Introduction

*Candida albicans* (C. albicans), a major human fungal pathogen, causes disorders ranging from mild infections to life-threatening diseases[1, 2]. *C. albicans* is often treated with fluconazole, which is a fungistatic drug. However, there is a rapid acquisition of resistance to azoles in *C. albicans*. This is due to a limited number of multidrug resistance related genes, which include *ERG11*, *CDR1*, *CDR2*, and *CaMDR1*[3–6]. Recently, DNA microarray and proteomic analyses have revealed many differentially regulated genes in either laboratory[7, 8] or clinical[9–12]azole-resistant *C. albicans* isolates. However, few of these new observations have been confirmed by genetic studies because *C. albicans* is difficult to study experimentally due to its asexual diploid nature and variant genetic code.

The calcineurin pathway is involved in the development ofazole resistance in *C. albicans*[13–15]. Calcineurin is a Ca2+ calmodulin-dependent serine/threonine phosphatase consisting of a catalytic subunit A (encoded by *CNA1*) and a regulatory subunit B (encoded by *CNB1*)[16]. The phosphatase activity of calcineurin is activated when calcineurin binds calmodulin in the presence of calcium ions. Activated calcineurin regulates downstream gene expression via transcription factors such as Crz1p[17].

In our previous study, we found that RTA2, *CaIPF14030*, and *MXR1* were involved in the calcineurin pathway. These three genes were overexpressed in the experimentally inducedazole-resistant *C. albicans* strain DSF28[18]. The *RTA2* and *ipf14030* null mutants from *C. albicans* were constructed along with deletions of *CDR1*, *CDR2*, and *CaMDR1*[18]. The disruption of *RTA2* increased the susceptibility of *C. albicans* to azoles, whereas the disruption of *CaIPF14030* did not influence the sensitivity of *C. albicans* to azoles[18]. Bioinformatic analysis also revealed that the promoters of both the *RTA2* and the *CaIPF14030* genes contained the calcineurin-dependent responsive element (CDRE) sequence, which is controlled by either calcineurin or Crz1p[17, 18]. However, the function of *CaIPF14030* was unknown. In the present study, we con-
structured the ipf14030 null mutant from the C. albicans wild-type strain to investigate the role of CalPF14030 during the development of azole resistance in C. albicans.

Materials and methods

Antifungal agents

Fluconazole was from Pfizer Inc (New York, NY, USA). Ketoconazole and econazole were purchased from Sigma (St Louis, MO, USA). Itraconazole was a gift from Prof Chao-mei LIU, and voriconazole was a gift from Prof Wan-nian ZHANG from the Second Military Medical University, Shanghai, China.

Candida albicans strains and culture media

Candida albicans strains used in this study are listed in Table 1; they were cultured in YPD medium or SC medium lacking uridine, methionine and cysteine.

RNA isolation and quantitative RT-PCR

All primer sequences are listed in Table 2. RNA isolation, cDNA synthesis, and PCR amplification were performed as described previously [18]. Triplicate independent quantitative RT-PCRs were performed using the LightCycler System (Roche Diagnostics, Mannheim, Germany). Gene expression levels relative to the calibrator were expressed as 2^-ΔΔCT^-.

Construction of mutant strains

All primer sequences are listed in Table 2. Construction of plasmid pUC-IPF14030-URA3 was performed as described previously [18]. Briefly, the fragment containing the 5’ and 3’ ends of the CaIPF14030 gene for homologous recombination was obtained according to the Fusion PCR method. The 4-kb hisG-URA3-hisG fragment was from the p5921 plasmid [19]. The two fragments were subsequently cloned into plasmid pUCm-t (Sangon, Shanghai, China). The XhoI-digested fragment of pUC-IPF14030-URA3 was transformed into the ura3 mutant strain (RM1000) by standard methods [20]. The hybridization probe primers are listed in Table 2. Southern blot analysis was used to confirm the absence of the CaIPF14030 gene.

Overexpression of CalPF14030 in ipf14030 mutants

The CalPF14030 ORF was amplified by PCR with Pyrobest polymerase (TaKaRa, Dalian, China). The BamHI/PstI-digested PCR fragment was ligated into pCaExp [21] to obtain recombinant plasmid pCaExp IPF14030.

