal growth.

To further investigate the role of *PFY1* in morphology transitions of *C. albicans*, late-log phase yeast cells were inoculated to fresh YPD medium with 0.02% MMS and 20 mM H_2O_2—agents known to cause DNA damage and promote pseudohyphal formation [23]. As expected, upon induction with MMS and H_2O_2, WT and *pfy1Δ/Δ+PFY1* cells produced pseudohyphae; however, most *pfy1Δ/Δ* remained as yeast forms (Figure 3A). Despite demonstrating defects in genotoxic stress-induced filamentous growth, *pfy1* mutants did not exhibit altered sensitivity to either MMS or H_2O_2 (Figure 3B).

Profilin interacts with Act1 in *C. albicans*

Polarized growth is essential for cell morphogenesis and development in *C. albicans*, which is tightly regulated by assembly of actin [24]. Profilin has been shown to modulate actin dynamics across various organisms, including fruit flies, yeast, and mammals [25]. To determine whether Pfy1 interacts with Act1, *PFY1-GFP* was introduced into *pfy1Δ/Δ* under its own promoter. Expression of the fusion protein Pfy1-GFP was validated through Western Blot analysis (Figure S3). Furthermore, defects in

hyphal development observed in *pfy1Δ/Δ* upon serum induction were rescued by the introduction of *PFY1* tagged with GFP (Figure 2A and B). These results demonstrated that Pfy1-GFP was fully functional. Subsequently, we tagged Act1 at its C terminus with mCherry and expressed it in the strain containing Pfy1-GFP. Immunoprecipitation of Act1-mCherry was able to pull down GFP-tagged Pfy1 in the yeast or hyphal cells (Fig. 4). This interaction was specific as it was not detectable in the control strain carrying only Pfy1-GFP (Fig 4). Our findings indicate that Pfy1 could interact with Act1 independently of the yeast-hyphal transition.

C. albicans PFY1 impacts biofilm formation

Next, the functions of *PFY1* in biofilm formation were examined. We found that the density of biofilm decreased significantly in *pfy1Δ/Δ* cells, compared with WT and *pfy1Δ/Δ+PFY1* cells (Figure 5A). Analyses with the 2,3-bis-(2-methoxy-4-nitro-5-sulfonyl)-2H-tetrazolium-5-carboxanilide salt (XTT) reduction assay revealed that the biofilm metabolic activity of *pfy1Δ/Δ* is reduced by~75% compared to WT and *pfy1Δ/Δ+PFY1* strains (Figure 5B). These results demonstrated that lacking *PFY1* is defective in biofilm formation in *C. albicans*.

PFY1 contributes to cell wall stress responses

As shown in Figure 1B, staining with CFW revealed an increased fluorescent signal associated with the cell wall in *pfy1Δ/Δ* compared to WT and *pfy1Δ/Δ+PFY1* strains. Moreover, qRT-PCR analyses revealed that the expression of chitin synthase (*CHS1, CHS2, CHS3, CHS8*) was elevated in *pfy1Δ/Δ* (Figure S4). This finding motivated us to further investigate cell wall integrity and function. Susceptibility of WT, *pfy1Δ/Δ*, and *pfy1Δ/Δ+PFY1* strains to a panel of cell wall stress agents (CFW, Congo Red, SDS) was assessed. Under these conditions, *pfy1Δ/Δ* had a dramatic growth defect compared to WT and *pfy1Δ/Δ+PFY1* strains (Figure 6A). Additionally, transmission electron microscopy revealed notable differences in cell wall structure among these strains; specifically, the thickness of the cell wall in *pfy1*Δ/Δ was substantially greater than that in either WT or *pfy1*Δ/Δ+*PFY1* (Figure 6B and C). These results suggested

that Pfy1 was involved in remolding the cell wall in response to cell wall stressor.

Furthermore, the susceptibility of *pfy1Δ/Δ* to caspofungin, a non-competitive inhibitor of β-1,3-glucan synthase, was evaluated. *C. albicans pfy1Δ/Δ* displayed a substantial growth defect under caspofungin treatment (64/128 ng/mL), while the growth defect observed for WT and *pfy1Δ/Δ+PFY1* strains were minor (Figure 6D).

The loss of *PFY1* attenuates the virulence of *C. albicans* in the systemic candidiasis mouse model.

To evaluate the role of Pfy1 in *C. albicans* pathogenicity, the virulence of the *pfy1Δ/Δ* was tested in a murine model of disseminated candidiasis. Over a 21-day observation period (Fig 7A), 3 of 10 BALB/c mice infected by intravenous tail vein injection with *pfy1Δ/Δ* survived. In contrast, all the mice infected with WT or *pfy1Δ/Δ+PFY1* died, and the median survival times were 4-5 days, respectively. Fungal burden was also measured 2 days post-infection (Fig 7B). The fungal burdens in kidney of mice inoculated with *pfy1Δ/Δ* were significantly lower than those in mice infected with either WT or *pfy1Δ/Δ+PFY1*. Moreover, shorter hyphae and milder inflammation were observed in PAS (Periodic Acid-Schiff) stained kidney sections from *pfy1Δ/Δ*-infected mice compared to those from WT and *pfy1Δ/Δ+PFY1*-infected mice (Fig 7C). In summary, Pfy1 contributes to the colonization and invasion of *C. albicans* in mice.

