Spf1 strongly influences calcium homeostasis, hyphal development, biofilm formation and virulence in *Candida albicans*

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The maintenance of cellular calcium homeostasis is associated with cellular signalling transduction and the functions of many membrane compartments, especially endoplasmic reticulum (ER) function. ER-localized proteins that serve to maintain ER and cellular calcium homeostasis in *Candida albicans* are still unclear. In this study, Spf1, the putative *C. albicans* homologue of the *Saccharomyces cerevisiae* ER-localized P-type calcium ATPase ScSpf1, was investigated for its roles in cellular calcium homeostasis, hyphal development and virulence. We constructed an Spf1 null mutant which showed decreased vegetative growth rate and hypersensitivity to EGTA, high-level calcium and antifungal drugs. Similar to treatments of ER stress agents, deletion of *SPF1* stimulated calcium influx in the presence of FK506, resulting in an increase in cellular calcium contents, and induced expression of the calcium-dependent response elements gene *CCH1*, which is essential for the cell calcium survival pathway. Moreover, the *spf1* null mutant had defects in hyphal development and biofilm formation, and was severely attenuated in virulence. These findings provided phenotypic evidence supporting roles for Spf1 in the maintenance of cellular calcium homeostasis, ER stress responses, hyphal development, biofilm formation and virulence in *C. albicans*.

**INTRODUCTION**

*Candida albicans* is the leading fungal pathogen in humans. It is responsible for epithelial infection and life-threatening systemic infections in immunocompromised hosts, ranking as the fourth most common cause of nosocomial bloodstream infections (Klepser, 2006; Pfaller & Diekema, 2007). This fungus can switch between yeast, pseudohyphal and hyphal growth patterns (Odds & Kerridge, 1985; Sudbery et al., 2004). The dimorphic property is beneficial to it as it enables the fungus to invade tissues and survive the host immune system, contributing significantly to its pathogenicity (Berman & Sudbery, 2002). Another property of this fungus is its ability to grow as a biofilm on implanted medical devices (Donlan & Costerton, 2002; Douglas, 2003). Like biofilms formed by bacterial pathogens, *C. albicans* biofilms are resistant to many antifungal agents, such as azoles, allyamines and polyenes, and are also responsible for its virulence (Finkel & Mitchell, 2011). Studies of functional proteins of *C. albicans* that influence dimorphic properties, biofilm formation and virulence will increase our understanding of its pathogenic mechanisms, and aid in the design of specific drugs for treatment of its infections.

The endoplasmic reticulum (ER) is the site of many essential cellular processes, including protein modification, lipid synthesis, polarized secretion and growth. These processes require stringent regulation of ER luminal ion levels, especially calcium (Scarborough, 1999). In mammal cells, the SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) pumps regulate ER calcium levels. *Saccharomyces cerevisiae*, in which SERCA pumps are absent, possesses ER-localized P-type ATPase ScSpf1 and Golgi-localized P-type ATPase ScPmr1 that are responsible for maintenance of calcium in the ER (Ando & Suzuki, 2005; Cronin et al., 2002; Marchi et al., 1999; Strayle et al., 1999). Deletion of *ScSPF1* disrupts cellular calcium homeostasis, resulting in increased expression of calcium-dependent response element (CDRE) genes. Furthermore, deletion of

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Abbreviations: CDRE, calcium-dependent response elements; CCS, cell calcium survival; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol.
both ScSPF1 and ScPMR1 stimulates calcium influx and leads to a consequent increase in cellular calcium (Cronin et al., 2002). Similarly, ER stress agents such as calcium chelators,azole drugs or tunicamycin also stimulate calcium influx mediated by the high affinity calcium influx system Cch1-Mid1, resulting in elevation of cytoplasmic calcium and activation of the calcium/calmodulin-dependent phosphatase calcineurin (Bonilla et al., 2002). Calcineurin then dephosphorylates the transcription factor Crz1, resulting in the translocation of Crz1 from cytoplasm to nucleus and consequent activation of expression of CDRE genes, such as CCH1, PMR1 and PMC1 (Karababa et al., 2006; Cronin et al., 2002). Moreover, calcineurin appears to inhibit calcium influx via a feedback loop by dephosphorylating Cch1, whose phosphorylation is essential for its activity (Bonilla & Cunningham, 2003; Martin, et al., 2011). This calcium signalling pathway, termed the cell calcium survival pathway, is required for tolerance to ergosterol biosynthesis inhibitors, survival of calcium depletion and other responses to ER stresses (Bonilla & Cunningham, 2003; Onyewu et al., 2004). In C. albicans, however, functional proteins maintaining ER calcium homeostasis and their roles remain unknown.

