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Affinity purification of *Candida albicans* *Ca*Cdc4-associated proteins reveals the presence of novel proteins involved in morphogenesis

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ABSTRACT

Candida albicans CDC4 is nonessential and plays a role in suppressing filamentous growth, in contrast to its evolutionary counterparts involved in the G1–S transition of the cell cycle. Genetic epistasis analysis has indicated that proteins besides Sol1 are targets of *C. albicans* Cdc4. Moreover, no formal evidence suggests that *C. albicans* Cdc4 functions through the ubiquitin E3 ligase of the Skp1-Cul1/Cdc53-F-box complex. To elucidate the role of *C. albicans CDC4*, *C. albicans* Cdc4-associated proteins were sought by affinity purification. A 6×His epitope-tagged *C. albicans* Cdc4 expressed from *Escherichia coli* was used in affinity purifications with the cell lysate of *C. albicans cdc4* homozygous null mutant. *Candida albicans* Cdc4 and its associated proteins were resolved by SDS–PAGE and visualized by silver staining. The candidate proteins were recovered and trypsin-digested to generate MALDI-TOF spectra profiles, which were used to search against those of known proteins in the database to reveal their identities. Two out of four proteins encoded by *GPH1* and *THR1* genes were further verified to interact with *C. albicans* Cdc4 using a yeast two-hybrid assay. We conclude that *in vitro* affinity purification using *C. albicans* Cdc4 generated from *E. coli* as the bait and proteins from cell lysate of *C. albicans cdc4* homozygous null mutant as a source of prey permit the identification of novel proteins that physically interact and functionally associate with *C. albicans* Cdc4.

1. Introduction

The opportunistic human fungal pathogen *Candida albicans*, a natural diploid lacking a conventional sexual cycle, causes disease in healthy and immunocompromised hosts. Considerable efforts have been made towards elucidating the molecular mechanism controlling morphogenesis in *C. albicans*, as it is associated with virulence and pathogenesis. Research has already revealed an unanticipated complexity in that at least three positive and five negative pathways control morphological transitions in *C. albicans* [1,2]. To add even more difficulty, Cdks and many cyclins, along with their regulators, have also been shown to play a role in controlling morphological transitions in *C. albicans* [3]. As such, a critical and underlying issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incompletely understood. Significantly, an intriguing question has recently been revealed by us and others in which key cell cycle genes such as *CDC4* and *GRR1*, which are conserved throughout evolution, play no essential role in cell cycle but affect morphogenesis in *C. albicans* [4–7].

Candida albicans CDC4 encodes a structural homologue of the *Saccharomyces cerevisiae* Cdc4, which is an F-box protein of the ubiquitin E3 ligase family and part of the Skp1-Cul1/Cdc53-F-box (SCF) complex, termed SCF^{Cdc4}. We and others have found that, in contrast to *S. cerevisiae CDC4* being essential for the progression through G1–S transition in the cell division cycle, *C. albicans CDC4* (*CaCDC4*) appears to be nonessential and is a negative regulator of filamentous growth [4,7]. The *C. albicans* homologue of Sic1, termed Sol1, has been isolated as a target of *Ca*Cdc4 [4]. However, the hyperfilamentation phenotype of the double mutant of *Cacdc4*−/− and *sol1*−/− has been shown to be similar to that of the single *Cacdc4*−/− mutant. This result suggests that the stability of Sol1 alone does not explain the constitutive hyphal morphology of *Cacdc4*−/−. We therefore postulated the presence of other *Ca*Cdc4 substrates and conducted affinity purification of *Ca*Cdc4 to identify *Ca*Cdc4-interacting proteins.

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In this report, we describe the identification of *Ca*Cdc4-associated proteins by affinity-purifying a recombinant *Ca*Cdc4 generated from *Escherichia coli* with cell lysate from a *Cacdc4* homozygous null mutant. MALDI-TOF analysis of the purified proteins and confirmation by yeast two-hybrid assays revealed two proteins encoded by the genes *GPH1* and *THR1*, which have not been known to be functionally associated with orthologs of *Ca*Cdc4 from other species. Our study uncovers new components that are functionally associated with *Ca*Cdc4 and will lead to a further understanding of the role of *CaCDC4* in regulating morphogenesis.

2. Materials and methods

2.1. Strains, growth conditions, and DNA methods

Escherichia coli DH5α (F$^-$, φ80d*lacZ*ΔM15, Δ (*lacZYA-argF*) U169, *deoR*, *recA*1, *endA*1, *hsdR*17 (rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1) was used as a host for the routine maintenance and amplification of plasmids. *Escherichia coli* BL21 (F$^-$ *ompT gal dcm lon hsd*S_B (r_B^- m_B^-) λ (DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]) was used to generate the recombinant *Ca*Cdc4 protein. Bacterial cultures were grown in L-broth, supplemented with 50 μg/ml ampicillin as required [8]. Plasmid DNA was purified using the Gene-Spin™-V^2 Miniprep Purification kit (PRO TECH, Taipei, Taiwan). The oligonucleotide primers used to construct the plasmids and in the diagnostic analysis of the strains are listed in Table 1.

The *Cacdc4* homozygous null mutant of *C. albicans* (*Cacdc4*−/−), *Cacdc4*::*dpl200*/*Cacdc4*::*dpl200* *ura3*Δ::λ*imm434*/*ura3*::λ*imm434* *arg4*::*hisG*/*arg4*::*hisG* *his1*::*hisG*/*his1*::*hisG* (Tseng and Shieh, unpublished data), was constructed from the auxotrophic *C. albicans* BWP17 (*ura3*Δ::λ*imm434*/*ura3*::λ*imm434* *arg4*::*hisG*/*arg4*::*hisG* *his1*::*hisG*/*his1*::*hisG*) [9], which was used for the *in vitro* affinity purification of *Ca*Cdc4-associated proteins. *Candida albicans* strains were grown at 30 °C in either complete media of yeast extract–peptone–glucose (YEPD) or synthetic minimal media with uridine [10].