Discussion

The actin cytoskeleton serves as a major cellular component that facilitates a plethora of essential functions [40]. The dynamic status of actin is modulated by accessory proteins that promote the rapid assembly and disassembly of filaments. In this study, we demonstrated that profilin, a key regulator of actin polymerization, was crucial for cell morphology, cell wall integrity and virulence in *C. albicans*. Another essential regulator of actin cytoskeletal dynamic is cofilin, which is involved in selective sorting, environmental stress responses (including sensitivity to azoles drugs) and mitochondrial morphology in *S. cerevisiaee* [41,42]. These findings underscore the

importance of actin cytoskeleton dynamics in determining the fate of budding yeast.

Profilin is a multi-ligand protein structurally conserved among eukaryotes [26,27]. In *S. cerevisiae*, it binds with actin to regulate the dynamic actin cytoskeleton [28,29], and coordinates with *SC*. Bni1p, Bem1p, Rho1p, Cdc24p, and Cla4p to regulate Ca^{2+} homeostasis and bud formation [10]. Additionally, it directly interacts with cyclase-associated protein (CAP, known in yeast as Srv2) to catalyze the nucleotide exchange on actin monomers [30]. Our research revealed that the disruption of *PFY1* resulted in striking morphological defects in both yeast and hyphal forms. Most *pfy1Δ/Δ* yeast cells were round instead of exhibiting a typical ellipsoidal shape and were enlarged in size (Figure 1B). Furthermore, the *pfy1Δ/Δ* mutant cells showed severe defects in hyphal growth under various induction conditions (Figure 2).We also confirmed the interaction between Pfy1 and Act1 (Figure 4), which localizes to polarized growth sites in budding and hyphal cells, indicating that profilin plays a role in polarized growth of *C. albicans* via binding with Act1. Future studies should investigate whether loss of *PFY1* could disturb the location of actin in *C. albicans*. In addition, profilin associated with polyproline helices that are present in a wide variety of proteins [31].

C. albicans can transform into different morphologies during commensalism and infection, such as yeast, hyphal, pseudohyphal, white, opaque, and gray cells [32]. Among them, the regulation of yeast-tohyphae transition and its influence on virulence have been studied widely. Here, we reported that deletion of *PFY1* in *C. albicans* resulted in abnormal morphogenesis characterized by enlarged cell size and impaired hyphal growth and reduced pathogenicity, suggesting that cell morphology is a key factor of virulence.The fungi switch between the different forms in response to external stimuli like pH, temperature, serum, nutrient starvation, *etc*. Apart from these, the cells also switch to alternate forms in response to certain genetic mutations or inhibition of cell cycle progression. Our findings indicated that *Ca.* Pfy1 is not only involved in hyphal development but also essential for pseudohyphal formation in response to DNA damage agents MMS and H_2O_2 (Figure 3A). These results highlighted the critical role of profilin in morphology transitions in *C. albicans*. Interestingly, we did not observe defective growth of *pfy1Δ/Δ* upon H_2O_2 treatment, consistent with the notion that

profilin function is dispensable for cell viability in response to oxidative stress in *S. cerevisiae* [33].

In *C. albicans*, the cell wall is composed of three primary layers: a basal chitin layer, followed by a central layer of β (1,3) and β (1,6)-glucan polymers, and an outer layer of mannosylated glycoproteins [34,35]. In agreement with previous studies demonstrating the impact of chitin content on cell wall integrity in *C. albicans* [36,37], we also observed chitin deposition and thicker cell wall in *pfy1Δ/Δ* cells (Figure 1B and Figure 6B, C), suggesting Pfy1 contributes to cell wall remodeling. Pfy1 has been predicted to be involved in carbohydrate transport [38], which may account for this phenomenon. There has been reported that overexpreesion Pfy1 suppressors Mid2, Rom2 and Syp1 in *S. cerevisiae* also resulted in an abnormally thick cell; however, the thickness of cell wall in *pfy1Δ* was similar to wild-type in *S. cerevisiae* [39].

Both hyphal growth and cell wall integrity are the important virulence factors of *C. albicans*. In a systemic candidiasis mouse model, we observed significantly reduced mortality rates in mice infected with *pfy1Δ/Δ* strains and found *C. albicans* hyphae only in the kidneys of mice infected with either the *PFY1* complemented strain or the WT strain (Figure 7 A, B). Besides, *pfy1Δ/Δ* confers hypersensitivity of caspofungin (Figure 6D), one of the first-line antifungals used to treat systemic candidiasis. These findings suggested that profilin could serve as a target for developing new antifungal drugs possibly for use in combination therapies with caspofungin for treating invasive candidiasis.

