Novel insight into the expression and function of the multicopper oxidases in *Candida albicans*

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Iron is an essential element required for most organisms. The high-affinity iron-uptake systems in the opportunistic pathogen *Candida albicans* are activated under iron-limited conditions and are also required for virulence. Here one component of high-affinity iron-uptake systems, the multicopper oxidase (MCO) genes, was characterized. We examined the expression of five MCO genes and demonstrated that *CaFET3* and *CaFET34* were the major MCO genes in response to iron deficiency. Complementation of the *Saccharomyces cerevisiae fet3* mutant showed that *CaFET34* could effectively rescue the growth phenotype in iron-limited medium. Deletion of *CaFET33* and *CaFET34* in *C. albicans* decreased cellular iron content and iron acquisition during iron starvation. However, the *fet33ΔΔ* and *fet34ΔΔ* mutants exhibited no obvious growth defect in solid iron-limited medium while the *fet34ΔΔ* mutant showed a slight growth defect in liquid medium. Further analysis shows that other members of the five MCO genes, especially *CaFET34*, would compensate for the absence of *CaFET33* and *CaFET34*. Furthermore, for the first time, we provide evidence that *CaFET34* is implicated in hyphal development in an iron-independent manner and is required for *C. albicans* virulence in a mouse model of systemic infection. Together, our results not only expand our understanding about the expression of the MCO genes in *C. albicans*, but also provide a novel insight into the role of *CaFET34* in iron metabolism, hyphal development and virulence.

INTRODUCTION

Iron is an essential nutrient for virtually all living organisms, including the opportunistic fungal pathogen *Candida albicans*. The redox potential of iron facilitates it as a pivotal cofactor for a variety of proteins, which are responsible for cellular respiration, electron transport, DNA synthesis and repair, oxygen transport, and many central metabolic pathways (Drakesmith & Prentice, 2008). However, hosts have evolved an elaborate iron-withholding defence system to saturate and limit the availability of iron, within which iron is mostly bound to storage proteins such as haemoglobin, transferrin and lactoferrin (Almeida et al., 2009; Ratledge & Dover, 2000). Therefore, iron availability of the host plays a crucial role in host–pathogen interactions. As a corollary, pathogens must have effective and specialized iron-uptake systems to survive in the harsh iron-limited host environment.

Most organisms have developed sophisticated strategies to extract iron from different environments (Kaplan & Kaplan, 2009; Kosman, 2003; Sutak et al., 2008). The high-affinity iron-uptake systems, as the major pathway for iron acquisition in response to iron limitation, have been extensively studied in *Saccharomyces cerevisiae* (Philpott & Prochenko, 2008). *S. cerevisiae* employs two distinct mechanisms to cope with iron-deficient conditions: a non-reductive uptake system mediated by the Arn/Sit siderophore transporters and a reductive uptake system. The reductive process entails at least two distinct steps. Firstly, cell-surface ferric reductases of the Fre family reduce insoluble ferric ion (Fe³⁺) into soluble ferrous ion (Fe²⁺). The reduced iron is then sequestrated by a high-affinity transport complex composed of a multicopper ferroxidase (Fet3) and a permease (Ftr1). In addition, copper availability and iron uptake are closely related (Dancis et al., 1994).