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

| Strain | Parental strain | Genotype | Reference |
|--------|-----------------|----------|-----------|
| CAF2-1 | SC5314          | ura3::imm434/URA3 | [19] |
| DSY2091 | CAF4-2          | cnad::hisG/cnad::hisG::URA3::hisG | [17] |
| DSY2115 | DSY2101         | cnad::hisG/cnad::hisG; LEU2::CNA::URA3 | [13] |
| DSY2195 | DSY2188         | crz1::hisG/crz1::hisG::URA3::hisG | [17] |
| MKY268  | MKY59           | crz1::hisG/crz1::hisG LEU2::CRZ1::URA3 | [17] |
| DSY1024 | CAF4-2          | cdr1::hisG/cdr1::hisGcdr2::hisG/cdr2::hisG camdr1::hisG/camdr1::hisGflu1::hisG/flu1::hisG::URA3-hisG | [32] |
| RM1000  | RM1000          | ura3::imm434/ura3::imm434, his1::HisG/his1::HisG, iro1::imm434/iro1::imm434 | [33] |
| MZP10   | RM1000          | RM1000* ipf14030::hisG/URA3::hisG/URA3::hisG | This study |
| MZP11   | MZP10           | RM1000* ipf14030::hisG/URA3::hisG | This study |
| MZP100  | MZP100          | RM1000* ipf14030::hisG/ipf14030::hisG | This study |
| MZP101  | MZP101          | RM1000* ipf14030::hisG/ipf14030::hisG | This study |
| Exp-14030 | MZP101     | RM1000* ipf14030::hisG/ipf14030::hisG RP10/rp10::pExpIPF14030::URA3 | This study |
| Exp-MZP | MZP101          | ipf14030::hisG/ipf14030::hisG RP10/rp10::pCaExp-URA3 | This study |
| Exp-RM  | RM1000          | RM1000* RP10/rp10::pCaExp-URA3 | This study |

* RM1000 background.
binant plasmid pEXP-IPF14030. DNA sequencing confirmed that the sequence of the insert was identical to the CaIPF14030 sequence reported in the Candida Genome Database (http://www.candidagenome.org/). The ipf14030 mutant (MZP101) was transformed with the Str I-digested and linearized pEXP-IPF14030 plasmid and selected on SC medium lacking uridine, methionine and cysteine.

Susceptibility testing
The sensitivities of the mutant strains to azoles were determined by testing the strains on agar plates containing different concentrations of azoles (fluconazole, ketoconazole, econazole, itraconazole, and voriconazole). Five microliters of ten-fold serial dilutions of each yeast culture (OD<sub>600</sub>=1.0) were spotted on plates of the appropriate medium and then incubated at 30 °C for the indicated time.

Measurement of intracellular ATP levels
C albicans strains were diluted to 1×10<sup>6</sup> or 1×10<sup>7</sup> cells/mL in YPD broth. A total of 100 μL of cell suspension was mixed completely with the same volume of BacTiter-Glo reagent (Promega Corporation, Madison, WI, USA) and incubated for 10 min at room temperature. Luminescent signals were measured with a TD 20/20 luminometer (Turner Biosystem, Sunnyvale, CA, USA) with a 1 s integration time per sample. A control tube without cells was used to obtain a value for background luminescence. The signal-to-noise ratio (S/N) was calculated as follows: [mean of signal - mean of background]/standard deviation of background. A standard curve for ATP increments from 10 pmol/L to 1 μmol/L was constructed. Signals represented the mean of three separate experiments. The ATP content was calculated from the standard curve.

Statistical analysis
Experiments were performed at least three times. Data are presented as mean±standard deviations, and data were analyzed using Student’s t test where indicated.