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Figure legends

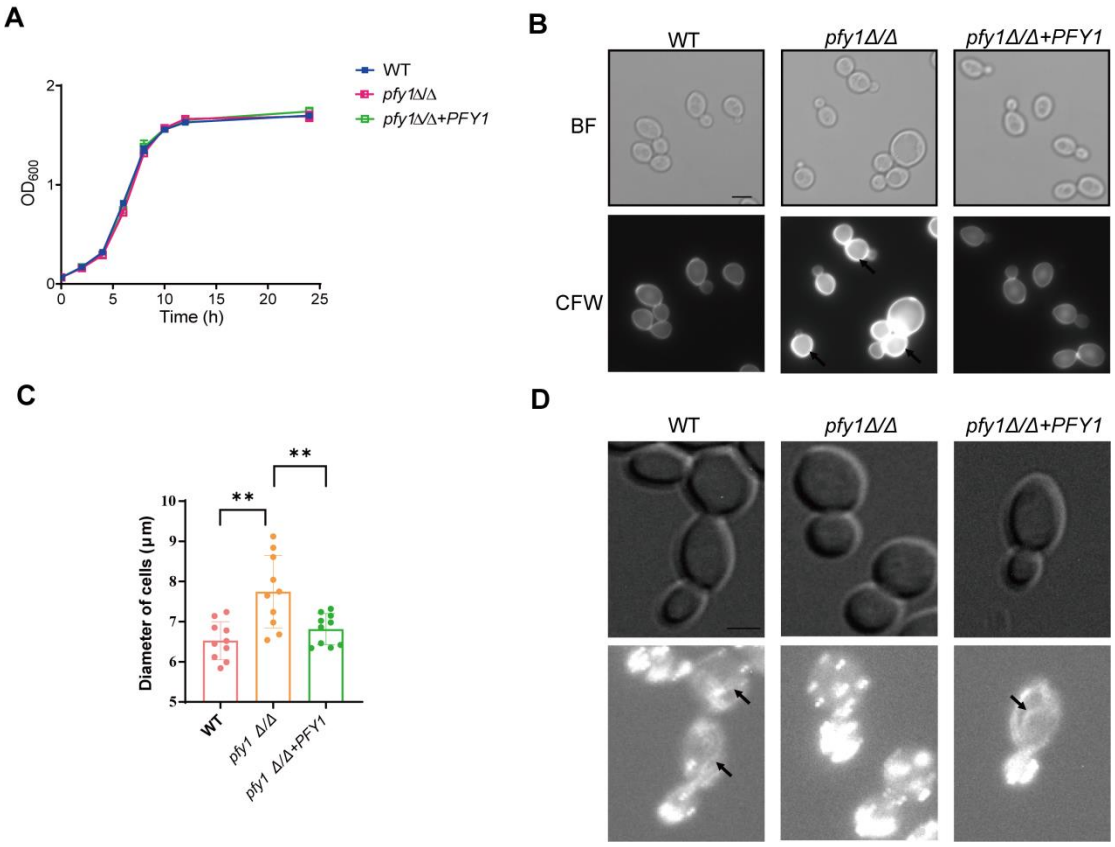

Figure 1. *pfy1Δ/Δ* has normal growth and abnormal cell morphology

A: Wild type (WT), *pfy1Δ/Δ* and *pfy1Δ/Δ+PFY1* strains were depicted in a growth curve. B: These same strains were visualized by bright field and fluorescence microscopy stained with calcofluor white (CFW). The arrows indicate delocalized chitin. Scale bar, 5 μm. C: Statistical results of diameter of cells in B. D: 20 μl log-phase yeast cells of indicated strains were stained using rhodamine-phalloidin to visualize actin. The arrows indicate actin cables. The scale bar indicates 5 μm. The images shown in panels B and D are representative of three different experiments. Error bars in panels C are the standard deviations of three different data sets. Each individual data point was run in triplicate and averaged. Statistical significance between control strains and *pfy1Δ/Δ* strain was determined with Student's *t*-test (** represents $p<0.01$).