### Abbreviations

- BCS, bathocuproinedisulfonic acid disodium salt
- BPS, bathophenanthroline disulfonate
- 5-FOA, 5-fluoroacoric acid
- MCO, multicopper oxidase
Copper, as an essential cofactor, is required for the oxidase activity of multicopper ferroxidase (MCO). It has been demonstrated that copper-dependent oxidase activity is indispensable for high-affinity iron uptake in *S. cerevisiae* (Askwith et al., 1994). *C. albicans* has also evolved the reductive iron-uptake system to scavenge iron from the host environment. Elements of the reductive iron-uptake system resemble those of *S. cerevisiae*, but are distinguished by the existence of multiple genes for each component (Almeida et al., 2009). There have been 17 putative ferric reductase genes identified in the *C. albicans* genome. CaFRE10 (orf19.1415/CFL95) was the major gene whose deletion caused a 75% decrease in cell-surface ferric reductase activity and a 77% decrease in the uptake of ferric iron (Knight et al., 2002, 2005). For the iron permease, CaFTR1, not CaFTR2, is essential for high-affinity iron uptake and required for the virulence in the mouse model of systemic infection (Stearman et al., 1996). In addition, five MCO homologues are present in the genome of *C. albicans* FET3 (orf19.4211), FET31 (orf19.4213), FET33 (orf19.943), FET34 (orf19.4215) and FET99 (orf19.4212). The transcriptional levels of some of these putative MCO genes have been shown to respond to a wide range of environmental changes, including ketoconazole, caspofungin, amphotericin B and hypoxia (Liu et al., 2005; Rogers & Barker, 2002; Synnott et al., 2010). A previous study demonstrated that CaFET3 (actually CaFET31) could partially restore the growth defect of a Scfet3A mutant in iron-limited medium, but had no effect on *C. albicans* virulence during mouse systemic infection (Eck et al., 1999). In addition, none of the candidate genes was found to complement the ScFET3 deletion by screening a *C. albicans* genomic library. Even the CaFET99, the MCO gene with the highest homology to ScFet3 (60% identity, 74% similarity), also failed to rescue the iron-deficient phenotype with the highest homology to ScFet3 (60% identity, 74% similarity). 

**METHODS**

**Strains and growth conditions.** All *C. albicans* strains used in this study are listed in Table 1. BW291 was used as the wild-type strain in the functional analysis; it was also used as the parental strain for the gene disruption. Cells were routinely cultivated in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with 80 µg ml⁻¹ uridine or synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acid, 2% glucose, 0.2% complete supplement mixture) lacking specific amino acid for selection of transformants. SC supplemented with 0.1% 5-fluoroorotic acid (5-FOA) and 80 µg ml⁻¹ uridine was used to counter-select for URA3, M199 medium (Gibco) containing 150 mM HEPEs was used to determine the growth curve. The iron chelator bathophenanthroline disulfonate (BPS) and the copper chelator bathocuproinedisulfonate (BCS) were added as stated to limit the availability of iron and copper, respectively. Liquid 'iron-replete' medium was YPD (Chen et al., 2011); 'high iron' was YPD with 200 µM ferric chloride (Fe³⁺); and 'low-iron' or 'iron-deficient' medium was YPD plus 500 µM BPS. Solid 'iron-replete' medium was YPD/2% agar, while 'low iron' or 'iron-deficient' was YPD/2% agar with 200 µM BPS. For copper, liquid YPD with 400 µM CuSO₄ was defined as 'high copper', and YPD with 200 µM BCS was defined as 'low copper'.

**Plasmid and strain construction.** The primers used in this study are listed in Table 2. All constructs were confirmed by PCR and DNA sequencing. The fet33/Δ mutant (NKX1) was constructed as follows. BW291 was consecutively transformed with the fet33/Δ::ARG4 and fet33/Δ::Ura3::dp200 cassettes, which were amplified by PCR using the primer pairs FET33-5DR and FET33-3DR from the pRS416·fet33/Δ::URA3-dpl200 plasmid. Then, a 2.6 kb fragment amplified from the BWP17 genome with the FET33-5com and FET33-3com primers was digested with *BglII* and *PvuII* and cloned into the yeast plasmid pRS416 containing a flanking URA3 selectable marker. The resulting plasmid was transformed into BW291, yielding NKX3. Moreover, deletion of CaFET3 and CaFET34 were the main MCO genes in response to iron starvation. The CaFET34 under the control of the strong *PGK1* promoter could effectively complement the fet33/Δ deletion in *S. cerevisiae*. Moreover, deletion of CaFET3 CaFET34 decreased cellular iron content in iron limitation, while fet33/Δ/Δ and fet34/Δ/Δ exhibited no obvious growth defect in iron-limited medium. Further research demonstrated that other members of the five MCO genes would compromise for the absence of CaFET33 and CaFET34. Finally, deletion of CaFET34, but not CaFET33, significantly attenuated the filamentous growth and virulence in the mouse model of systemic candidiasis.