In this study, we identified a C. albicans homologue of ScSpf1 by BLASTP search of the Candida genome database. We constructed a Spf1 null mutant and found that the mutant showed low growth rate and hypersensitivity to EGTA, high-level calcium and antifungal drugs. The mutant also exhibited severe defects in hyphal development and biofilm formation, and was significantly attenuated in virulence. These results revealed that Spf1 is required for growth, cellular calcium homeostasis and pathogenicity of C. albicans.

METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. C. albicans cells were grown at 30 or 37 °C in YPD medium (adding 80 μg uridine ml⁻¹) or, when indicated, in synthetic complete (SC) (adding 80 μg uridine ml⁻¹) or synthetic drop-out (SD) medium.

Plasmid construction. Plasmids used in this study are shown in Table 1. For generating the reconstituted plasmid pSPF1-HIS1, the SPF1 reconstituted cassette containing the 3672 bp ORF plus ~800 bp of promoter sequence and ~700 bp of terminator sequence was amplified by PCR using the primers SPF1-5W (5′-GGACTAGTG-CCTTGTTCAATTGCATGG-3′) and SPF1-3W (5′-GGACTAGTG-CCATCGATT-3′), the calcineurin inhibitor FK506 (Sigma, 2 μg ml⁻¹), tunicamycin (BBI, 2.5 μg ml⁻¹) plus FK506 (2 μg ml⁻¹), or tunicamycin (2.5 μg ml⁻¹) plus FK506 (2 μg ml⁻¹). Cells were harvested, washed with HBSS buffer three times, and incubated with Fluoro-3AM (Sigma, 5 μg ml⁻¹) in the dark at 37 °C for 4 h. Cells were harvested, washed with HBSS buffer three times again, then suspended with 500 μl HBSS buffer. A 100 μl aliquot of the suspension was added into a well of a 96-well plate (Denmark). The fluorescence density (excitation wave 485 nm, emission wave 510 nm) and the absorbance at 570 nm were determined for evaluating cytoplasmic calcium levels. For evaluating total cellular calcium levels, cells were prepared and treated with the agents as described above at 37 °C for 3 h. Cells were harvested, washed with HBSS buffer three times, and digested overnight in 33 % (w/v) nitric acid. Total calcium content of the digestion liquid was measured by using an atomic absorption spectrophotometer (HITACHI, 180-80).

β-Galactosidase assays. β-Galactosidase assays were performed as described by Giarante (1983). Briefly, overnight cultures were diluted to OD600 0.1 and incubated at 37 °C for 4 h, then treated with the agents

Candida albicans strain construction. All C. albicans strains were generated in the wild-type strain DAY1 background. For deletion of the SPF1 gene, DAY1 was transformed with the PCR product amplified from the plasmid pRS-ArgAsp with the deletion primers SPF1-5DR (5′-TTCAACATCTTCTTTGCATGATTAACAC-GATCACAACCTCAGCTATCAATACGTC-TT-3′) and SPF1-3DR (5′-TGAAATCTAATGCGATTGAACAATA-TGATCAAACCTTCATGTTGATGTTGCTCAGCG- TT-3′) and SPF1-3det (5′-TGGAATCTAATGCGATTGAACAATA-TGATCAAACCTTCATGTTGATGTTGCTCAGCG- TT-3′); and the heterozygous mutant NKF110 was confirmed by PCR with the detection primers SPF1-5det (5′-TG-TGAGGTTGTTGTTGTTCT-3′) and SPF1-3det (5′-TCAGGC-ATCTCATCAAC-3′). NKF110 was then transformed with the PCR product amplified from the plasmid pDB857 with the deletion primers, and the homozygous mutant NKF111 was confirmed by PCR using the detection primers and by using Southern blot analysis. The specific probe pSPF1 used for Southern blotting was amplified from the DAY1 genome with the detection primers; the product was gel-purified and DIG-labelled with random primers. To construct the SPF1 reconstituted strain NKF112 and the control strain NKF113, NKF111 was transformed with the Nrdl-digested pSPF1-HIS1 and pDB78, respectively. To evaluate the effect of SPF1 deletion on expression of CDRE genes, DAY1 and NKF111 were transformed with the Nrdl-digested plasmid pCC1H-lacZ, in which the promoter of the CDRE gene CCH1 is fused with the reporter gene lacZ encoding β-galactosidase (Guarente, 1983). The generated strains NKF116 and NKF117 were used for β-galactosidase assays.

