Identification and functional characterization of a novel *Candida albicans* gene *CaMNN5* that suppresses the iron-dependent growth defect of *Saccharomyces cerevisiae* *aft1*Δ mutant

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In *Saccharomyces cerevisiae*, the transcription factor Aft1p plays a central role in regulating many genes involved in iron acquisition and utilization. An *aft1*Δ mutant exhibits severely retarded growth under iron starvation. To identify the functional counterpart of *AFT1* in *Candida albicans*, we transformed a *C. albicans* genomic DNA library into *aft1*Δ to isolate genes that could allow the mutant to grow under iron-limiting conditions. In the present paper, we describe the unexpected discovery in this screen of *CaMNN5*. *CaMnn5p* is an α-1,2-mannosyltransferase, but its growth-promoting function in iron-limiting conditions does not require this enzymatic activity. Its function is also independent of the high-affinity iron transport systems that are mediated by Ftr1p and Fth1p. We obtained evidence suggesting that *CaMnn5p* may function along the endocytic pathway, because it cannot promote the growth of *end4*Δ and *vps4*Δ mutants, where the endocytic

pathway is blocked at an early and late step respectively. Neither can it promote the growth of a *fth1*Δ *smf3*Δ mutant, where the vacuole–cytosol iron transport is blocked. Expression of *CaMNN5* in *S. cerevisiae* specifically enhances an endocytosis-dependent mechanism of iron uptake without increasing the uptake of Lucifer Yellow, a marker for fluid-phase endocytosis. *CaMnn5p* contains three putative Lys–Glu–Xaa–Xaa–Glu iron-binding sites and co-immunoprecipitates with ⁵⁵Fe. We propose that *CaMnn5p* promotes iron uptake and usage along the endocytosis pathway under iron-limiting conditions, a novel function that might have evolved in *C. albicans*.

Key words: *Candida albicans*, endocytosis, iron-binding protein, iron uptake, mannosyltransferase.

INTRODUCTION

Iron is an essential nutrient for almost all organisms. The facile nature of iron to gain and lose electrons enables it to participate as a co-factor in diverse enzymatic reactions [1,2]. Keeping free iron away from invading microbial pathogens is an important element of host innate immunity. To overcome this host defence, pathogens must develop efficient ways to compete for iron. Thus specialized iron acquisition tactics often constitute virulence factors of many pathogens such as *Candida albicans*, one of the top human fungal pathogens [3,4]. In human body fluids, largely because of the presence of iron-chelating proteins such as transferrin and lactoferrin, free iron concentration is ~10^{−18} M, far below the minimal nutritional requirement of 10^{−6}–10^{−7} M for growth [5]. On the other hand, iron reacts readily with oxygen-containing compounds to generate cytotoxic free radicals. Thus iron uptake requires tight regulation.

Iron transport has been studied extensively in *Saccharomyces cerevisiae*. The iron-uptake systems found so far can be divided into two categories on the basis of affinities for iron. Among the low-affinity transporters is a plasma membrane protein Fet4p that has a *K_m* of 30 μM and functions under iron-replete conditions [6]. Fet4p has a broad specificity for bivalent metal ions, such as copper, cobalt, manganese and zinc, as well as iron [7]. Another low-affinity metal ion transport system is encoded by *SMF1*, 2 and 3 [8,9], orthologues of the *Nramp* family that play key roles in iron transport in mammals [10]. Under metal starvation, Smf1p accumulates at the cell surface, while Smf2p localizes to intracellular vesicles. Smf3p constitutively resides at the vacuolar membrane, transporting vacuolar iron into the cytosol [11].

Fet3p–Ftr1p is a plasma membrane high-affinity iron transporter with a *K_m* of 0.15 μM [12]. Fet3p is a ferroxidase that converts ferrous into ferric iron and Ftr1p is a permease that translocates the ferric iron into cytoplasm [13,14]. Ftr1p contains a putative iron-binding motif Arg–Glu–Gly–Leu–Glu at amino acid positions 157–161 that is essential for iron uptake. Substituting alanine for either one of the glutamate residues completely abolishes the iron transport activity [14,15]. Another ferroxidase–permease complex resides at the vacuolar membrane, encoded by *FET5* and *FTH1*, which are homologues of *FET3* and *FTR1* respectively. This complex performs vacuole–cytosol iron transport [16]. Many microbes produce ferric chelators called siderophores with high affinities for iron [17]. Microbes can take up siderophore–iron complexes via specific transporters. *S. cerevisiae* contains four siderophore–iron transporters, Arn1, 2, 3 and 4p, with distinct specificities [18,19]. Recently, Fischbach et al. [20] reported that the IroB protein of pathogenic *Escherichia coli* and *Salmonella enterica* strains catalyses the C-glucosylation of a siderophore, enterobactin, increasing the availability of enterobactin to the pathogen in serum.

Most genes involved in high-affinity iron transport in *S. cerevisiae* are activated when the iron supply is limiting and repressed when replete. This regulation is primarily controlled by a transcription factor Aft1p [21], which activates approx. 20 genes [21–23]. Thus *aft1*-null mutants cannot grow in iron-limiting media [21].

Endocytosis also has a role in iron uptake in *S. cerevisiae*. The *end4* mutant, which is defective in endocytosis, exhibits delayed growth under iron starvation [24]. A fraction of molecules brought into cells via endocytosis end up in the vacuoles for storage, usage,

Abbreviations used: BPS, bathophenanthroline sulphonate; GMM, glucose minimal medium; GaMM, galactose minimal medium; HA, haemagglutinin; LY, Lucifer Yellow; ORF, open reading frame.