**RNA isolation and quantitative real-time PCR.** For RNA preparation, cells were grown under appropriate conditions to an
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype and description</th>
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<tbody>
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<td><strong>C. albicans</strong></td>
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<td>BWP17</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</td>
<td>Wilson et al. (1999)</td>
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<td>BWP17 +</td>
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<td>This study</td>
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<td>NKX2</td>
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<td>This study</td>
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<td><strong>S. cerevisiae</strong></td>
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<td>DY150</td>
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<td>Askwith et al. (1994)</td>
</tr>
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<td>Askwith et al. (1994)</td>
</tr>
<tr>
<td>Scfet3A + FET33</td>
<td>MATα ade2-1 ura3-52 leu2-3,112 rpl1-1 can1-100(oc) fet3::HIS3, YEplac195-P&lt;sub&gt;PCK1&lt;/sub&gt;-CaFET33</td>
<td>This study</td>
</tr>
<tr>
<td>Scfet3A + FET34</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pRS-ARG4ASpel</td>
<td>Containing ARG4 marker, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Wilson et al. (1999)</td>
</tr>
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<td>pDBBS7</td>
<td>Containing URA3 marker, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Wilson et al. (1999)</td>
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<td>pBES116</td>
<td>ADE2-URA3-ADE2, AscI fragment in pBluescript II KS (+)</td>
<td>G. Fink</td>
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<td>pBES116-FET34</td>
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<td>This study</td>
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<td>pLUBP</td>
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<td>pFA-HA-URA3</td>
<td>Containing a 1.8 kb HA-URA3 cassette</td>
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OD600 of 0.6–0.8. Then, cultures were harvested by centrifugation and the cell pellet was frozen in liquid nitrogen. Total RNA was extracted by the phenol/chloroform method as previously described (Baek et al., 2008). The overall quality of the RNA was analysed by agarose gel electrophoresis. Quantitative real-time PCR was carried out with the Mastercycler ep realplex system. The primers used are listed in Table 2. The SYBR Green qPCR SuperMix (TransGen Biotech) was used for the real-time PCR analysis. All samples were taken in triplicate independent experiments. The EFBI transcripts were used as an internal control for RNA input and quality after reverse transcription (Schaller et al., 1998), and the ACT1 transcripts were used as an endogenous control for the quantitative PCR. Expression was calculated by the ΔΔCT method (2<sup>−ΔΔCΤ</sup>) as described previously (Livak & Schmittgen, 2001).

**Protein extraction and immunoblotting.** Strains producing HA-tagged Fet proteins were incubated under appropriate conditions to an optical density of 0.6–0.8. Protein extracts were prepared by breakage with glass beads according to the previously described method (Petrovská & Kumamoto, 2012). Lysates were analysed by SDS-PAGE and immunoblotted with mouse anti-HA high-affinity antibody (Sigma) for HA-tagged proteins. Anti-alpha tubulin antibody (Novus Biologicals) was used as a loading control.

**Intracellular iron content assay.** Intracellular iron levels were quantified by the atomic absorption spectroscopy and BPS-based colorimetric method as described previously (Hsu et al., 2011; Tamarat et al., 2006; Xu et al., 2012). Overnight cultures were pre-grown in YPD or YPD + 500 μM BPS medium at 30 °C with
shaking for at least 24 h, respectively. These steady-state cells were harvested, and resuspended in fresh YPD medium at an optical density of 2.0. Iron-uptake activities were evaluated by measuring the increase in intracellular iron levels after 60 min of iron uptake in fresh YPD medium. The increase in cellular iron content of the mutants was expressed as the percentage relative to control wild-type cells.