Sensitivity testing. EGTA sensitivity tests were set up in 96-well polystyrene flat-bottom microtitre plates (Denmark). Cell suspension (100 μl of 1 × 10⁶ cells ml⁻¹) in SC medium, containing the calcium chelator EGTA (BBI) ranging from 0 to 30 mM, was added to wells of a microtitre plate. The plate was covered with its lid, sealed with Parafilm and incubated at 37 °C for 24 h. The optical density at 595 nm of each well was determined by using a microplate reader (Bio-Rad), and the growth as a percentage of control (% of control) was calculated as ((OD600 in the presence of EGTA/OD600 in the absence of EGTA) × 100). Calcium-rich growth tests were set up in 40 ml SC medium per flask (250 ml) with calcium chloride (BBI, 1 M) from 0 to 160 mM. Strains were incubated with shaking at 37 °C for 6 h, and the optical density at 600 nm of the incubated cultures were determined. The growth as a percentage of the control (% of control) was also calculated as ((OD600 in the presence of calcium chloride/OD600 in the absence of calcium chloride) × 100). c.f.u. counts of the cultures were calculated after 48h of incubation on solid YPD medium for assessing viability of these strains. In drug sensitivity tests, strains were spotted on solid YPD media containing fluconazole (Sigma, 8 μg ml⁻¹), tunicamycin (BBI, 2.5 μg ml⁻¹) or hygromycin B (BBI, 50 μg ml⁻¹) and incubated at 30 °C for 48–72 h.

Calcium measurements. Evaluation of cytoplasmic calcium was carried out by using a microplate reader (Synergy 4, Biotek). Briefly, strains were incubated in YPD medium with an initial OD600 0.1. After growing with shaking at 37 °C for 4 h, the cultures were supplemented with EGTA (0–30 mM), calcium chloride (0–160 mM), fluconazole (8 μg ml⁻¹), the calcineurin inhibitor FK506 (Sigma, 2 μg ml⁻¹), tunicamycin (2.5 μg ml⁻¹), fluconazole (8 μg ml⁻¹) plus FK506 (2 μg ml⁻¹), or tunicamycin (2.5 μg ml⁻¹) plus FK506 (2 μg ml⁻¹) and incubated at 37 °C for a further 3 h. Cells were harvested, washed with HBSS buffer (pH 7.0) three times, and incubated with Fluo-3AM (Sigma, 5 μg ml⁻¹) in the dark at 37 °C for 50 min. Cells were harvested and washed with HBSS buffer three times again, then suspended with 500 μl HBSS buffer. A 100 μl aliquot of the suspension was added into a well of a 96-well polystyrene flat-bottom polystyrene plate (Denmark). The fluorescence density (excitation wave 485 nm, emission wave 510 nm) and the absorbance at 595 nm were determined for evaluating cytoplasmic calcium levels. For evaluating total cellular calcium levels, cultures were prepared and treated with the agents as described above at 37 °C for 3 h. Cells were harvested, washed with deionized water three times, and digested overnight in 33 % (w/v) nitric acid. Total calcium content of the digestion liquid was measured by using an atomic absorption spectrophotometer (HITACHI, 180-80).
Table 1. *C. albicans* strains and plasmids in this study

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<th>Strain or plasmid</th>
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mentioned above. Cultures were incubated at 37 °C for a further 3 h. Cells were harvested and suspended in 1 ml working Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 2.7 ml β-mercaptoethanol 1<sup>-1</sup>, pH 7.0). The OD<sub>600</sub> of 50 μl suspensions was determined. The suspensions (150 μl) were permeabilized, equilibrated at 37 °C for 10 min, mixed with 700 μl ONPG (BBI, 1 mg ml<sup>-1</sup>), and incubated at 37 °C for a given time (T). Reactions were stopped by the addition of 500 μl Na<sub>2</sub>CO<sub>3</sub> (BBI, 1 M). The OD<sub>420</sub> was determined. Miller units of activity were calculated as (A<sub>420</sub> × 1000)/(A<sub>600</sub> × T × 3). The fold change in Miller units was calculated as (Miller units of NKFI16 or NKFI17 in the presence of the agent/Miller units of NKFI16 in the absence of the agent) × 100.