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Table 1 *S. cerevisiae* and *C. albicans* strains used in the present study

Strain	Relevant characteristics	Source or reference
<i>S. cerevisiae</i>		
CRY2 α	<i>trp1 his3 leu2 ura3-52</i>	
MBY3	Same as SEY6210, except <i>his3 leu2 ura3-52 vps4</i> Δ :: <i>TRP1</i>	Gift from G. Odorizzi
MBY3-1	Same as MBY3, except <i>2</i> μ <i>PtFTR1-CaMNN5 URA3</i>	This study
RH1965	<i>his4 lys2 ura3-52 end4</i> Δ :: <i>LEU2</i>	Gift from J. Kaplan
RH1965-1	Same as RH1965, except <i>2</i> μ <i>PtFTR1-CaMNN5 URA3</i>	This study
ScWYBC1	Same as CRY2 α , except <i>ftr1</i> Δ :: <i>HIS3</i>	[4]
ScWYBC1-1	Same as ScWYBC1, except CEN <i>GAL-CaMNN5 URA3</i>	This study
ScWYBC2	Same as CRY2 α , except <i>ftr1</i> Δ :: <i>HIS3 fet3</i> Δ :: <i>LEU2</i>	[4]
ScWYBC2-1	Same as ScWYBC2, except CEN <i>GAL-CaMNN5 URA3</i>	This study
ScWYBC3	Same as CRY2 α , except <i>aft1</i> Δ :: <i>LEU2</i>	This study
ScWYBC3-1	Same as ScWYBC3, except <i>2</i> μ <i>CaMNN5 URA3</i>	This study
ScWYBC3-2	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5 URA3</i>	This study
ScWYBC3-21	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{D282A} URA3</i>	This study
ScWYBC3-22	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{D284A} URA3</i>	This study
ScWYBC3-23	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{E132A} URA3</i>	This study
ScWYBC3-24	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{E230A} URA3</i>	This study
ScWYBC3-25	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{E588A} URA3</i>	This study
ScWYBC3-26	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{E132A E230A} URA3</i>	This study
ScWYBC3-27	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5^{E132A E230A E588A} URA3</i>	This study
ScWYBC3-3	Same as ScWYBC3, except CEN <i>PtGAL-CaMNN5-HA URA3</i>	This study
ScWYBC3-4	Same as ScWYBC3, except CEN <i>PtGAL-MNN2-HA URA3</i>	This study
ScWYBC3-5	Same as ScWYBC3, except CEN <i>PtGAL-MNN5-HA URA3</i>	This study
ScWYBC4	Same as CRY2 α , except <i>ftr1</i> Δ :: <i>HIS3 fth1</i> Δ :: <i>LEU2</i>	This study
ScWYBC4-1	Same as ScWYBC4, except CEN <i>PtGAL-CaMNN5 URA3</i>	This study
ScWYBC5	Same as CRY2 α , except <i>fth1</i> Δ :: <i>LEU2</i>	This study
ScWYBC5-1	Same as ScWYBC5, except <i>2</i> μ <i>PtFTR1-CaMNN5 URA3</i>	This study
ScWYBC6	Same as CRY2 α , except <i>smf3</i> Δ :: <i>HIS3</i>	This study
ScWYBC6-1	Same as ScWYBC6, except <i>2</i> μ <i>PtFTR1-CaMNN5 URA3</i>	This study
ScWYBC7	Same as CRY2 α , except <i>fth1</i> Δ :: <i>LEU2 smf3</i> Δ :: <i>HIS3</i>	This study
ScWYBC7-1	Same as ScWYBC7, except <i>2</i> μ <i>PtFTR1-CaMNN5 URA3</i>	This study
<i>C. albicans</i>		
SC5314	Wild-type	[38]
CaWYNR1	<i>ura3/ura3 Caftr1</i> Δ :: <i>hisG/Caftr1</i> Δ :: <i>hisG</i>	[4]
CaWYBCR	Same as CaWYNR1, except <i>CIP10-CaFTR1 URA3</i>	This study
CaWYBCR-1	Same as CaWYNR1, except <i>CIP10-CaFTR1^{KEYLE} URA3</i>	This study
CaWYBCR-2	Same as CaWYNR1, except <i>CIP10-CaFTR1^{KEFCE} URA3</i>	This study
CaWYBCR-3	Same as CaWYNR1, except <i>CIP10-CaFTR1^{KETA} URA3</i>	This study
<i>P. pastoris</i>		
KM71	<i>arg4 his4 aox1</i> :: <i>ARG4</i>	Invitrogen
KMCON	Same as KM71, except pPIC9 <i>HIS4</i>	This study
KMCAmNN5	Same as KM71, except pPIC9- <i>CaMNN5 HIS4</i>	This study
KMCAmNN5-1	Same as KM71, except pPIC9- <i>CaMNN5^{D282A} HIS4</i>	This study
KMCAmNN5-2	Same as KM71, except pPIC9- <i>CaMNN5^{D284A} HIS4</i>	This study

recycling or degradation. The vacuole was designated as an iron-storage organelle [25], from which iron is exported into cytosol by Fet5p–Fth1p and Smf3p [11,16].

In comparison with *S. cerevisiae*, much less is known about iron transport in *C. albicans*. A high-affinity system was found consisting of CaFet3p and CaFtr1p [4,26], homologues of Fet3p and Ftr1p of the budding yeast. *Cafet3* Δ mutants exhibit growth defects in iron-limiting media, but remains virulent [26], whereas *CaFTR1* deletion causes severe iron-dependent growth defects and a complete loss of virulence in mice [4]. A single siderophore–iron transporter CaArn1p has also been found and characterized [27]. *Caarn1* Δ cannot use siderophore–iron, but is still virulent. Although *CaFTR1* and *CaARN1* expression is regulated by iron concentration [4,27], the transcription factors that are involved are not clear. A transcription repressor CaTup1p was shown to have a role, but the functional counterpart of *S. cerevisiae* Aft1p has not been found.

In the present study, we conducted a genetic screen to identify *C. albicans* genes that may suppress the iron-dependent growth

defects of *S. cerevisiae* *aft1* Δ mutants. We present the discovery and functional characterization of *CaMNN5* that promotes cell growth under iron-limiting conditions by a previously unknown mechanism.

EXPERIMENTAL

Strains and growth conditions

S. cerevisiae and *C. albicans* strains are listed in Table 1. *S. cerevisiae* strains were routinely grown in YPD medium (1.0 % yeast extract, 2.0 % peptone and 2.0 % glucose), minimal medium containing yeast nitrogen base without amino acids and 2.0 % glucose (GMM or glucose minimal medium) or 2.0 % galactose (GaMM or galactose minimal medium). All strains were grown at 30 °C, except *end4* mutants which were grown at 22 °C. Iron-limiting media were prepared in most cases by adding 1 mM ferrozine and 50 μ M ferrous ammonium sulphate to the minimal media. To create stringent iron-limiting conditions, 200 μ M BPS (bathophenanthroline sulphate) was added to GMM or GaMM.