**Filamentous growth assays.** Spider, RPMI 1640, SD-GlcNAc and YPD + 10% serum plates were used for filamentous growth analysis. SD-GlcNAc medium was prepared as previously described (Du et al., 2012). K$_2$HPO$_4$ (2.5 g l$^{-1}$) was added for pH maintenance. For the phenotypic experiments of the colony morphology, cells from each strain were diluted in water to an OD$_{600}$ of 0.1. The colonies were imaged after 5–6 days of incubation at 30 and 37$\, ^\circ$C.

### Table 2. List of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')</th>
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<td>FET33-5DR</td>
<td>TCTTCTTCTGTGTTGTTATGTTGTTTTTTTTCCTGCTCCCTTCTGACATATACATTTCCAGTACGAGTGT</td>
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<td>FET33-3DR</td>
<td>ATATGTTAGCTTGATTTGTTTTCTTCTTTCTTTCCCTTTATTGAAACTACACCATGTTGTGGGGGATTGA</td>
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<td>FET33-5detect</td>
<td>GGGGTACCCCGAGAGTCACTACCTAGCAAC</td>
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<td>FET33-5RT</td>
<td>GGTAGACCAAGAGACATCAAGG</td>
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<tr>
<td>FET33-3RT</td>
<td>CCGGTGTCGGTTGTTGGGTATG</td>
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<tr>
<td>FET33-3RT</td>
<td>CCGGTGTCGGTTGTTGGGTATG</td>
</tr>
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</table>

Restriction sites are underlined.
Systemic infection of mice and virulence assay. The relevant C. albicans strains were cultured overnight in SC liquid medium at 30°C. Then cells were harvested, washed, counted and resuspended to a density of 5 x 10^6 cells ml^{-1} in 0.9% NaCl solution. Aliquots (100 μl) of each suspension were inoculated into lateral tail veins of 6–7-week-old ICR female mice (ten mice per group). The survival of mice was monitored daily for 20 days after inoculation. Kidneys were excised on day 5, fixed in 10% phosphate-buffered formalin and then stained with haematoxylin–eosin for histological examination.

RESULTS

Iron and copper availability affect the expression of C. albicans MCO genes

In S. cerevisiae, iron availability modulated the expression of FET genes (ScFET3, ScFET5 and ScFET4) (Askwith et al., 1994; Dix et al., 1994; Spizzo et al., 1997). To determine whether the expression of the five MCO genes in C. albicans was affected by iron availability, we performed quantitative real-time PCR to assay their mRNA levels in both iron-deficient and iron-replete conditions. Our results suggested that iron deficiency dramatically increased the expression of CaFET3 and CaFET34 (Fig. 1). The transcripts of CaFET3 displayed 25-fold higher expression under iron-deficient conditions in comparison with iron-replete conditions, and mRNA levels of CaFET34 were slightly higher-expressed, by 29-fold. However, there was no difference or little difference (<2-fold change) in mRNA abundance for the other putative MCO genes, during either iron repletion or deficiency (Fig. 1). Thus, these results suggested that the expression of CaFET3 and CaFET34 was induced in iron deficiency and likely involved in high-affinity iron uptake in C. albicans.

Copper is a necessary cofactor for the iron transporter component Fet3 in S. cerevisiae. Copper deficiency or mutation of genes involved in delivery of copper to Fet3 resulted in profound decrease in ferrous iron uptake (Askwith et al., 1994). Thus, we further examined the effect of copper availability on the expression of MCO genes in C. albicans. The transcription of MCO genes remained unchanged during copper loading (Fig. 1). Copper deprivation mildly upregulated the expression of CaFET3 and CaFET34, by 5-fold and 3.6-fold respectively, while the expression of the other three MCO genes changed little (Fig. 1). In addition, we also evaluated the expression of MCO genes when cells were exposed to iron-deficient and copper-deficient conditions simultaneously (Fig. 1). Iron deprivation, along with copper deprivation, markedly increased the mRNA levels of CaFET3, CaFET34 and CaFET99, implying a significant role of iron and copper in the regulation of MCO genes.