**Hyphal induction.** Hyphal development ability was tested on solid YPD with 10% (v/v) fetal bovine serum (FBS, Sigma), Spider or SLAD at 37 °C for the indicated time, in liquid YPD with 10% (v/v) FBS at 37 °C for 3–6 h, or in molten YPS agar at 30 °C for 4 days.

**Biofilm development assays.** *C. albicans* biofilms were formed under static conditions (Nobbs et al., 2010; Uppuluri et al., 2010). Briefly, cells were grown in SC medium overnight, washed with PBS and adjusted to OD<sub>600</sub> 0.1 in SC medium. An inoculum (100 μl) was added to each well of a 96-well flat-bottom polystyrene plate. The plate was covered with its lid, sealed with Parafilm and incubated at 37 °C for 24 h. The wells were washed with PBS several times to remove any non-adherent cells, and examined under a light microscope. The wells were then submerged in 1% (w/v) crystal violet for 2 min, washed with distilled water to remove excess stain, and submerged in 100 μl 10% (w/v) acetic acid for 15 min. Suspensions were diluted, and OD<sub>495</sub> was determined as a measurement of biomass.

**Virulence assays.** ICR female mice (25–30 g, 7 weeks old) were used to assay the virulence of *C. albicans* strains. Overnight cultures were grown for 5–6 h with shaking in YPD medium at 30 °C. Cells were washed twice, counted and resuspended to a density of 5 × 10<sup>6</sup> cells ml<sup>-1</sup> with 0.9% (w/v) NaCl solution. Groups of 10 mice were inoculated intravenously with 0.1 ml cell suspension per mouse. Each group was monitored over 30 days, and the survival rate was recorded. Data were analysed by using SPSS software (Version 17.0). For histological analysis, mouse kidneys were collected at the time of sacrifice, cut longitudinally and fixed in 10% (w/v) formalin (alcohol solution). The fixed materials were embedded in paraffin, sectioned and stained with haematoxylin and eosin. Stained sections were examined microscopically (Nikon E200) for *C. albicans* morphology and infiltration. These protocols were approved by the Institutional Animal Care and Use Committee of Nankai University.

**RESULTS**

**Identification of Spf1 in *C. albicans***

To identify homologous proteins of the *S. cerevisiae* ER-localized P-type ATPase ScSpf1 in *C. albicans*, a BLAST search of this protein against the *C. albicans* genome was carried out at the Candida Genome Database web server (http://www.candidagenome.org). A homologue of ScSpf1,
which is encoded by orf19.30 (named SPF1 below), was found and named Spf1, which is a protein of 1223 amino acids. The deduced amino acid sequence of Spf1 demonstrated high homology to other type V subfamily P-type ATPases that are conserved in eukaryotes, all of which are ATP-fuelled ion pumps with a single catalytic subunit (Palmgren & Axelsen, 1998). Ten putative transmembrane regions are predicted by TMHMM (http://cbs.dtu.dk/services/TMHMM), indicating that Spf1 is a membrane protein.

**The spf1Δ/Δ mutant shows growth defects and drug hypersensitivity**

In order to study the role(s) of Spf1 in *C. albicans*, both SPF1 alleles were deleted by PCR-mediated gene disruption. Successful deletion of SPF1 was confirmed by PCR detection and Southern blotting (Fig. 1). To explore the effects of Spf1 on growth rate, we measured the growth curves of the wild-type strain DAY1, the spf1Δ/Δ mutant NKF111 and the SPF1 reconstituted strain NKF112 in liquid YPD (adding uridine) at 30 and 37 °C. Compared with the wild-type strain and the reconstituted strain, the mutant showed decreased growth rate, and its growth defect was more significant at 37 °C (Fig. 2a).