Gene disruption

AFT1-, *FTH1*- and *SMF3*-deletion mutants were generated from *CRY2 α* by following the strategy described in [4]. A gene-deletion cassette was constructed by flanking a marker gene, *HIS3* or *LEU2*, with two DNA fragments corresponding to the 5'- and 3'-untranslated regions of the target gene. The primers used for PCR-amplification of the DNA fragments are given in the Supplementary Data (available at <http://www.BiochemJ.org/bj/389/bj3890027add.htm>). Strain genotype was verified by Southern blotting.

Site-directed mutagenesis

Mutations of the Lys-Glu-Xaa-Xaa-Glu and Asp-Xaa-Asp motifs of CaMnn5p were created by using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene). The oligonucleotides used for mutagenesis are listed in the Supplementary Data (available at <http://www.BiochemJ.org/bj/389/bj3890027add.htm>). Mutations were verified by DNA sequencing.

Indirect immunofluorescence staining and Western blotting

Mouse monoclonal antibody 12CA5 (Roche Applied Science) was used as a primary antibody in both indirect immunofluorescence staining of HA (haemagglutinin)-tagged proteins and Western blot analysis. For Western blotting, horseradish-peroxidase-conjugated sheep anti-mouse IgG antisera (Amersham Biosciences) was used, and, for indirect immunofluorescence staining, Cy3-conjugated goat anti-(mouse IgG) antibody was used. For Western blotting, yeast cells were grown to 1×10^7 cells/ml in 10 ml of GaMM. The culture was centrifuged at 3000 g for 5 min. The cell pellet was resuspended in 300 μ l of ice-cold lysis buffer containing 1 % (v/v) Triton X-100, 0.1 % (w/v) SDS, 50 mM Tris/HCl (pH 7.2), 1 % (w/v) sodium deoxycholic acid and one protease inhibitor mixture tablet (Roche Molecular Biochemicals)/25 ml. The cell suspension was then transferred to a 2-ml screw-cap tube. An equal volume of glass beads was added, and the cells were broken by using the Mini-Beadbeater (Biospec Products) at the maximum speed for two cycles of 2-min beating on ice. The cell lysate was spun at maximum speed in an Eppendorf microfuge for 5 min and the supernatant was transferred to a new 1.5-ml tube. Protein concentration was determined by the Bradford assay (Bio-Rad). Proteins were resolved by SDS/10 % PAGE, blotted on to Hybond-C membrane (Amersham Biosciences) and detected by using the ECL® (enhanced chemiluminescence) Western blot detection kit (Amersham Biosciences). Indirect immunofluorescence staining was performed as described in [30].

Cell fractionation

Subcellular fractionation was performed as described in [31] with some modifications. Cells were grown in 500 ml of GaMM to a D_{600} of ~ 0.9 before centrifugation at 3000 g for 10 min. The cell pellet was resuspended in 50 ml of 200 mM Tris/HCl (pH 8.0), 1 mM EDTA and 1 % (w/v) 2-mercaptoethanol. The cell suspension was centrifuged again at 3000 g for 10 min, and the pellet was resuspended in 50 ml of 1.2 M sorbitol solution, containing 20 mg of lyticase, and digested at 30°C for 60 min. The spheroplasts were washed once with 1.2 M sorbitol and then lysed with a Dounce homogenizer in 4 ml of an ice-cold buffer containing 50 mM potassium phosphate buffer (pH 7.5), 200 mM sorbitol, 1 mM EDTA and protease inhibitor mixture. The cell lysate was centrifuged at 500 g for 5 min, and then the supernatant was cleared by centrifugation at 13000 g for 15 min, loaded on to the top of a 22–60 % sucrose gradient and centrifuged at 170000 g at 4°C for 18 h. Serial fractions of 0.75 ml were collected, and 40 μ l of each was resolved by SDS/10 % PAGE.

⁵⁹Fe uptake and ⁵⁵Fe protein-binding assays

⁵⁹Fe uptake was measured as described in [4] with some modifications. *S. cerevisiae* strains were first grown to saturation in GMM or GaMM according to strain background and then washed twice with 10 mM EDTA and once with GMM or GaMM containing 200 μ M BPS. The cells were then resuspended in the GMM/BPS or GaMM/BPS to a density of 1×10^7 cells/ml and grown for 6 h to deplete intracellular iron. Cells were then counted, spun down at 3000 g for 5 min, and divided into 50 μ l aliquots in GaMM or GaMM/BPS medium, each containing $\sim 2 \times 10^7$ cells. ⁵⁹Fe was added to a final concentration of 2 μ M, and the cells were incubated at 30°C. One such aliquot of each strain was kept on ice to estimate background ⁵⁹Fe binding. After incubation, cells were collected and washed twice with ice-cold 10 mM EDTA to remove free ⁵⁹Fe. The cell pellet was then resuspended in 3 ml of scintillation fluid for radioactivity counting in an Amersham γ -counter. After subtracting the background, the iron-uptake rate is expressed as femtomoles of ⁵⁹Fe/10⁶ cells/min. For the assays in the presence of 200 μ M BPS, iron uptake is expressed as total femtomoles of ⁵⁹Fe/10⁶ cells at the end of incubation.

For ⁵⁵Fe-binding assay, the strains expressing HA-tagged proteins were first grown in the presence of 200 μ M PBS for 6 h to deplete intracellular iron and then in GaMM containing 5 μ M ⁵⁵Fe for 12 h. Total protein was extracted as described above, and the cell lysate was mixed with 6 μ l of anti-HA antibody-coated (ProFound™ HA Tag Co-IP kit; Pierce) or naked agarose beads in a Handee™ Mini-spin column. The mixtures were incubated at 4°C for 3 h with gentle mixing. Then the beads were spun down and washed twice with TBS-T buffer (25 mM Tris/HCl, pH 7.2, 0.15 M NaCl and 0.05 % Tween-20). The HA-tagged protein was eluted with 50 μ l of elution buffer (from the kit) and mixed with 3 ml of scintillation fluid. An aliquot of the whole-cell lysate was used to determine ⁵⁵Fe uptake into the cell and an aliquot of the eluted protein was examined by anti-HA Western blotting.

LY (Lucifer Yellow) endocytosis assay

To assay fluid-phase endocytosis, LY (Sigma) was added to exponential-phase yeast cells to a final concentration of 1 mg/ml. After 2 h, cells were washed three times with PBS containing 10 mM sodium azide and three times with PBS. Cells were then digested in 1.2 M sorbitol containing 0.2 mg/ml zymolase at 30°C for 30 min. The spheroplasts were washed once with 1.2 M sorbitol before lysis by sonication for 30 s in ice-cold PBS containing 0.05 % (v/v) Triton X-100. Fluorescence was determined by using a PerkinElmer LS-55 spectrofluorimeter with $\lambda_{\text{excitation}}$ at 423 nm and $\lambda_{\text{emission}}$ at 530 nm, normalized to a LY standard curve.