Results

CalIPF14030 up-regulation by Ca<sup>2+</sup> in a calcineurin-dependent manner
Bioinformatic analysis revealed that the CalPF14030 promoter contains the CDRE sequence, which can be controlled by either calcineurin or Crz1<sup>p</sup> [17]. In our study, expression levels of CalPF14030 were examined by quantitative RT-PCR in wild-type (CAF2-1), cnaΔ/Δ mutant (DSY2091), crz1Δ/Δ mutant (DSY2195), CNA revertant (DSY2115) and CRZ1 revertant (MKY268) strains after exposure to 200 mmol/L CaCl<sub>2</sub> for 2 h (Figure 1A). CalPF14030 was up-regulated by 6.51 fold in the CAF2-1 strain (Figure 1A). Depletion of CNA or CRZ1 inhibited the effects of CaCl<sub>2</sub> on CalPF14030 up-regulation by 1.53 fold in the DSY2091 strain and by 0.96 fold in the DSY2195 strain (Figure 1A). However, reintroduction of CNA or CRZ1 in the respective null mutant strains restored the effect of 200 mmol/L CaCl<sub>2</sub> on CalPF14030 up-regulation. There was a 3.62-fold change in the DSY2115 strain and a 3.19-fold change in the MKY268 strain (Figure 1A). Taken together, these data demonstrate that CalPF14030 is up-regulated by Ca<sup>2+</sup> in a calcineurin-dependent manner.

CalPF14030 is overexpressed during the stepwise acquisition of azole resistance in DSY1024
It has been well documented that calcium can activate the calcineurin pathway and modulate azole activity in C albicans [22, 23]. Because the CalPF14030 gene of C albicans is up-regulated by Ca<sup>2+</sup> in a calcineurin-dependent manner, we investigated the dynamic expression of CalPF14030 in the DSF7, DSF14, DSF21, and DSF28 strains obtained in our previous study [18]. As shown in Figure 1B, the CalPF14030 gene was up-regulated in the DSF7, DSF14, DSF21, and DSF28 strains obtained in our previous study [18]. As shown in Figure 1B, the CalPF14030 gene was overexpressed in the DSF7, DSF14, DSF21, and DSF28 strains. These results are similar to those found for other known resistance-related genes such as ERG11, CDR1, and CmMDR1 [4, 24]. These data suggest that CalPF14030 is involved in the development of azole resistance in C albicans.

Disruption and ectopic overexpression of the CalPF14030 gene in C albicans
To investigate the role of CalPF14030 during the development
of azole resistance in \textit{C. albicans}, two alleles of \textit{CalPF14030} were sequentially disrupted in the RM1000 strain using the URA-blast method and 5-FOA selection. This yielded the following strains: Ura\textsuperscript{+} \textit{ipf14030/IPF14030} (MZP10), Ura\textsuperscript{+} \textit{ipf14030/ IPF14030} (MZP11), Ura\textsuperscript{+} \textit{ipf14030/ipf14030} (MZP100), and Ura\textsuperscript{+} \textit{ipf14030/ipf14030} (MZP101) (Table 1). The strategy to disrupt both copies of \textit{CalPF14030} is depicted in Figure 2A and 2B.

Southern blot analysis of DNA from the constructed strains revealed that the recombination patterns were as expected (Figure 2C). To further characterize the generated mutants, RM1000 and MZP101 growth curves were obtained. They showed identical growth rates.

To investigate whether other deletions were introduced into the \textit{C. albicans} genome, the \textit{CalPF14030} ORF was placed under

Figure 2. Schematic representation of the plasmid construction and disruption of \textit{CalPF14030} using the \textit{URA3} selection marker. (A) Diagrammatic representation of the plasmid construction of pUC-\textit{IPF14030}-\textit{URA3} containing \textit{hisG-URA3-hisG} with flanking portions on either side for homologous recombination. Ba: \textit{BamH} I, X: \textit{Xho} I, N: \textit{Nde} I. (B) Sequential targeted disruption of the two \textit{CalPF14030} alleles in \textit{C. albicans} with the disruption cassette. Strain designation is shown on the right. (C) Southern blot analysis of the genomic DNA digested with \textit{Kpn} I and \textit{Sac} I. The exact size and genotype of the expected hybridizing DNA fragment are indicated on the right. The hybridization probe was a 0.528-kb PCR fragment from -432 to 96 of the \textit{CalPF14030} gene amplified by Pyrobest polymerase (TaKaRa, Dalian, China) from genomic DNA extracted from RM1000.
the control of the MET3 promoter in the pCaEXP vector, and it was successfully integrated into the ipf14030Δ/Δ mutant (MZP101) at the RP10 locus as determined by PCR (data not shown). Ectopic overexpression of CalPF14030 in the Exp-14030 strain was confirmed by quantitative RT-PCR with CalPF14030 expression in the parental strain (RM1000) carrying empty vector pCaEXP, which served as a control (Figure 1C). The growth rates of the RM1000 and Exp-14030 strains were similar, suggesting that there were no other gene disruptions that affected the growth of the strains.