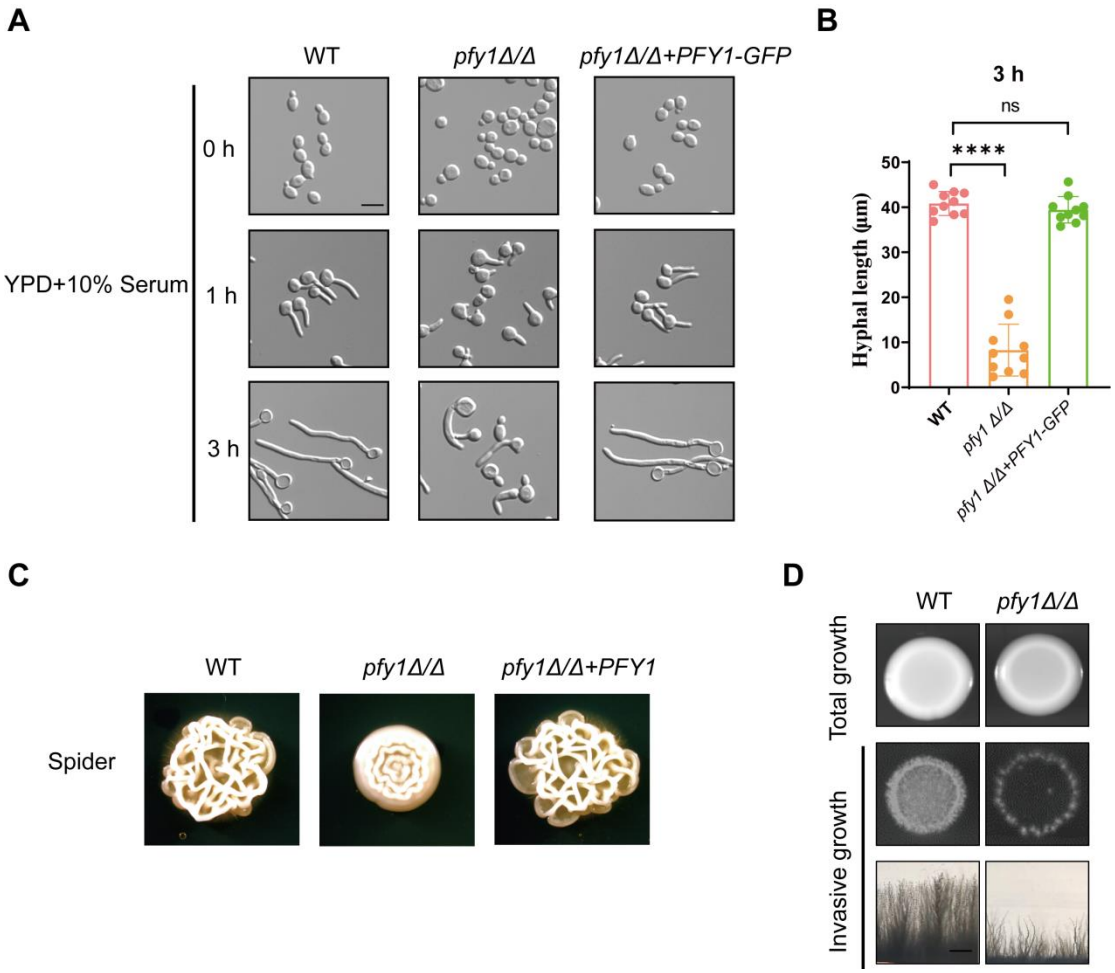

Figure 2. The disruption of *PFY1* results in defective filamentous growth in *C. albicans*

A: WT, *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1-GFP* strains were re-inoculated in YPD medium containing 10% serum and incubated at 37 ℃ for 3 h to take photos. Scale bars = 5 μm. B: Statistical analysis of hyphal length in the same strains as described in (A) following a 3-hour induction with 10% serum. C: Colony morphology of WT, *pfy1Δ/Δ* and *pfy1Δ/Δ+PFY1* strains grown on Spider plates at 30 ℃ for 7 days. D: Invasive growth of WT and *pfy1Δ/Δ* strains. Strains were grown on YPD plates at 30 ℃ for 2 days. After this period the plates were photographed before (Total growth) and after (Invasive growth) thoroughly washing the cells from the surface. Scale bars = 1 mm.

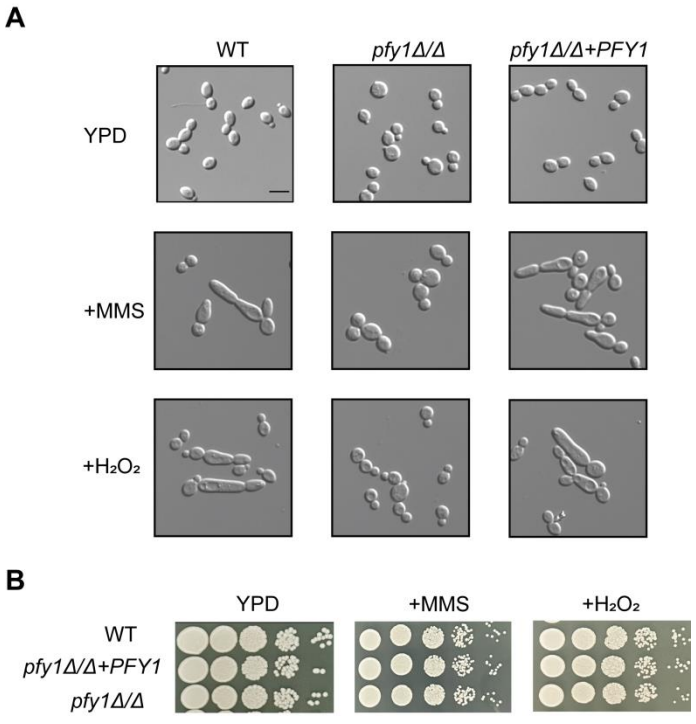

Figure 3. *pfy1Δ/Δ* cells are defective in formation of pseudohyphae

A: WT, *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1* strains were re-inoculated in YPD medium containing 0.02% MMS or 20 mM H_2O_2 and incubated at 30 ℃ for 6 h to take photos. Size bars = 5 μm. B: Log-phase yeast cells of the same strains as described in (A) were serially diluted 10-fold and spotted onto YPD plates supplemented with 0.02% MMS or 20 mM H_2O_2. The YPD plates were incubated at 30 ℃ for 48 h.

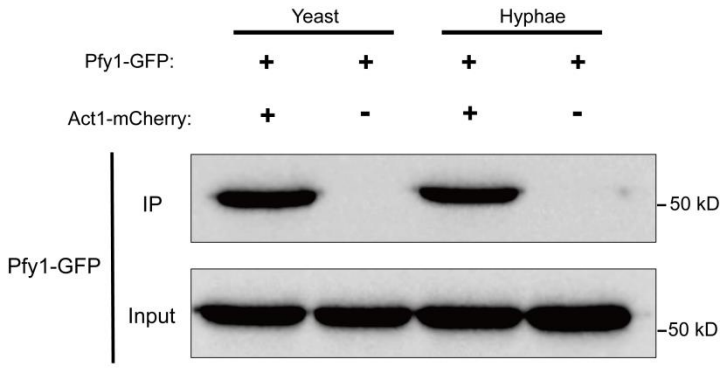

Figure 4. Profilin interacts with Actin

Co-IP of Pfy1 with Act1. Anti-mCherry conjugated beads were used for pull-down from log-phase yeast cells' and hyphae cells' lysates, and the pull-down products were probed with anti-GFP in WB analysis. As an input control, cell lysates were analysed by Western blotting with the anti-GFP antibody.