Expression of CaMnn5p in Pichia pastoris

The expression of CaMnn5p was carried out using the *Pichia* Expression kit (Invitrogen) following the manufacturer's instructions.

Assay of CaMnn5p enzyme activity

Mannosyltransferase activity was assayed as described in [32]. The *P. pastoris* culture supernatant was used directly as the enzyme source in a buffer containing 50 mM Tris/HCl (pH 7.2), 10 mM MnCl₂, 0.23 μ M GDP-[¹⁴C]mannose (62 nCi; specific radioactivity 289 mCi/mmol), 20 mM 6-O- α -D-mannopyranosyl-D-mannopyranose acceptor (Sigma) and 5 mg/ml BSA. Standard reactions were performed for 60 min at 30°C in a final volume of 50 μ l. To terminate the reaction, the mixtures were passed through 0.8 ml of QAE-Sephadex anion-exchange resin

Figure 1 *CaMNN5* enhances *aft1* Δ growth on iron-limiting media

(A) *CaMNN5* on a 2 μ vector with its native promoter (*aft1* Δ :*CaMNN5*) suppressed the growth defect of *aft1* Δ on the iron-limiting GMM plate containing 1 mM ferrozine and 50 μ M ferrous ammonium sulphate (FAS). The wild-type strain and *aft1* Δ transformed with the empty vector (CRY2 α :vector and *aft1* Δ :vector respectively) were included for comparison. The GMM plate without ferrozine is iron-sufficient. (B) *aft1* Δ transformed with a CEN plasmid harbouring *CaMNN5* driven by the Gal1-10 promoter (*aft1* Δ :*CaMNN5*) grew on the iron-limiting GaMM, but not on GMM plates. CRY2 α and *aft1* Δ transformed with the empty plasmid were used as controls. Approx. 10^4 , 10^3 and 10^2 cells of each strain were spotted on to the plates and incubated at 30 $^{\circ}$ C for 5 days.

(Amersham Biosciences) to remove unlabelled GDP-mannose. The 14 C-labelled products were eluted with 0.8 ml of water, and radioactivity was counted in 3 ml of scintillation fluid.

RESULTS

Identification of *C. albicans* genes that suppress the growth defects of *S. cerevisiae* *aft1* Δ under iron-limiting conditions

We transformed a *C. albicans* 2 μ genomic DNA library into the *aft1* Δ mutant and screened for clones that grew on iron-limiting GMM plates. After 1 week of incubation at 30 $^{\circ}$ C, colonies were picked and grown for the recovery of the transforming plasmids and DNA sequencing of the inserts. The plasmids from 80 % of the plasmids contained an identical ORF (open reading frame) of 1791 base pairs (bp). In the *C. albicans* sequence database (<http://www-sequence.stanford.edu/group/candida>), this ORF (19.2347) is annotated as *CaMNN5*, a homologue of *S. cerevisiae* *MNN5*, encoding a Golgi α -1,2-mannosyltransferase. *CaMnn5p* is approx. 30 % identical with *Mnn5p* and another *S. cerevisiae* homologue *Mnn2p*. To confirm that *CaMNN5* is indeed responsible for suppressing the defects of *aft1* Δ , the ORF, together with $\sim$ 600 bp of its native promoter, was cloned in the 2 μ vector. Figure 1(A) shows that this construct was able to allow *aft1* Δ to grow on the iron-limiting plate. *CaMNN5* was also expressed from the Gal1-10 promoter in a CEN plasmid. This plasmid exhibited the same growth-promoting effect on *aft1* Δ on GaMM only, but not GMM iron-limiting plate (Figure 1B). The results confirmed that *CaMNN5* is responsible for rescuing the growth defect of *aft1* Δ . When *CaMNN5* with its own promoter was cloned in a CEN plasmid, it did not rescue the defects of *aft1* Δ (results not shown). However, when driven by the *FTR1* (see Figure 6) or *ADH1* (results not shown) promoter from the 2 μ vector, it could effectively rescue the defects of *aft1* Δ . The results indicate that *CaMNN5* expression level is important for the observed growth-promoting function.

Like other known Golgi mannosyltransferases, *CaMnn5p* contains a predicted transmembrane domain near the N-terminus.

Figure 2 *CaMnn5p* contains three potential iron-binding Lys-Glu-Xaa-Xaa-Glu motifs

(A) The Lys-Glu-Xaa-Xaa-Glu motifs of *CaMnn5p* were aligned with some conserved motifs of several known iron-binding proteins: *S. cerevisiae* (Sc) Ftr1p (amino acids 157–161) and Fth1p (amino acids 242–246), *C. albicans* (Ca) Ftr1p (amino acids 157–161), *Salmonella enterica* (Se) PmrB (amino acids 38–42, 63–67), *Escherichia coli* (Ec) PmrB (amino acids 38–42, 60–64), *Klebsiella pneumoniae* (Kp) PmrB (amino acids 36–40, 62–66) and *Yersinia pestis* (Yp) PmrB (amino acids 29–33, 54–58). (B) Each of the Lys-Glu-Xaa-Xaa-Glu motifs of *CaMnn5p* could functionally replace the Arg¹⁵⁷-Glu-Gly-Leu-Glu¹⁶¹ motif of *CaFtr1p*. All strains were grown on iron-limiting GMM plates at 30 $^{\circ}$ C for 5 days. (C) The Lys-Glu-Xaa-Xaa-Glu motifs of *CaMnn5p* were mutated by site-directed mutagenesis. Cells of each strain were spotted on to iron-limiting GMM and GaMM plates for incubation at 30 $^{\circ}$ C for 5 days. The table summarizes the ability of each mutated gene in promoting *aft1* Δ growth on the GaMM plates. None of the strains grew on the GMM plates.