It is well known that ScSpf1 has an effect on calcium-related growth (Dean, 1995). In this study, we also tested growth rate of the spf1Δ/Δ mutant in calcium-related conditions. Similar to the *cch1Δ/Δ* mutant NKF101, the spf1Δ/Δ mutant was more hypersensitive to EGTA than the wild-type strain DAY1, the *pmr1Δ/Δ* mutant NKF102 and the *yvc1Δ/Δ* mutant NKF103 (Fig. 2b, left). Its growth was completely blocked by the addition of 10 mM EGTA, while growth of the wild-type strain was only blocked by more than 20 mM EGTA. Moreover, the mutant was more sensitive to high levels of extracellular calcium than other strains. Its biomass dropped by nearly 30% in the presence of 160 mM calcium, while that of other strains did not drop or dropped by <10% (Fig. 2b, right). The c.f.u. count of the spf1Δ/Δ mutant also significantly decreased with the treatment of high-level calcium when compared with other strains (data not shown). These results suggested that Spf1 function is associated with the maintenance of cellular calcium homeostasis.

Since ER functions are essential for fungal survival of antifungal drug treatment (Bonilla *et al.*, 2002), we hypothesized
that SPF1 deletion may increase the sensitivity to these drugs in C. albicans. As expected, the mutant showed hypersensitivity to all of the antifungal drugs tested, such as fluconazole, tunicamycin and hygromycin B (Fig. 2c), implying an essential role of Spf1 in the functioning of the ER.

**SPF1 plays a role in maintaining cellular calcium homeostasis**

Defective calcium transport can result in a disturbance of calcium homeostasis, which is associated with hypersensitivity to this ion, its chelators and other ER stress agents (Dean, 1995; Lapinskas et al., 1995; Bonilla et al., 2002; LaFayette et al., 2010). The sensitivity of the spf1Δ/Δ mutant to EGTA, calcium and antifungal drugs suggests an essential role of Spf1 in the maintenance of calcium homeostasis. To further confirm this role of Spf1, we evaluated both cytoplasmic and total cellular calcium levels in the mutant with the treatments of these ER stress agents, calcium and the calcineurin inhibitor FK506. There was little difference in cytoplasmic and total cellular calcium levels between the mutant and the wild-type strain in the absence of these agents (the control). However, the mutant showed significantly higher cytoplasmic and total cellular calcium levels in the presence of these agents alone or together (Fig. 3). The results demonstrated that the mutant

![Figure 2](https://www.microbiologyresearch.org/content/images/2018/12/fig2.png)

**Fig. 2.** Effect of SPF1 deletion on cell growth and sensitivity to antifungal drugs. (a) Growth curves of the wild-type strain DAY1 (●), the spf1Δ/Δ mutant NKF111 (▲) and the reconstituted strain NKF112 (▲). Cells were incubated in YPD (uridine added) at 30 °C (left) or 37 °C (right), and growth (OD600) was determined at the indicated times. (b) Effects of the deletion of several calcium homeostasis-associated genes on calcium-related growth. DAY1 (●), NKF111 (▲), the cch1Δ/Δ mutant NKF101 (○), the pmrΔ/Δ mutant NKF102 (×) and the yvc1Δ/Δ mutant NKF103 (■) were grown in SC medium overnight. Cell suspensions (100 μl, 1×10⁶ cells ml⁻¹) in SC medium (control) or SC medium with different concentrations of EGTA were inoculated into microtitre plates at 37 °C. OD₅₉₅ was measured to evaluate growth of these strains after 24 h of incubation (left). Strains were incubated in SC medium with different concentrations of calcium chloride. OD₆₀₀ was measured after 6 h of incubation with shaking at 37 °C (right). The growth as a percentage of the control was calculated as (OD₅₉₅ in the presence of EGTA/OD₅₉₅ in the absence of EGTA)×100. (c) Serial 10-fold dilutions of DAY1, NKF111 or NKF112 cells were spotted onto YPD medium plates or YPD medium plates containing fluconazole (8 μg ml⁻¹), tunicamycin (2.5 μg ml⁻¹) or hygromycin B (50 μg ml⁻¹). Plates were incubated at 30 °C for 48–72 h. Error bars on (a) and (b) show sd.
is defective in maintaining calcium homeostasis under ER stress or high-level extracellular calcium treatments.