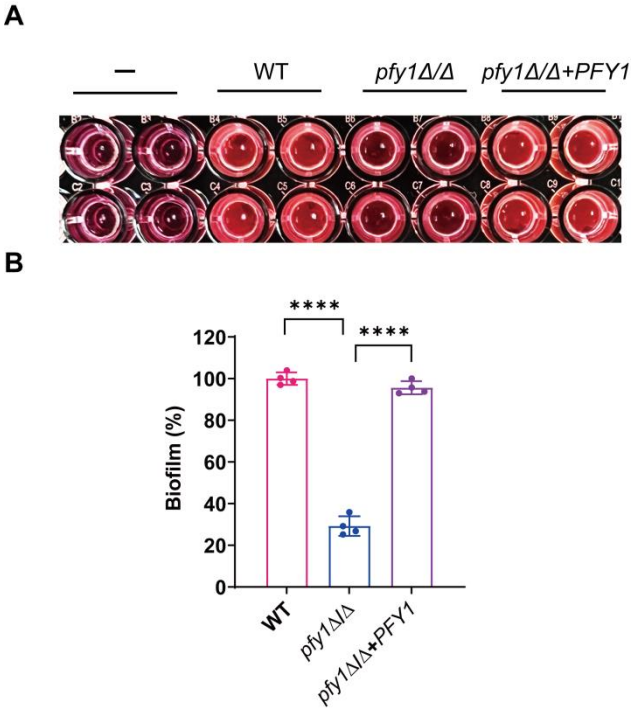

Figure 5. *pfy1Δ/Δ* forms an aberrant biofilm

A:WT, *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1* strains were re-inoculated in YPD medium and incubated at 30 ℃ for 72 h. The biofilms formed was determined through XTT reduction assay. B: Quantitatively measure the biofilm formation by a colorimetric change resulting from XTT reduction was measured using a microtiter reader at 450 nm. **** represents $p<0.0001$.

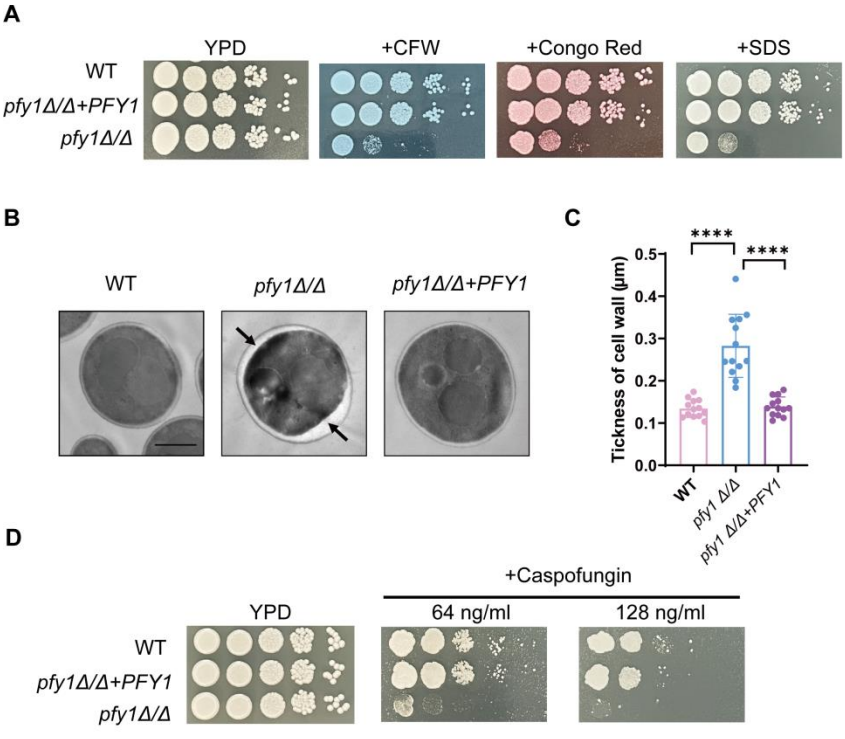

Figure 6. *pfy1Δ/Δ* cells have thicker cell wall and are hyper-sensitivity to cell wall stress

A: WT, *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1* strains were re-inoculated in YPD medium containing 80 μg/mL CFW, 100 μg/mL Congo Red or 0.02% SDS and incubated at 30 ℃ for 48 h. B-C: Cell wall thickness from the same strains were taken photos and analyzed. The black arrows show the cell wall thickness. Size bars = 2 μm. **** represents $p<0.0001$. D: These same strains were serially diluted tenfold and spotted onto YPD plates supplemented with 64 or 128 ng/mL Caspofungin. The plates were incubated at 30 ℃ for 48 h.