CaFET34 plays an important role in iron metabolism

S. cerevisiae FET3 is required for high-affinity iron accumulation and is essential for growth under iron-limited conditions. To investigate the role of C. albicans MCO genes in iron metabolism, plasmids containing C. albicans FET33 and FET34 under the control of the S. cerevisiae strong PGK1 promoter were constructed and transformed into the Scfet3A mutant. The results showed that CaFET34 under
the PGK1 promoter could efficiently rescue the iron-related phenotypes of S. cerevisiae, revealing a functional role of CaFET34 in iron acquisition (Fig. 2a). However, CaFET33 failed to complement the iron-deficient phenotype of the ScFET3 mutant. To further explore the role of the two genes in C. albicans, we constructed deletion mutants for CaFET33 and CaFET34. Then, the cellular whole iron content of the mutants was quantified. There was no significant difference in whole iron content between fet33Δ/Δ, fet34Δ/Δ and wild-type strains in YPD medium (Fig. 2b). Nevertheless, the fet33Δ/Δ and fet34Δ/Δ mutants exhibited decreased cellular iron accumulation under iron-deficient conditions. Especially, the fet34Δ/Δ mutant showed a 45% reduction (Fig. 2b). To understand the function of CaFET33 and CaFET34 in iron metabolism, the 60 min iron-uptake assay was performed (Fig. 2c). When compared with wild-type cells, deletion of CaFET33 or CaFET34 resulted in a decrease in iron-uptake activity under both no starvation and pre-iron starvation conditions. In addition, the fet34Δ/Δ mutant showed a more significant reduction in iron acquisition, especially under iron-deficient conditions. These results showed that deletion of CaFET33 and CaFET34 decreases cellular iron content and iron-uptake activity during iron starvation, indicating a potential role of CaFET33 and CaFET34 in iron acquisition.

**Impact of CaFET33 and CaFET34 deletion in C. albicans**

To further elucidate the role of CaFET33 and CaFET34 in iron homeostasis, we then evaluated the growth of the mutants in iron-limited medium. No obvious growth defect was observed for both the fet33Δ/Δ and fet34Δ/Δ mutants in solid medium, while the fet34Δ/Δ mutants showed a slight growth defect in liquid medium (Fig. 3a). In view of the fact that C. albicans harbours five MCO homologues, we speculated that there may exist a functional redundancy and/or compensatory upregulation mechanism among these genes under iron-deficient conditions. Firstly, we examined the expression levels of the other four MCO genes in the fet33Δ/Δ and fet34Δ/Δ mutants. As expected, CaFET33 and CaFET34 were upregulated 13-fold and 16-fold, respectively, under the iron-deficient condition when CaFET33 was absent (Fig. 3b). Similarly, the deletion of CaFET34 resulted in a remarkable increase in CaFET3 and CaFET99 mRNA levels. The protein levels of these three MCO genes were further analysed by Western blotting (Fig. 3c). Interestingly, Fet3-HA migrates as two bands after prolonged electrophoresis, with an apparent molecular mass greater than its theoretical 74 kDa mass, indicating that Fet3 was modified extensively by a post-translational mechanism. Given the fact that C. albicans Fet3 is predicted to have ten N-linked glycosylation sites (Ziegler et al., 2011), the changes in molecular mass might be due to additional glycan chains. In addition, our results showed that iron-deficient stimuli remarkably increased modified-Fet3 protein levels in both fet33Δ/Δ and fet34Δ/Δ, which was presumed to be a functional product and critical to cellular activity. Taken together, these results indicated that CaFET33 and CaFET34 play crucial roles in C. albicans iron acquisition under iron-deficient conditions.
together, these results confirm that cognate MCO genes could mask the effects of the absence of another MCO gene and that CaFET3 might play an essential role in this process.