Deletion of SPF1 induces expression of the CDRE gene CCH1

Cch1 is an important plasma membrane protein that consists of the high affinity calcium influx system with Mid1, and plays an essential role in the cell calcium survival (CCS) pathway (Karababa et al., 2006). CCH1 is one of the CDRE genes known to be upregulated in response to increased cytoplasmic calcium concentration (Viladevall et al., 2004; Karababa et al., 2006). For further confirming the role of Spf1 in cellular calcium homeostasis, we investigated the effect of SPF1 deletion on the expression of CCH1, using the β-galactosidase reporter system, following treatment with different agents, as mentioned above.

Consistent with previous observations that ER stress agents stimulated calcium influx and induced expression of CDRE genes via calcineurin (Bonilla et al., 2002; Losev et al., 2008), the expression of CCH1 increased when wild-type cells (NKF116) were treated with EGTA or other ER stress agents, but decreased when treated with FK506 alone and in combination with fluconazole or tunicamycin. Interestingly,
the expression of this reporter was more than threefold higher in the spf1Δ/Δ mutant (NKF117) than in the wild-type strain in the absence of ER stress agents, which is similar to the effect of these agents (Fig. 4). Likewise, addition of FK506 diminished the effect of SPF1 deletion on β-galactosidase activity, indicating that the increased expression of CCH1 in the spf1Δ/Δ mutant was mainly dependent on calcineurin.

**The spf1Δ/Δ mutant is deficient in hyphal development**

To study the role of Spf1 in *C. albicans* hyphal development, we tested the ability of the spf1Δ/Δ mutant in response to inducers of filamentous growth. On solid serum-containing YPD, Spider and SLAD media, the wild-type strain DAY1 and the SPF1 reconstituted strain NKF112 showed filamentous colonies, while the spf1Δ/Δ mutant NKF111 and the control strain NKF113 with a null reconstitution plasmid failed to form filaments (Fig. 5a). In the liquid serum-containing YPD medium, NKF111 and NKF113 underwent hyphal transition in 3 h, but their hyphal cells were short and swollen compared with those of DAY1 or NKF112. After 6 h of hyphal induction, the mutant cells showed a pseudohyphal cell shape, while the wild-type and reconstituted strains produced elongated and regular hyphal cells (Fig. 5b). Furthermore, we tested the effect of SPF1 deletion on *C. albicans* filamentation under microaerophilic conditions. DAY1 and NKF112 produced filaments in 4 days when grown embedded in YPS agar. In contrast, the colonies of NKF111 and NKF113 remained smooth (Fig. 5c). These results demonstrated that Spf1 is essential for hyphal development under various hyphal-inducing conditions.

**The spf1Δ/Δ mutant is deficient in biofilm formation**

Within the host, *C. albicans* can attach to host tissues or medical devices and form biofilms, which contribute to its virulence and drug resistance. Here, we used 96-well polystyrene microtitre plates to investigate the role of Spf1 in biofilm formation. The spf1Δ/Δ mutant NKF111 was shown to be defective in biofilm formation after 24 h of incubation, and only a monolayer of yeast and pseudohyphal cells was seen attached to the polystyrene surface (Fig. 6a). In contrast, the wild-type strain DAY1 and the reconstituted strain NKF112 showed significant development of thick biofilms on the polystyrene surface with abundant hyphae and complex structure, and their biomass levels were approximately eightfold higher than the biomass level of NKF111 (Fig. 6b).

![Fig. 5. Effect of SPF1 deletion on hyphal development in *C. albicans*. (a) The wild-type strain DAY1, the spf1Δ/Δ mutant NKF111, the reconstituted strain NKF112 and the control strain NKF113 with the null plasmid pDDB78 were spotted on solid YPD containing 10 % (v/v) fetal bovine serum, solid SLAD medium and solid Spider medium, and incubated at 37 °C for the indicated time. (b) Strains were incubated in liquid YPD + 10% (v/v) serum at 37 °C for 3 or 6 h. (c) Cells were plated on molten YPS agar and incubated at 30 °C for 4 days.](image1)