Interestingly, it also contains three Lys-Glu-Xaa-Xaa-Glu motifs, Lys¹³¹-Glu-Tyr-Leu-Glu¹³⁵, Lys²²⁹-Glu-Phe-Cys-Glu²³³ and Lys⁵⁸⁷-Glu-Thr-Ala-Glu⁵⁹¹ (referred to as motif I, II and III). Arg/Lys/His-Glu-Xaa-Xaa-Glu motifs are present in diverse iron-binding proteins, such as mammalian ferritin light chain, fungal iron permeases and the bacterial iron sensor PmrB (Figure 2A). Evidence for direct interaction between these motifs and iron has been reported for amphibian ferritin light chain and *Salmonella* PmrB [28,29]. Mutation of any one of the glutamate residues to alanine in a conserved Arg-Glu-Gly-Leu-Glu motif in Ftr1p and CaFtr1p completely abolishes the iron-uptake function of the protein [14,15]. *S. cerevisiae* *Mnn2p* and *Mnn5p* do not contain any such motifs.

The Lys-Glu-Xaa-Xaa-Glu motifs of *CaMnn5p* can functionally replace the Arg¹⁵⁷-Glu-Gly-Leu-Glu¹⁶¹ motif of *CaFtr1p*

Statistically, Lys-Glu-Xaa-Xaa-Glu motifs occur only once every 8000 amino acids. The presence of three such motifs in *CaMnn5p* suggests that they might have a role in suppressing the iron-dependent growth defect of *aft1* Δ . To evaluate the importance of these motifs in *CaMnn5p*'s activity, we first determined whether each of the motifs can functionally replace the essential putative iron-binding motif Arg¹⁵⁷-Glu-Gly-Leu-Glu¹⁶¹ of *CaFtr1p* [4]. *CaFTR1* was cloned in plasmid C1p10 and then the Arg¹⁵⁷-Glu-Gly-Leu-Glu¹⁶¹ motif was replaced by each of the three Lys-Glu-Xaa-Xaa-Glu motifs of *CaMnn5p*, yielding three mutated genes *CaFtr1*-I, II and III. The three constructs were transformed into *CaFtr1* Δ , and transformants were grown on the iron-limiting plates. We found that all three constructs were able to rescue the

growth defect of *Caft1Δ* as effectively as the wild-type gene (Figure 2B).

Next we asked whether these motifs are required for CaMnn5p to rescue the growth defects of *aft1Δ*. We systematically introduced mutations into the Lys-Glu-Xaa-Xaa-Glu motifs, individually or in combination, and tested each for its ability to promote *aft1Δ* growth under iron-limitation conditions. We mutated the first glutamate residue of each motif to alanine. Five mutated genes were generated. *Camnn5-I_{mu}*, *-II_{mu}* and *-III_{mu}* each had a single motif mutated, *Camnn5-(I + II)_{mu}* had I and II mutated, and *Camnn5-(I + II + III)_{mu}* had all three mutated. Each mutated gene was cloned in a CEN plasmid driven by the Gal1-10 promoter and transformed into *aft1Δ*. Figure 2(C) shows that the *aft1Δ* mutant expressing the CaMnn5p with a single motif mutated grew equally well as the one expressing the wild-type CaMnn5p on the iron-limiting plate. However, the *aft1Δ* mutants expressing the CaMnn5p with both I and II or all three motifs mutated exhibited retarded and no growth respectively. The results suggest that these three potential iron-binding sites in CaMnn5p may have a direct role in suppressing the growth defects of *aft1Δ*, although they appear to be functionally redundant. However, we cannot rule out the possibility that the mutations may have caused a structural change of the protein, which led to the loss of its activity.

CaMnn5p functions independent of the known high-affinity iron transporters of *S. cerevisiae*

We next set out to investigate the mechanism by which *CaMNN5* promotes *aft1Δ* growth. The most likely mechanism responsible for iron uptake under the iron-limiting conditions we used is the high-affinity iron transporter Fet3p–Ftr1p. To determine whether *CaMNN5* expression may increase the cellular level or activity of Fet3p–Ftr1p, we first used Northern blotting to examine *FET3* and *FTR1* expression, but did not detect any increase of the mRNA levels as a result of *CaMNN5* expression (results not shown). We then tested whether *CaMNN5* can enhance the growth of mutants defective in the high-affinity iron transport systems, including *ftr1Δ*, *ftr1Δ fet3Δ* and *ftr1Δ fth1Δ*. None of these mutants grew on the iron-limiting plates, whereas introducing *CaMNN5* in these mutants enabled all of them to grow (Figure 3A). Since there is no high-affinity iron permease in *ftr1Δ fth1Δ*, we conclude that the function of CaMnn5p in promoting *aft1Δ* growth in iron-limiting conditions is independent of the high-affinity iron transport systems.

CaMNN5 can also enhance the growth of wild-type *S. cerevisiae* cells under iron-limiting conditions

We consistently noted that the *aft1Δ* mutant expressing *CaMNN5* grew better than the isogenic wild-type strain on the iron-limiting media used (results not shown). Next we tested whether *CaMNN5* would allow the wild-type cells to grow under the iron-limiting conditions where the high-affinity uptake system cannot function. We established previously that there is little iron uptake into the cells in GMM containing 200 μ M BPS, a strong ferrous chelator [4]. Figure 3(B) shows that, under these conditions, the wild-type strain grew much more slowly than the strain expressing *CaMNN5*.

CaMnn5p has α -1,2-mannosyltransferase activity

We next examined whether CaMnn5p has α -1,2-mannosyltransferase activity. We used the *P. pastoris* expression system to produce soluble secreted protein. The region of *CaMNN5* encoding amino acids 28–597 was cloned in an expression vector in-frame with the α -factor secretion signal peptide sequence, transformed into *P. pastoris*, and *CaMNN5* integrated behind the *AOX1* pro-

Figure 3 *CaMNN5* promotes cell growth under iron-limiting conditions by a mechanism independent of the high-affinity iron transporters

(A) *CaMNN5* expression promoted the growth on iron-limiting plates of *S. cerevisiae* mutants deleted of high-affinity iron transporter genes, such as *ftr1Δ*, *ftr1Δ fet3Δ* and *ftr1Δ fth1Δ*. *CaMNN5* was transformed into each mutant on the CEN plasmid driven by the Gal1–10 promoter. The cells were spotted on to iron-limiting GaMM plates. (B) *CaMNN5* promoted the growth of the wild-type strain under more stringent iron-limiting conditions. The same plasmid expressing *CaMNN5* described in (A) was transformed into *CRY2α*, and the transformants were grown on GaMM containing 200 μ M BPS. The plate was incubated at 30 °C for 5 days.