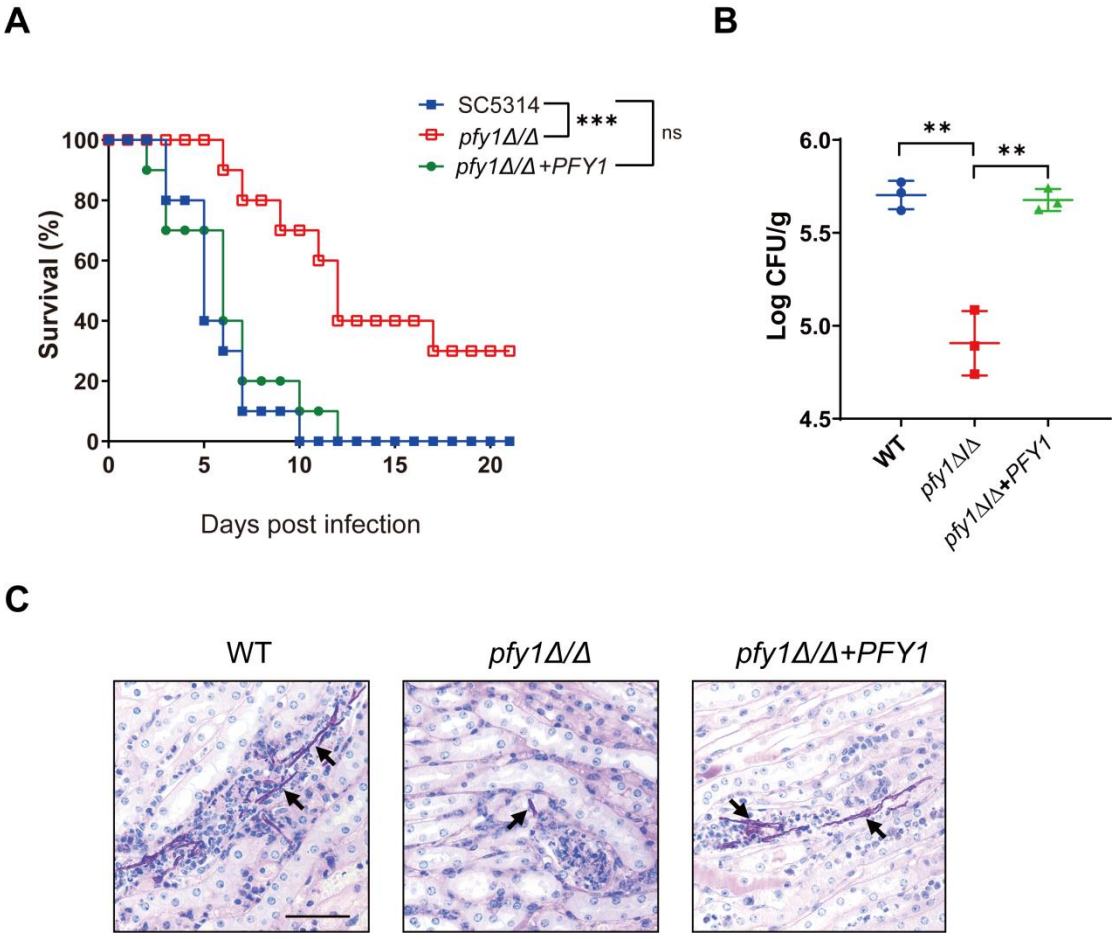

Figure 7. *pfy1Δ/Δ* shows impaired virulence in the systemic candidiasis mouse model

A: BALB/c mice (n=10) were injected via the tail vein with 10^6 yeast cells of SC5314(WT), *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1* strains, and monitored for survival over a period of 21 days. B: Three mice were sacrificed after 48 h of the injection to determine the fungal loads in the kidney. C: Conduct histological examinations of kidney sections. Size bars = 0.13 mm. Arrows indicate *C. albicans* cells in the renal tissues. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. ** represents $p<0.01$; *** represents $p<0.001$.

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Table 1. *C.albicans* strains used in this study.

Strain	Relevant genotype	Source
SC5314	Wild-type, clinical isolate.	Fonzi and Irwin, 1993
pfy1Δ/Δ	SC5314 *pfy1Δ::FRT/pfy1Δ::FRT*	This study
pfy1Δ/Δ+PFY1	SC5314 *pfy1Δ/pfy1Δ, RP10:: PFY1p-PFY1-FRT*	This study
PFY1-GFP	SC5314 *pfy1Δ/ PFY1-GFP-FRT*	This study
Pfy1-GFP Act1-mCherry	SC5314 *pfy1Δ/ PFY1-GFP, ACT1/ACT1-mCherry-FRT*	This study

Table 2. Primers used in this study.

Name	Sequence (5' to 3')	Description
PFY1-A	TTTGTTCAATCTTTGGATTAAATATAC AACAGATCTATTAAATACAATTGCAG AATCATTGCTGGGTACCGGGCCCCCC TCGAG	To delete *PFY1*
PFY1-B	CTTAACATCAACCATTAAATCTGGAC AGGTATTGCACGTCCATGGATGCAAT GAAAATATGGGCGAATTGGAGCTCC ACCGCGG	To delete *PFY1*, and tag *PFY1* with *GFP*
PFY1-GFP-A	CCAGGTGAAGCTACCACTCTTGTTG AAAAATTAGCCGATTACTTGATCAAT GTCGGTTATGGGCCCATGTCTAAAGG TGAAGAA	To tag *PFY1* with *GFP*
Primer 01	TTTCCAGTGTTAAGCAACACCTGG	To verify the mutations by PCR
Primer 02	TATAACTGTGCTAAAAGCCACGTA	
Primer 03	ATAACCGACATTGATCAAGTAATC	
ACT1-mCherry-A	TGGATTTCAAAACAAGAATACGACG AATCTGGTCCATCCATTGTTCACCAC AAATGTTTCATGGTTTCAAAAGGTG AAGAAGAT	To tag *ACT1* with *mCherry*
ACT1-mCherry-B	AACAAAAAGAAGAATAACAAGAATA CAAAACCAGATTTCCAGATTTCCAG AATTTCACTCGGGCGAATTGGAGCT CCACCGCGG	