![Fig. 6. Effect of SPF1 deletion on biofilm formation in *C. albicans*. The wild-type strain DAY1, the spf1Δ/Δ mutant NKF111 and the reconstituted strain NKF112 were incubated with SC medium in 96-well polystyrene microtitre plates at 37 °C for 24 h. Wells were washed to remove non-adherent cells and examined by using a light microscope (a). Crystal violet-stained wells were washed with acetic acid, and the A595 of the suspensions was measured to evaluate biofilm biomass (b). Control, no strain was inoculated. The mean ± SD of 12 repeats is shown.](image2)
The \( spf1/\Delta \) mutant is attenuated in virulence

The virulence of the \( spf1/\Delta \) mutant NKF111 was tested in a mouse model. All mice injected with the wild-type strain DAY1 or the \( SPF1 \) reconstituted strain NKF112 died after 30 days. In contrast, all mice injected with the \( spf1/\Delta \) mutant NKF111 survived until the end of the experiments (Fig. 7a). The virulence of the mutant was significantly lower than that of the wild-type and reconstituted strains (\( P<0.01 \)).

In view of the defect in virulence, we examined the ability of the \( spf1/\Delta \) mutant to grow invasively in mouse kidneys. While the wild-type strain showed extensive hyphal invasion into renal pelvis (Fig. 7b, i), the mutant exhibited little fungal masses in the same structure (Fig. 7b, ii), indicating that the mutant is defective for invasion. In the renal cortex, both strains caused infiltration of lymphocytes (Fig. 7b, iii, iv). This indicated that they were capable of eliciting an immune response in the host.

**DISCUSSION**

Calcium not only has a significant role in cellular signal transduction but also is essential for many functions of cell compartments, especially ER function. Therefore, it is of key importance to cells to maintain cellular calcium homeostasis. In \( C. \ albicans \), three calcium channels, plasma membrane Cch1-Mid1 complex, Fig1 and vacuolar Yvc1, and two P-type calcium ATPases (calcium pumps), vacuolar Pmc1 and Golgi Pmr1, were known to be associated with cellular calcium homeostasis (Bates et al., 2005; Bennett & Johnson, 2006; Brand et al., 2007; Sanglard et al., 2003; Yang et al., 2011). In this study, we identified another protein associated...
with the maintenance of calcium homeostasis, Spf1, which is the homologue of \textit{S. cerevisiae} ER-localized P-type calcium ATPase Spf1/Cod1. By creating a null mutant in \textit{SPF1}, we initially analysed the role of Spf1 on cell growth and cellular calcium homeostasis. In contrast with the wild-type strain, the \textit{spf1}Δ/Δ mutant demonstrated a low growth rate in YPD, indicating the important role of Spf1 in maintaining normal growth of \textit{C. albicans}. Furthermore, the mutant is hypersensitive to all tested drugs, including fluconazole, tunicamycin and hygromycin B. Fluconazole is one of the most commonly prescribed antifungal drugs that is widely used for treatment of fungal infections. This drug targets the \textit{tunicamycin} and \textit{hygromycin B}. Fluconazole is one of the hypersensitive to all tested drugs, including fluconazole, indicating the important role of Spf1 in maintaining normal growth of \textit{C. albicans}.

In this study, the \textit{spf1}Δ/Δ mutant showed most sensitivity to the calcium chelator EGTA and calcium among all tested mutants of calcium homeostasis-associated proteins, indicating an important role of Spf1 in calcium-related growth and maintenance of cellular calcium homeostasis. As mentioned above, cellular calcium homeostasis is controlled by a complicated system that is composed of pumps, channels and exchangers for calcium transport. This system regulates cellular calcium levels by calcium influx and release from calcium stores, such as vacuolar, ER and Golgi. Therefore, the defect in Spf1 function alone did not significantly affect cytoplasmic or total cellular calcium levels. However, in contrast with the wild-type strain, the \textit{spf1}Δ/Δ mutant exhibited a significant increase in cytoplasmic and total cellular calcium in the presence of ER stress agents, FK506 or high-level calcium. It is well known that ER stress agents stimulate calcium influx in the presence of FK506, an inhibitor relieving the inhibition of calcineurin on Cch1 activity (Bonilla \& Cunningham, 2003). The effect of \textit{SPF1} deletion on cellular calcium levels is similar to that of ER stress agents, suggesting that disruption of Spf1 also leads to ER stress and a consequent stimulation of calcium influx. Likewise, the mutant exhibited enhancement in \textit{CCH1} expression, which is attributed to the elevation of cytoplasmic calcium and activation of the CCS pathway. Taken together, our study demonstrated that Spf1 is essential for maintenance of cellular calcium homeostasis under ER stresses and high-level calcium conditions.