moter. A copy of the empty vector was also transformed into *P. pastoris* for a negative control. *CaMNN5* expression was induced for 24 h, and a single protein band of CaMnn5p was detected from 20 μ l of culture supernatant by SDS/PAGE, whereas no protein band was detected from the negative control supernatant (Figure 4A). Aliquots of 10 μ l (approx. 0.1 μ g of CaMnn5p) of the supernatants were assayed directly for α -1,2-mannosyltransferase activity. This assay detects the transfer of 14 C-labelled GDP-mannose to an acceptor 6-*O*- α -D-mannopyranosyl-D-mannopyranose, which is specifically catalysed by α -1,2-mannosyltransferases [33,34]. For control, 0.1 μ g of BSA was added to the negative control supernatant. Figure 4(B) shows that the control supernatant produced a background 14 C count of approx. 5×10^6 c.p.m./mg of protein per h, while the supernatant of the CaMnn5p-expressing strain resulted in a count of approx. 1.5×10^8 c.p.m./mg of protein per h, 30 times that of the control. The results confirm that CaMnn5p has α -1,2-mannosyltransferase activity. Two transferase-dead forms of the enzyme exhibited only a background level of 14 C counts (see below, and Figures 4A and 4B).

Mannosyltransferase activity of CaMnn5p is not required for suppressing the growth defect of *aft1Δ*

We examined whether the mannosyltransferase activity is required for CaMnn5p to suppress the growth defects of *aft1Δ*. It is well-established that mannosyltransferases contain a conserved Asp-Xaa-Asp motif in the catalytic domain, and the mutation of either one of the aspartate residues completely inactivates the enzyme [34]. In CaMnn5p, this motif (amino acids 282–284) is present in a highly conserved region (Figure 4C). We substituted an alanine residue for the first or the second aspartate (D282A or D284A) and transformed the mutated genes into *aft1Δ* under the control of Gal1-10 promoter. We found that both mutated forms could still suppress the defects of *aft1Δ* (results not shown), and, when expressed from *P. pastoris*, they did not exhibit any detectable enzymatic activity (Figures 4A and 4B). The results indicate

Figure 4 Mannosyltransferase activity of CaMnn5p is not required for promoting cell growth

(A) CaMnn5p and two mutant forms were expressed in *P. pastoris*. Aliquots of 20 μ l of culture supernatant from each of the strains expressing CaMnn5p, CaMnn5p (D282A) or CaMnn5p (D284A) [see below for the description of the mutants in (C)] were examined by SDS/10% PAGE. The gel was stained with Coomassie Blue. (B) The supernatants from the same cultures described in (A) were assayed directly for mannosyltransferase activity. The assay was conducted three times for each sample and results are means $\pm$ S.D. The results are expressed as activity/mg of protein per h. Protein concentration was determined by the Bradford assay. (C) Amino acid sequence alignment of a segment of the catalytic region of three α -1,2-mannosyltransferases, *S. cerevisiae* Mnn2p and Mnn5p and *C. albicans* CaMnn5p. The arrows indicate two essential aspartate residues. (D) Expression of MNN2 and MNN5 did not promote the growth of *aftr1* Δ on the iron-limiting plates. CaMNN5, MNN2 and MNN5 were cloned on the same CEN plasmid driven by the Gal1-10 promoter. Each was transformed into *aftr1* Δ , and the transformants were spotted on to iron-limiting GaMM plates. FAS, ferrous ammonium sulphate. (E) HA-tagged CaMNN5, MNN5 and MNN2 were transformed into *aftr1* Δ . The transformants were grown in liquid GaMM before total protein was extracted for Western blot analysis.

that the mannosyltransferase activity of CaMnn5p is not required for the promotion of cell growth under iron-limiting conditions.

We next asked whether *S. cerevisiae* MNN5 and MNN2 are able to rescue the growth defect of *aftr1* Δ when similarly expressed. We found that neither one could enhance *aftr1* Δ growth on the iron-limiting plates (Figure 4D). To ensure that the proteins were expressed to similar levels in the transformants, we constructed a new set of constructs by adding a HA tag to the C-terminus of CaMnn5p, Mnn2p and Mnn5p. Again, only the construct expressing CaMnn5p-HA rescued the defects of *aftr1* Δ (results not shown). Western blot analysis showed comparable levels of expression for all three proteins (Figure 4E). These results suggest further that it is not the mannosyltransferase activity, but some novel activity unique to CaMnn5p, that is responsible for promoting cell growth under iron-starvation conditions.

CaMnn5p enhances a slow process of iron uptake

Next we tested whether CaMnn5p can enhance iron uptake. We compared iron uptake in the wild-type, *aftr1* Δ and *aftr1* Δ mutant expressing CaMNN5 driven by the Gal1-10 promoter. Iron uptake

Figure 5 CaMNN5 enhances a slow process of iron uptake

(A) Strains CRY2 α :vector, *aftr1* Δ :vector and *aftr1* Δ :CaMNN5 driven by the Gal1-10 promoter were first grown in GaMM to exponential phase. Then the same number of cells of each strain was assayed for iron uptake in the same medium supplemented with 2 μ M ⁵⁹Fe for 30 min. ⁵⁹Fe uptake was determined by scintillation counting and expressed as fmol/10⁶ cells per min. (B) The same strains used in (A) were incubated in GaMM containing 200 μ M BPS and 2 μ M ⁵⁹Fe for 1, 4 and 16 h. The iron uptake is expressed as the total amount of ⁵⁹Fe accumulated in 10⁶ cells at the end of incubation. (C) Strains CRY2 α and *aftr1* Δ transformed with CaMNN5 driven by the Gal1-10 promoter or the empty vector were incubated in GaMM containing 200 μ M BPS and 2 μ M ⁵⁹Fe for 0.5, 2 and 8 h. Each assay was repeated three times.

was first assayed in GaMM containing 2 μ M ⁵⁹Fe for 30 min. Figure 5(A) shows that *aftr1* Δ and the *aftr1* Δ mutant expressing CaMNN5 exhibited a similar low ⁵⁹Fe-uptake rate of less than 3 fmol/10⁶ cells per min. In comparison, the wild-type exhibited an uptake rate of approx. 150 fmol/10⁶ cells per min, which is largely due to the activity of Ftr1p-Fet3p. This experiment shows that, under these conditions, CaMnn5p did not result in any significant increase in iron uptake. We next tested whether CaMnn5p might contribute to a less efficient iron-uptake process. Considering that the basal level of Ftr1p-Fet3p activity in *aftr1* Δ might mask the low activity of CaMnn5p in iron uptake, we added 200 μ M BPS to the assay medium and repeated the assay over an extended period of time. Under these conditions, the high-affinity iron uptake was blocked as shown by the drastically reduced iron uptake into the wild-type (Figure 5B). The amounts of ⁵⁹Fe accumulated in the CaMNN5-expressing *aftr1* Δ cells at 4 and 16 h were approx. 9 and 24 fmol/10⁶ cells respectively, which are $\sim$ 3.2