RePFY1-A (Xho I)	CCGCTCGAGCCAGCTGAAAATTGTG CCAGTGAT	To clone *PFY1-GFP* into the *Xho*I-*Hind*III sites of *pAG6*
RePFY1-B (Hind III)	CCCAAGCTTTTATTTGTAC AATTCATCCA TACC	
CHS1-A	AAAAGTGTTGACCAGAACCGAG	qRT-PCR analysis
CHS1-B	ATGGCGTGAGCACAAATGA	
CHS2-A	TGATTTGGCAGCGATTAGTTAT	
CHS2-B	TCTTGTTGTGGAGGAGGTTCTT	
CHS3-A	GCTTGTAAGACTGTTGTCCCCG	
CHS3-B	AAATAGTAAATGTAATGGCTGCTGG	
CHS8-A	ATGGATGATGGTTCTCTTGTTG	
CHS8-B	GAATGTCTCTTCTTGATGGTGG	

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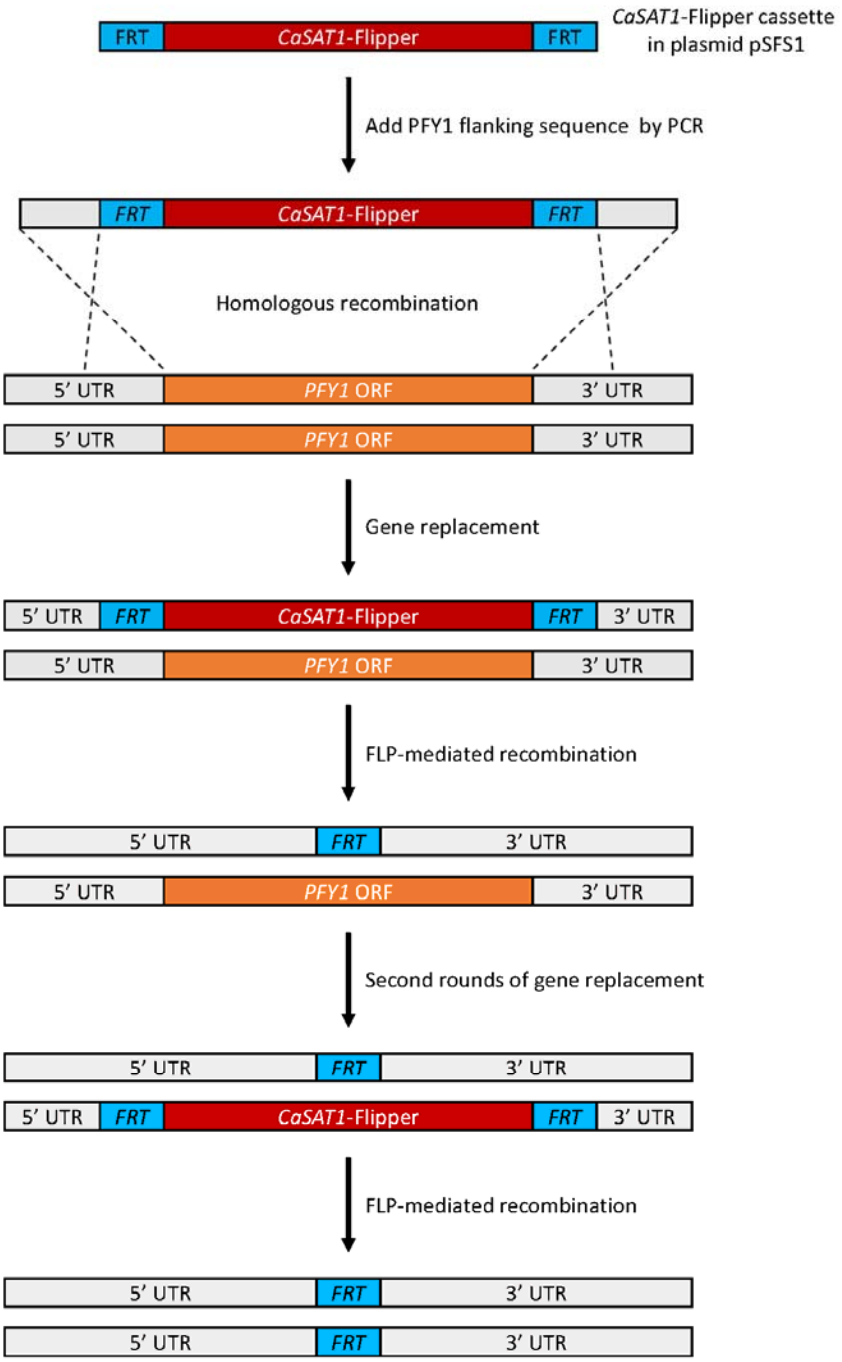