The CaFET34 mutant exhibits a filamentous growth defect on hyphal induction medium

Recent studies showed a correlation between hyphal morphogenesis and iron-uptake genes in *C. albicans* (Braun et al., 2000; Hameed et al., 2008). Given the importance of hyphal development in pathogenicity, we hypothesized that MCO genes may play a role in filamentous growth. So we examined the hyphal growth ability of the fet33Δ/Δ and fet34Δ/Δ strains on hypha-inducing agar at 30 and 37 °C (Fig. 4). In Spider and serum plates at 37 °C, wild-type strains formed wrinkled colonies with strong peripheral filaments, whereas the CaFET34 mutant only exhibited wrinkling morphology with hardly any peripheral invasion. Deletion of CaFET34 resulted in smooth colonies with almost no invasion in Spider medium at 30 °C. The fet34Δ/Δ mutant also showed attenuated filamentous growth ability compared to the reference strain in RPMI 1640 solid medium at the indicated temperature. In addition, deletion of CaFET34 impaired filamentous growth induced by GlcNAc at 30 °C, but not at 37 °C. The same experiments were also done with the fet33Δ/Δ mutant and a similar but slightly weaker filamentous growth defect was observed for this mutant. Taken together, the fet34Δ/Δ mutant showed an obvious reduction in the peripheral invasive filaments, indicating the potential role of CaFET34 in filamentous growth. However, addition of exogenous iron could not reverse this phenotype defect. The fet34Δ/Δ mutant still abolished peripheral filamentous growth in *C. albicans*. These observations indicate that CaFET34 is involved in filamentous growth in an iron-independent manner.

Loss of CaFET34 impedes virulence of *C. albicans* in a mouse model of systemic infection

Accumulated evidence suggested that availability of iron plays a critical role in microbial infections (Bullen et al., 2006). A defect in iron-acquisition ability may decrease the fitness of pathogens to survive within the host. To determine whether CaFET33 and CaFET34 contribute to virulence, we performed intravenous infections of ICR mice with fet33Δ/Δ, fet34Δ/Δ and wild-type *C. albicans*. To avoid positional effects on URA3 expression, we reintroduced the URA3 gene at its common locus in all tested
strains. After intravenous inoculation, nearly all mice displayed symptoms of infection. The ten mice injected with the wild-type control strain died within 7 days, as expected (Fig. 5a). Similar results were observed for mice infected with the \textit{fet33}\textsuperscript{D/Δ} and the corresponding complemented strain (\( P = 0.96 \), Fig. 5a, left). In contrast, mice infected with the \textit{fet34}\textsuperscript{D/Δ} mutant had a significant reduction of virulence compared to the wild-type strain (\( P < 0.0001 \)) and 40% remained alive at 3 weeks post infection (Fig. 5a, right). Histological examination of kidneys infected with the wild-type strain or the \textit{fet33}\textsuperscript{Δ/Δ} mutant clearly demonstrated a high level of penetration by hyphal filaments (arrows). In contrast, no cells from the \textit{fet34}\textsuperscript{Δ/Δ} mutant strain could be visualized (Fig. 5b). All these results suggest that \textit{CaFET34} is an important virulence determinant and that deletion of \textit{CaFET34} attenuates the virulence of \textit{C. albicans}.

**DISCUSSION**

Iron is required for most microorganisms, primarily owing to its role as a cofactor in essential metabolic processes. The human pathogen \textit{C. albicans} possesses at least three different iron-uptake systems (Almeida \textit{et al.}, 2009). Each of these iron-uptake systems is specially adapted for an environmental niche and helps \textit{C. albicans} to deal with the variability of iron abundance and form. High-affinity iron-uptake systems are induced by iron limitation and require a
great many components to function. Of special interest is
the multicopper oxidase, which forms a complex with the
ferric iron permease and supports high-affinity Fe uptake
(Wang et al., 2003). Three major MCO genes have been
characterized in S. cerevisiae, that is, FET3, FET4 and FET5.
FET4 is involved in a low-affinity iron-uptake pathway (Dix
et al., 1994), while FET3 and FET5 are induced under iron-
deficient conditions and are required for high-affinity iron
uptake (Askwith et al., 1994; Spizzo et al., 1997). Five
putative MCO genes exist in the C. albicans genome.
Nevertheless, there are no systematic studies about the role
of MCOs in high-affinity iron uptake of C. albicans and the
correlation among these genes is still unclear.