Figure 6 *CaMnn5p*-mediated iron uptake in mutants defective of the endocytic pathway

(A) *CRY2α*, *end4Δ* and *vps4Δ* mutants were transformed with a 2 μ plasmid carrying *CaMNN5* under the control of *FTR1* promoter or with the empty plasmid. The cells were spotted on to GMM plates containing no chelator or 200 μ M BPS and incubated at 25 °C for 7 days. (B) *CRY2α*, *fth1Δ*, *smf3Δ* and *fth1Δ smf3Δ* strains were transformed with the same plasmids described in (A) and grown on GMM plates containing 200 μ M BPS at 30 °C for 5 days. (C) ⁵⁹Fe uptake assay was performed in GMM containing 200 μ M BPS and 2 μ M ⁵⁹Fe for 30 and 120 min. The total amount of ⁵⁹Fe accumulated at the end of incubation is shown. The amount of ⁵⁹Fe associated with the cells kept on ice was subtracted. (D) LY uptake into *CRY2α* or *end4Δ* cells transformed with *CaMNN5* or vector alone. There was no detectable LY uptake when the cells were kept on ice. The levels of cellular LY are expressed as μ g of LY/mg of protein. (E) Co-immunoprecipitation of ⁵⁵Fe with *CaMnn5p*-HA and *Mnn5p*-HA. The left-hand panel shows the amount of cellular ⁵⁵Fe accumulation as c.p.m./g of cell pellet, and the inset shows the expression levels of the two HA-tagged proteins determined by Western blot analysis. The right-hand panel shows the amount of ⁵⁵Fe (c.p.m./g of cell pellet) co-immunoprecipitated with *CaMnn5p*-HA and *Mnn5p*-HA. The HA-tagged protein was precipitated by mixing cell lysate with anti-HA antibody-coated agarose beads (open bars) or with naked agarose beads (closed bars). All assays were repeated three times.

and 3.1 times of those detected in *aft1Δ* cells at the same time points. A similar increase of iron uptake was also detected in the *ftr1Δ* and *CRY2α* strains expressing *CaMNN5* (Figure 5C). The results demonstrate that *CaMnn5p* may enhance iron uptake by a mechanism much less efficient than and independent of the high-affinity iron-uptake system.

The enhancement of cell growth by *CaMNN5* depends on endocytosis

A significant fraction of vacuolar iron was brought in via endocytosis in *S. cerevisiae* [24]. The *end4Δ* mutant, which is defective in both receptor-mediated and fluid-phase endocytosis, accumulated much less vacuolar iron than the wild-type [24]. We next asked whether *CaMnn5p* function might be dependent on endocytosis. We expressed *CaMNN5* in *end4Δ* under the control of the *FTR1* promoter, which is activated by iron shortage [14], to see whether *CaMNN5* can promote *end4Δ* growth in iron-limiting media. As shown in Figure 6(A), while *CaMNN5* markedly enhanced the growth of the wild-type strain on the medium containing 200 μ M BPS, it had no such effect on *end4Δ*. The results indicate that *CaMnn5p*'s function is End4p- or possibly endocytosis-dependent. *VPS4* encodes an AAA-type ATPase,

which is required for efficient transport out of the pre-vacuolar endosome, a late step of endocytosis [35]. *vps4Δ* mutants are impaired in the transport of endocytosed fluorescent dyes, plasma membrane receptors and ligands from early to late endosomes [36]. We found that *CaMNN5* expression in *vps4Δ* also failed to enhance cell growth (Figure 6A). Together, the results show that the intact endocytosis pathway is essential for *CaMnn5p* function.

Iron acquired through endocytosis is likely to reach the vacuole before being exported to the cytosol for cell use. We thought that if *CaMnn5p*'s function is endocytosis-dependent, it may not be able to enhance the growth of mutants blocked in the vacuole-cytosol iron transport. The Fet5p-Fth1p complex and Smf3p are known to be responsible for iron export from the vacuoles. We created *smf3Δ*, *fth1Δ* and *smf3Δ fth1Δ* mutants to determine whether *CaMNN5* expression can enhance their growth on iron-limiting plates. As shown in Figure 6(B), *CaMNN5* expression in the wild-type, *smf3Δ* and *fth1Δ* strains enhanced cell growth to a similar extent, but had no such effect on *smf3Δ fth1Δ*. All of the strains grew equally well on iron-sufficient plates. Together, the results support our view that *CaMnn5p* may function along the endocytic pathway. We also examined ⁵⁹Fe uptake into the *CaMNN5*-expressing *end4Δ* and *smf3Δ fth1Δ* strains and found that it enhanced ⁵⁹Fe uptake into *smf3Δ fth1Δ* to the

Figure 7 Subcellular localization of CaMnn5p

(A) CaMnn5p was tagged with HA at the C-terminal end and expressed from a CEN plasmid under the control of the Gal1-10 promoter. (B) Indirect immunofluorescence staining of the CaMnn5p-HA-expressing cells. a, anti-HA antibody was used as the primary antibody; b, the primary antibody was omitted. (C) The subcellular localization of CaMnn5p-HA was determined by using sucrose gradient fractionation and Western blot analysis. The fractionation patterns of Mnn2p and Pep12p were also determined for comparison.

same extent as it did to the wild-type, *aft1*Δ and *ftr1*Δ strains (Figure 6C). However, it did not increase iron uptake into *end4*Δ. The results indicate that *END4*-deletion blocked the endocytosis-mediated iron uptake, while deletion of *SMF3* and *FTH1* impaired iron export from the vacuole without any detectable effect on iron uptake into the cell.

Next we addressed the possibility that the CaMnn5p-dependent iron uptake may be a result of increased liquid-phase endocytosis. We used LY as a marker for fluid-phase endocytosis. We found that, while *end4*Δ showed much less cellular LY accumulation than the wild-type, *CaMNN5* expression did not cause any detectable increase in LY uptake in the wild-type or *end4*Δ cells (Figure 6D), indicating that *CaMNN5* does not enhance the overall fluid-phase endocytosis.