Figure S1. Schematic diagram of the construction of *pfy1* mutant

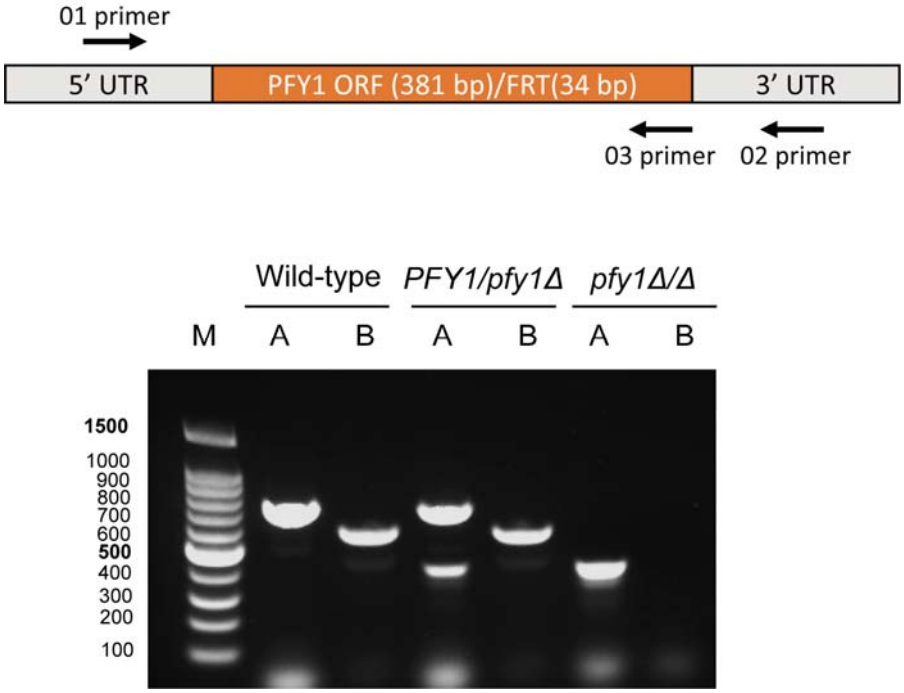

Figure S2. Verification of correct construction of *pfy1Δ/Δ*, *pfy1Δ/Δ+PFY1* strains by PCR

The PCR primers used are shown on the top. Genomic DNA was extracted from each strain and used as the template for PCR. (A: Primers 01 and 02 , B: Primers 01 and 03)

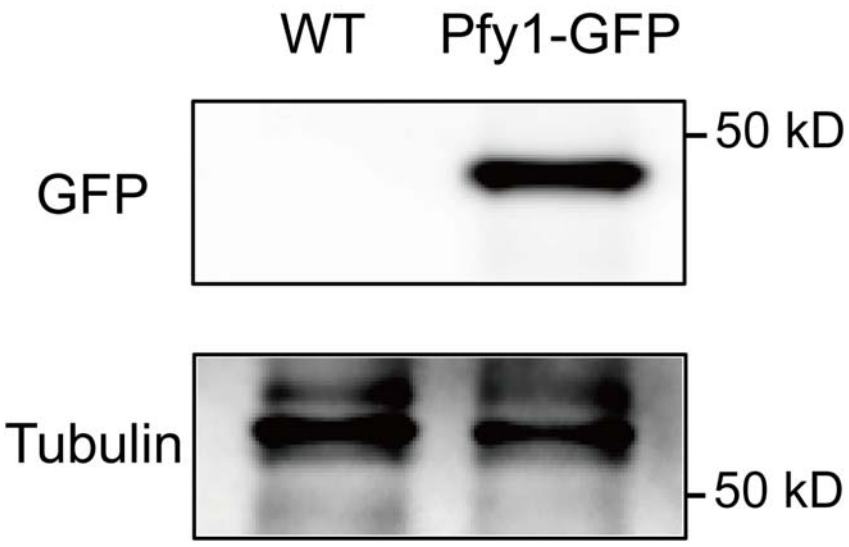

Figure S3. Protein level of Pfy1-GFP was analysis by Western Blot

Expreesion of the fusion protein was analysis by Western blot. Representative blots of three independent experiments are shown.

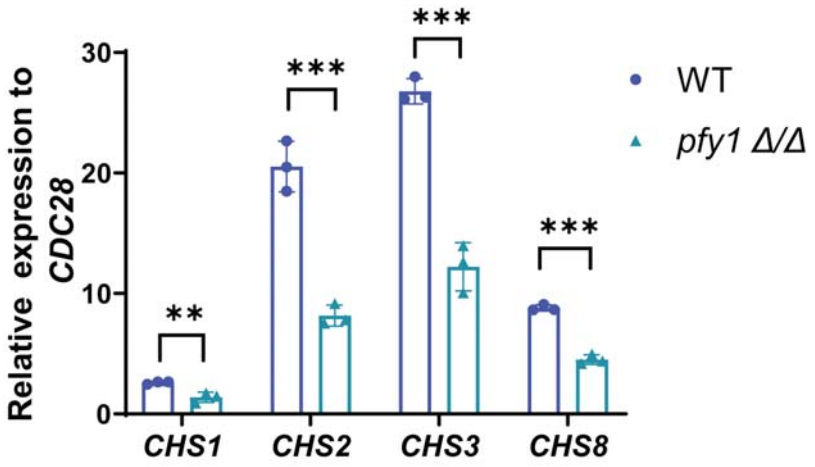

Figure S4. Expression levels of chitin synthase were increased in *pfy1Δ/Δ*

qRT-PCR analysis of expression levels of chitin synthase (*CHS1, CHS2, CHS3,CHS8*) in *pfy1Δ/Δ* and WT cells. Data shown as means ± SD of three independent experiments. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. ** represents $p<0.01$; *** represents $p<0.001$.