**Disruption or ectopic overexpression of CalPF14030 does not affect the sensitivity of C albicans to azoles**

Because the CalPF14030 gene was overexpressed during the stepwise acquisition ofazole resistance in DSY1024, a drug susceptibility assay was performed to determine whether CalPF14030 is associated with enhanced azole resistance in C albicans. Spot assay results demonstrated that neither disruption nor ectopic overexpression of CalPF14030 affected the sensitivity of C albicans to the azoles, including fluconazole, ketoconazole, econazole, itraconazole, and voriconazole (Figure 3A, 3B). Taken together, these data support the hypothesis that CalPF14030 does not play an important role in the development of azole resistance in C albicans.

**CalPF14030 negatively controls the intracellular ATP content of C albicans**

It has been well documented that lower intracellular ATP levels are found in C albicans strains that are azole resistant or have been treated by azoles[25, 26]. Because overexpression of CalPF14030 occurred during the development of azole resistance in C albicans, intracellular ATP concentrations were measured in the following strains: wild-type (RM1000), ipf14030Δ/Δ mutant (DSY2091), cnaΔ/Δ mutant (DSY2195), CNA revertant (DSY2115) and CRZ1 revertant (MKY268) strains after exposure to 200 mmol/L CaCl₂. As shown in Figure 4, the level of intracellular ATP in the MZP101 strain was significantly higher than that in the RM1000 strain (P<0.01), whereas the level of intracellular ATP in the Exp-14030 strain was significantly lower than that in the RM1000 strain (P<0.01). These results suggest that CalPF14030 plays a critical role in the generation of intracellular ATP during the development of azole resistance in C albicans.

**Discussion**

Bioinformatic analysis have revealed that the CalPF14030 promoter contains a CDRE sequence, which can be controlled by either calcineurin or Crz1p[17]. The expression levels of CalPF14030 were examined by quantitative RT-PCR in the wild-type (CAF2-1), cnaΔ/Δ mutant (DSY2091), crz1Δ/Δ mutant (DSY2195), CNA revertant (DSY2115) and CRZ1 revertant (MKY268) strains after exposure to 200 mmol/L CaCl₂.

**Figure 3.** Drug susceptibility profiles of C albicans strains (Table 1) determined by spot assays. (A) The ipf14030 heterozygous and homozygous mutant strains from RM1000 were spotted on YPD agar plates supplemented with 0.0025% uridine with or without different antifungal agents at the indicated concentrations. Plates were incubated for 48 h at 30°C. (B) The ipf14030 mutant strains (MZP101) carrying pCaEXP or pCaEXP-IPF14030 and the parental strain (RM1000) carrying pCaEXP were spotted on SC medium plates lacking methionine, cysteine, and uridine with or without drugs. Plates were incubated for 72 h at 30°C.

**Figure 4.** Intracellular ATP content in the wild-type (RM1000), RM1000 carrying pCaEXP (Exp-RM), ipf14030 mutant (MZP101) and MZP101 carrying pCaEXP-IPF14030 (Exp-14030) strains. ATP levels represent the mean±standard deviation for three independent experiments. aP<0.05 when compared to the control strains (RM1000 or Exp-RM); bP<0.01 when compared to the control strains (RM1000 or Exp-RM).
CalPF14030 was up-regulated by Ca\textsuperscript{2+} in a calcineurin-dependent manner. It is well documented that calcium can activate the calcineurin pathway and modulate azole activity in C. albicans\textsuperscript{[22, 23]}. In addition, the dynamic expression of CalPF14030 was investigated in the DSF7, DSF14, DSF21, and DSF28 strains obtained in our previous study\textsuperscript{[18]}. The CalPF14030 gene shows a steady overexpression state in the DSF7, DSF14, DSF21, and DSF28 strains. These results are similar to those involving other known resistance-related genes such as ERG11, CDR1, and CaMDR1\textsuperscript{[6, 24]}. Taken together, these data suggest that CalPF14030 is involved in the development of azole resistance in C. albicans.