In \textit{C. albicans}, hyphal cells contain more chitin and β-1,6-glucan, and less mannoproteins than yeast cells, indicating a link between hyphal development and cell wall composition, which is dependent on cell wall protein homeostasis (Herrero \textit{et al.}, 2004; Umeyama \textit{et al.}, 2006). Our study showed that the \textit{spf1}Δ/Δ mutant had a significant defect in hyphal development. We propose that this defect is associated with malfunction of the ER and consequent defects in cell wall biosynthesis. In \textit{S. cerevisiae}, many functions of the secretory pathway, such as protein glycosylation, glycosylphosphatidylinositol (GPI)–anchor biosynthesis, quality control of secretory proteins and delivery of cell wall proteins, are essential for maintenance of cell wall protein homeostasis and for alteration of cell wall composition in response to diverse environmental conditions (Lesage \& Bussey, 2006; Scrimale \textit{et al.}, 2009). However, long-term ER calcium depletion results in uncompensated ER stress and consequent malfunction of the ER, which leads to defects in those functions associated with the secretory pathway and in regulation of cell wall composition. Similarly, deficiency of Spf1 results in malfunction of the ER and a disturbance of cell wall protein delivery (our unpublished data), which influences the alteration of cell wall composition and contributes to the defect of the \textit{spf1}Δ/Δ mutant in hyphal development.

The \textit{spf1}Δ/Δ mutant has defects in not only hyphal development but also biofilm formation, which may also be attributed to a disturbance in ER function. Biofilm formation begins with adherence of yeast cells to a substrate. Biofilm adhesins and several other cell wall proteins, such as GPI protein Eap1 (Li \& Palecek, 2008; Li \textit{et al.}, 2007) and Als family members Als1 and Als3 (Hoyer, 2001; Sheppard \textit{et al.}, 2004), play key roles in adherence. Mutants of these proteins reduced adherence to surfaces, and are defective in biofilm formation. Deletion of \textit{SPF1} results in malfunction of the ER and a defect in the secretory pathway. Consequently, cells are unable to sufficiently synthesize and transport functional cell wall proteins essential for cell adherence, and have defects in biofilm formation. In addition, the defect of the mutant in hyphal development is likely to be associated with defects in biofilm formation.

Deletion of \textit{SPF1} also leads to a significant reduction in virulence in the mouse model. In contrast with the wild-type, the \textit{spf1}Δ/Δ mutant produced little biomass and is defective for invasion in mouse kidneys. We propose that a series of defects resulting from deletion of \textit{SPF1}, such as those in vegetative growth, hyphal development, biofilm formation and cell wall integrity, contribute to the attenuated virulence. The same phenotype is observed in the \textit{pmr1}Δ/Δ mutant (Bates \textit{et al.}, 2005). These results underline the importance of the secretory pathway in \textit{C. albicans} virulence.

In conclusion, our study demonstrates that Spf1 is essential for many physiological processes in \textit{C. albicans}, such as cell growth, tolerance to antifungal drugs, the maintenance of cellular calcium homeostasis, hyphal development, biofilm formation and infection. Further studies of its biochemical characteristics may illuminate the mechanisms by which it functions in these processes and provide new evidence of the function of the ER in the virulence of \textit{C. albicans}. 
ACKNOWLEDGEMENTS

We thank Dana Davis (University of Minnesota, USA) for generously providing strains and plasmids. We are grateful to Yan Jiang, Xinqiao Xie, Min Yu and Xue Yang for calcium measurement, Professor Yuehao Li for histological assays, Tieliang Pang for colony photography. We also thank the reviewers for critical reading and helpful suggestions. This work was supported by the National Natural Science Foundation of China (grant nos 81171541 and 31070126) and Natural Science Foundation of Tianjin (10JCYBJC09700).

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Edited by: K. Kuchler