Our comprehensive research shows that CaFET3 and
CaFET34 are the main MCO genes induced by iron
deficiency. Previous genomic profiling identified CaFET3
or CaFET34 as the iron-responsive gene in C. albicans
(Chen et al., 2011; Lan et al., 2004). Despite slight
discrepancies (perhaps due to differences in experimental
conditions or the culture conditions), these results, along
with our results, suggest the significance of CaFET3 and
CaFET34 in response to iron limitation. Structure–
function studies have identified that C. albicans Fet34
protein supports Fe uptake along with the native yeast
ScFtr1 protein in S. cerevisiae (Ziegler et al., 2011).
Although the potential oxidase–permease complex was
not well characterized in C. albicans, our results provide a
possibility that the CaFet3 protein, which was likely
involved in high-affinity Fe uptake like CaFet34, might
also function with an alternative permease (CaFtr1 or
CaFtr2) homologue that conferred iron sequestering or
iron detoxifying activity. In addition, we also detected an
increased mRNA level for CaFET99 under iron-limited
conditions, which correlates with previous observations
and indicates its potential role in iron uptake (Knight
et al., 2002). Interestingly, no obvious differential expression
of other putative MCO genes was detected under tested
conditions. We hypothesized that these functionally similar
genes may contribute to various degrees of MCO activity in
response to different iron stress conditions. Another
possibility is that the functional redundancy and/or

Fig. 5. Virulence assay of C. albicans strains. (a) ICR mice were injected via tail veins with 5×10⁶ cells of the indicated strains. Survival curves for mice infected with the designated strains demonstrated the greatly reduced virulence of the fet34 Δ/Δ mutant. (b) Infected mice were sacrificed on the fifth day, and the kidneys were collected for histological examination. Haematoxylin–eosin staining was used to observe the colonization. The wild-type and fet33 Δ/Δ cells were observed as filamentous forms (arrows). Kidney section of the fet34 Δ/Δ mutant-infected mice showed no fungal colonization (×400 magnification).
compensatory upregulation of other MCOs may alleviate the negative effects when one of them is absent. Copper, as an essential cofactor, is required for the oxidase activity of MCOs. In humans and other mammals, copper deficiency affects the MCO (ceruloplasmin, GPI-ceruloplasmin, hephaestin and zykopen) protein levels rather than mRNA expression (Prohaska, 2011). Limitation of copper does not appear to limit synthesis of MCOs, but rather their stability and turnover. Homology analysis with the S. cerevisiae Fet3 indicates that all five MCOs possess four Cu-binding motifs specific to MCO. MCOs require adequate copper to form holoenzymes and stabilize protein structure. Our results indicate that copper deficiency results in slightly altered changes in some MCO mRNA levels, including CaFET3 and CaFET34. Limitation in available copper has no apparent impact on the other three MCO genes. These results also support the hypothesis that copper availability may primarily influence the turnover rate of/and stability of MCO protein in C. albicans. However, further research is needed to confirm this speculation.