Both copies of CalPF14030 were successfully disrupted, and the role of CalPF14030 during the development of azole resistance was investigated. A drug susceptibility assay demonstrated that the disruption or ectopic overexpression of CalPF14030 did not affect the sensitivity of the tested C. albicans strains to azoles. Therefore, these data support the hypothesis that CalPF14030 does not play an important role in the development of azole resistance in C. albicans. Recent studies have shown that lower intracellular ATP levels were observed in C. albicans strains that were azole-resistant or had been treated with azoles\textsuperscript{[25, 26]}. Our data showed that disruption of CalPF14030 significantly increased intracellular ATP content, whereas ectopic overexpression of CalPF14030 significantly decreased intracellular ATP content. Therefore, CalPF14030 may play a critical role in regulating intracellular ATP levels during the development of azole resistance in C. albicans. Further analysis is required to elucidate the precise relationship between the generation of intracellular ATP that is negatively controlled by CalPF14030 and the lower intracellular ATP levels in C. albicans strains that are azole resistant or have been treated with azoles.

It has been previously reported that there was a strong correlation between azole susceptibility and intracellular ATP levels in C. albicans\textsuperscript{[27]}. It has also been documented that several peptides have synergistic activity against C. albicans when combined with fluconazole due to inhibition of production and release of intracellular ATP\textsuperscript{[28–30]}. Because CalPF14030 plays a critical role in regulating intracellular ATP levels, the protein encoded by CalPF14030 may be targeted by the inhibitors of intracellular ATP. Further studies must be performed to unravel the function of CalPF14030 in C. albicans.

A BLAST search of the C. albicans genome (available at http://www.genolist.pasteur.fr/CandidaDB/) revealed that the CalPF14030 gene (also known as orf19.851) encoded a putative polypeptide of 839 amino acids with a calculated molecular mass of 97.2 kDa. The SOSUI\textsuperscript{[31]} program predicted that the protein encoded by CalPF14030 was an integral membrane protein with one membrane-spanning segment. There are two CalPF14030 homologs (Mnn4p and YJR061Wp) in the S. cerevisiae genome database (available at http://www.yeastgenome.org/) that have 30%–37% identity and 44%–55% similarity with the C. albicans protein. Mnn4p has been shown to be a positive regulator of mannansphosphate transferase, and it is involved in mannosylphosphorylation of N-linked oligosaccharides. Expression of MNN4 increases in late-logarithmic and stationary growth phases\textsuperscript{[32–33]}. It has been shown that YJR061W is a putative membrane protein involved in glycosylation, and it has been shown to be repressed by Rim101p\textsuperscript{[34, 35]}. Further studies are needed to verify whether CalPF14030 is involved in mannosylphosphorylation or glycosylation.

Our data show that CalPF14030 is up-regulated by Ca\textsuperscript{2+} in a calcineurin-dependent manner as well as during the stepwise acquisition ofazole resistance in C. albicans. Disruption or ectopic overexpression of CalPF14030 did not affect the sensitivity of C. albicans to azoles, although disruption of CalPF14030 significantly increased intracellular ATP content, and ectopic overexpression significantly decreased intracellular ATP content. In conclusion, our findings suggest that CalPF14030 plays a critical role in the generation of intracellular ATP during the development of azole resistance in C. albicans.

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Author contribution

Xin-ming JIA and Jun GU designed the experiments; Xin-ming JIA performed the experiments and wrote the paper; and Jun-dong ZHANG, Ying WANG, Yuan-ying JIANG, and Hong-yue TAN critically read the manuscript.

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