To assess the possibility that CaMnn5p may be involved in iron binding, we expressed HA-tagged CaMnn5p in *aft1*Δ and examined whether iron can be co-immunoprecipitated with CaMnn5p-HA. A strain of *aft1*Δ expressing *S. cerevisiae* Mnn5p-HA was included for comparison. The strains were grown in the presence of 5 μM ⁵⁵Fe for 12 h before immunoprecipitation of the HA-tagged proteins for radioactivity counting. Figure 6(E) shows that, while the cellular iron uptake and expression levels of the HA-tagged proteins are similar in the two strains, the amount of ⁵⁵Fe co-immunoprecipitated with CaMnn5p-HA was approx. 8 times that co-immunoprecipitated with Mnn5p-HA, suggesting that CaMnn5p may bind iron directly or through an interacting protein.

Subcellular localization of CaMnn5p in *S. cerevisiae*

Next we examined the subcellular localization of HA-tagged CaMnn5p in *aft1*Δ. The HA-tagged protein is fully functional in promoting cell growth (Figure 7A). Indirect immunofluorescence microscopy revealed that CaMnn5p-HA localized to some intracellular vesicles with a punctate staining pattern (Figure 7B). On sucrose gradients, the CaMnn5p-containing organelles demonstrated a distribution pattern similar to that of Pep12p (Figure 7C), which is involved in Golgi–vacuole transport and localizes to the Golgi apparatus and endosome [37]. Although this experiment does not have a resolution to pinpoint the exact organelles

for CaMnn5p, the results are consistent with our hypothesis of CaMnn5p functioning along the endocytic pathway.

DISCUSSION

How does CaMnn5p provide a solution to the lack of sufficient iron uptake in *aft1*Δ under iron-limiting conditions? We have addressed several possibilities. First, CaMnn5p activity may indirectly increase the expression or activities of high-affinity iron transporters through glycosylation; secondly, it may function as an iron transporter; and thirdly, we thought that, under the iron-limiting conditions we used, there might still be a low level of iron uptake through processes such as liquid-phase endocytosis, albeit not sufficient to support cell growth. CaMnn5p may help the cells use this source of iron more efficiently.

We ruled out the first possibility, first because we did not detect increased *FTR1* or *FET3* expression in *CaMNN5*-expressing cells. Secondly, *CaMNN5* expression was able to promote the growth of several mutants deleted of iron permease or ferroxidase genes or both, including *ftr1*Δ, *ftr1*Δ *fet3*Δ and *ftr1*Δ *fth1*Δ. Thus we conclude that CaMnn5p functions completely independently of the high-affinity iron transporters.

To determine whether CaMnn5p functions through glycosylation of other proteins, we first confirmed that CaMnn5p expressed in *P. pastoris* possesses α-1,2-mannosyltransferase activity and then created transferase-dead CaMnn5p mutants. We found that the mutated forms of CaMnn5p fully retained the ability to enhance *aft1*Δ growth under iron-limiting conditions. Furthermore, overexpression of either *MNN2* or *MNN5* in *aft1*Δ did not promote cell growth. Thus we conclude that CaMnn5p's growth-enhancing activity in iron-limiting environments does not require the mannosyltransferase activity and is a novel function that may have evolved in *C. albicans*.

To determine whether CaMnn5p enhances iron uptake, we measured ⁵⁹Fe uptake into *aft1*Δ, *ftr1*Δ and wild-type cells transformed with *CaMNN5*. We used a medium containing 200 μM BPS where the Ftr1p–Fet3p complex does not function. Under these conditions, a slow gradual increase in ⁵⁹Fe cellular accumulation was detected in all the strains tested, and all the strains expressing *CaMNN5* consistently exhibited approx. 3-fold more ⁵⁹Fe accumulation than the corresponding control strain lacking *CaMNN5*, indicating that CaMnn5p plays a role in enhancing cellular iron uptake.

The observation of a slow increase of iron accumulation over time in *aft1*Δ and *ftr1*Δ under iron-limiting conditions suggests the presence of other mechanisms for iron entry into the cells. We thought that constitutive endocytosis may mediate this slow iron uptake. Supporting this view, we found that *CaMNN5* expression in *end4*Δ and *vps4*Δ, which are defective in different steps of endocytosis, did not promote cell growth, indicating that an intact endocytosis pathway is required for CaMnn5p's function. The vacuole is thought to be the end point of endocytosis and the iron storage organelle in *S. cerevisiae* [24,25]. Two mechanisms are responsible for the vacuole–cytosol iron transport: one involves the iron permease Fth1p and the other the *Nramp* family bivalent metal transporter Smf3p. If our hypothesis of CaMnn5p working along the endocytosis pathway is correct, it may be predicted that *CaMNN5* expression would not be able to enhance the growth of mutants where the vacuole–cytosol iron transport is completely blocked. Indeed, *CaMNN5* expression did not enhance the growth of the *smf3*Δ *fth1*Δ double mutant, whereas it promoted the growth of *smf3*Δ and *fth1*Δ single mutants. Moreover, CaMnn5p does not increase cellular accumulation of LY, a commonly used marker for fluid-phase endocytosis. Together with the observed co-immunoprecipitation of ⁵⁵Fe with CaMnn5p, these results

suggest that CaMnn5p may have a specific role in iron uptake. Thus we propose the following model. Constitutive endocytosis continuously brings into the cell from the environment a small amount of iron leading to vacuolar iron accumulation. The vacuolar iron can be exported to cytosol by the iron transporters Fet5p–Fth1p and Smf3p on the vacuolar membrane. Although this pathway serves as a source of iron supply, it alone may not be sufficient to support cell growth, especially when the external iron concentration is far below the K_m of the Fet3p–Ftr1p high-affinity iron transporter. CaMnn5p may work along this endocytic iron-supply route, possibly by increasing the efficiency of iron uptake, transport or mobilization, resulting in cell growth under iron-limiting environments. However, the exact molecular mechanism needs further investigation.

The present study takes advantage of the power of *S. cerevisiae* genetics to discover and characterize a *C. albicans* protein that performs a novel function in iron metabolism. We have also characterized the functions of CaMNN5 in *C. albicans*. Our results show that CaMnn5p's activity is regulated by iron, and the deletion of the gene led to the loss of virulence in mice (C. Bai, F. Y. Chan and Y. Wang, unpublished work).

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