The FET3 gene of S. cerevisiae affects the high-affinity transport of ferrous iron and plays an indispensable role in cellular iron accumulation (Askwirth et al., 1994). The possible function of CaFET31 (orf19.4213) and CaFET99 in Fe uptake has been examined in C. albicans (Eck et al., 1999; Knight et al., 2002) and the fet31Δ/Δ mutant was shown to have a growth defect in low-iron medium. In addition, CaFET31 was able to complement the FET3 deletion in S. cerevisiae. However, CaFET99 failed to complement the Fe-deficient phenotype. Our results show that CaFET34, but not CaFET33, can effectively rescue the iron-related growth defects of the Sfet3A mutant. In C. albicans, deletion of CaFET34 dramatically decreased cellular whole iron content and iron-uptake activity under iron-deficient conditions, as did, to a lesser extent deletion of CaFET33, indicating a role of CaFET33 and CaFET34 in maintaining iron homeostasis. However, further research revealed that the two mutants exhibited no significant growth limitation in low-iron solid medium, while the growth rate of fet34Δ/Δ mutant was slightly slower in iron-chelated liquid medium. We speculate that there exist other MCO genes in place of the deleted genes for function. As will be discussed, this assumption was validated by RT-PCR and Western blotting. Deletion of CaFET34 resulted in increased gene expression of CaFET3 and CaFET99. Likewise, the transcripts of CaFET3 and CaFET34 were induced in the fet33A/Δ mutant. In addition, our results showed that Fet3 protein was glycosylated and/or modified by other yet-undefined post-translational modifications in C. albicans. Glycosylation is recognized as an important post-translational modification, which is believed to be essential for protein function, folding and stability. Previous studies have showed that N-linked glycosylation of yeast multicopper ferroxidase was critical to its cellular activity and assembly into a functional iron-uptake complex (Ziegler et al., 2010, 2011). Therefore, we speculate that the increased protein levels of modified Fet3 product were mainly responsible for the masking effects of the absence of another MCO gene under iron-limited conditions. Surprisingly, there were paradoxical results about CaFET34 and CaFET99 in mRNA and protein levels. The possibility is that glycosylation influences the stability of proteins, and unglycosylated proteins are more rapidly degraded in response to iron deficiency (Hessa et al., 2011; Quan et al., 2008). Taken together, our results provide evidence that there exists a mutual complementary mechanism among the MCO genes in C. albicans. This may explain the observations that deletion of CaFET99 and CaFET33, partly for CaFET31 and CaFET34, has no obvious phenotypes in iron deprivation.

The transcriptome analysis performed by Lan et al. (2004) displayed an inverse correlation between hyphal morphogenesis and iron availability in C. albicans. In addition, iron deprivation caused by BPS had a capacity to promote EFG1-mediated hyphal development (Hameed et al., 2008). Interestingly, haemoglobin, as a major source of iron in the host environment, is an effective inducer of infective specific genes and hyphal development (Braun et al., 2000). In this study, our phenotypic assays for colony morphology show that deletion of CaFET34 obviously attenuates filamentous growth ability with almost no peripheral hyphae. Interestingly, addition of exogenous iron cannot rescue the filamentous growth defect on hyphal-inducing medium, supporting a possibility that the role of CaFET34 in filamentous growth might be performed in an iron-independent manner. Recent studies have also established a link for iron in systemic infections. The efficient iron-acquisition tactics constitute important virulence factors for many microbial pathogens, including C. albicans. Deletion of the high-affinity iron permease gene, CaFTR1, abolished the capacity of C. albicans to establish systemic infection in mice (Ramanan & Wang, 2000). Similarly, siderophore uptake mediated by the Arn1 transporter is required for epithelial invasion (Heymann et al., 2002). Furthermore, C. albicans invades endothelial cells and oral epithelial cells in an iron-dependent manner (Almeida et al., 2008; Fratti et al., 1998). These data all imply that iron availability may play a role in signalling or facilitating the commenceal-to-pathogenic transition in C. albicans. In accordance with the filamentous phenotype, the virulence of the fet33A/Δ mutant was comparable to that of the wild-type strain, whereas deletion of CaFET34 exhibited significantly attenuated virulence in a mouse systemic infection model.

Taken together, these results indicate that CaFET34, as a prominent contributor, is required for C. albicans virulence. These findings will shed a new light on MCO genes and provide a novel therapeutic target for the treatment of candidiasis. However, the regulation and function of these MCO genes still need to be further investigated. Exploration of these factors may better define the role of iron acquisition in virulence.
ACKNOWLEDGEMENTS

We are grateful to Dr Dana Davis (University of Minnesota, USA), Dr Gerald Fink (Whitehead Institute for Biomedical Research, MIT, USA), Dr Julia R. Koehler (Boston Children’s Hospital, USA) and Dr Jerry Kaplan (University of Utah, USA) for the generous gift of strains and plasmids. We would also like to thank the reviewers for critical reading and helpful suggestions. This study was supported by the National Natural Science Foundation of China (nos 31070126 and 81171541) and the Tianjin Research Program of Application Foundation and Advanced Technology (no. 13JCYBJC20700).

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Edited by: J. Morschhäuser