2.2. Expression and purification of recombinant CaCdc4 in bacteria

To express the recombinant protein in *E. coli*, the coding region of *Ca*Cdc4 was PCR amplified with the primers CaCDC4_XhoI_F and CaCDC4_XhoI_R (Table 1), and cloned into the vector pET-29b (+) at the XhoI site. The construct was transformed into *E. coli* BL21 and selected for kanamycin resistant cells. The transformant was pre-cultured in L-broth at 37 °C with 25 μg/ml kanamycin, and the induction of expression of the C-terminally 6×histidine-tagged *Ca*Cdc4 in a total culture of 500 ml was optimized with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 25 °C for 3 h. The cells were lysed in binding buffer containing 0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9, and 0.1% lysozyme (Sigma) by incubating on ice for 30 min followed by successive sonication for 10 min. The lysate containing histidine-tagged *Ca*Cdc4 proteins was centrifuged and the supernatant was filtered through a 0.45-μm filter before purification with HIS-Select nickel affinity gel (Sigma) essentially as described [11]. Protein concentration was determined by the Protein Assay (Bio-Rad) method according to the manufacturer's instruction.

Table 1
Synthetic oligonucleotide primers used in this study.

Name	Sequence
CaCDC4_XhoI_F	5′-GAACTCGAGATGGATAAGAAATCAAAG-3′
CaCDC4_XhoI_R	5′-GAACTCGAGCTGTAAAAGTGGTTGACT-3′
CaCDC4_NcoI_F	5′-TGCGCCATGGTGGATAAGAAATCAAAGCTA-3′
CaCDC4_NcoI_R	5′-TGCGCCATGGTCACTGTAAAAGTGGTTG-3′
CaGPH1_NcoI_F	5′-CATGCCATGGAGATGCCAATGGATTATCTTACC-3′
CaGPH1_BamHI_R	5′-GCGGGATCCCTAAACATTGGATGGTTCAAC-3′
CaSTI1_BglII_F	5′-GGAAGATCTGTATGACAACAGCTGACGAATA-3′
CaSTI1_BglII_R	5′-GGAAGATCTTTATCTGGTACGAATAACACC-3′
CaHMT1_BglII_F	5′-GGAAGATCTGTATGTCTGAATCAGCTACTGAT-3′
CaHMT1_BglII_R	5′-GGAAGATCTCTAACGTAAAAAGTAAGTGTATC-3′
CaTHR1_NcoI_F	5′-CATGCCATGGAGATGAGCGTTATTTCATTTAAAAT-3′
CaTHR1_BamHI_R	5′-GCGGGATCCTTATCGTAAGACATTTAATTTTTA-3′
pACT2_F_(1)	5′-CTATTCGATGATGAAGATACC-3′
pACT2_R_(1)	5′-AGATGGTGCACGATGCAC-3′

Note: Sequences underlined donate site of restriction enzyme.

2.3. Affinity purification of CaCdc4-interacting proteins in vitro

To purify *Ca*Cdc4-associated proteins *in vitro*, the total cell lysate from a *C. albicans Cacdc4*−/− culture in 500 ml YEPD was first prepared as described previously [12]. The cell lysate was centrifuged and the supernatant was filtered through a 0.45-μm filter before being applied to the HIS-Select nickel affinity gel column (Sigma) pre-bound with 6×histidine-tagged *Ca*Cdc4 prepared from *E. coli* cell lysate, and the *Ca*Cdc4-associated proteins were eluted with the 6×histidine-tagged *Ca*Cdc4 by HIS-Select nickel affinity chromatography (Sigma) according to the manufacturer's instructions. Briefly, the column containing the HIS-Select nickel affinity gel bound with 6×histidine-tagged *Ca*Cdc4 proteins was subjected to binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9) twice before applying the proteins from the *Cacdc4*−/− cell lysate. The column was then subjected to binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9) twice, wash buffer (0.5 M NaCl, 20 mM Tris–HCl, pH 7.9 with either 20, 40, 60, or 80 mM imidazole) four times, and elution buffer (0.5 M NaCl, 20 mM Tris–HCl, and 1 M imidazole, pH 7.9) five times.

The proteins were resolved by SDS–PAGE and visualized with silver nitrate staining using a modified version of a previously described method [13]. Briefly, after electrophoresis, gels were washed twice with ddH_2O and then fixed with 50% methanol and 25% glacial acetic acid in ddH_2O for 2 h, followed by washing with 30% methanol for 15 min and subsequently washing three times with ddH_2O for 5 min each. The gels were incubated with a sensitizing solution of 0.8 mM sodium thiosulphate for 2 min, washed twice with dH_2O for 30 s each, followed by incubation in a 0.2% silver nitrate solution for 25 min at room temperature in the dark. After washing twice with dH_2O for 30 s each, the gels were developed with a solution containing 0.28 M sodium carbonate, 0.185% formaldehyde, and 0.016 mM sodium thiosulphate for about 10 min or until the appropriate visualization of proteins was achieved. The reaction was terminated by the addition of 0.042 M EDTA.

2.4. In-gel tryptic digestion and mass spectrometry

The proteins were recovered from the gels and in-gel digested with trypsin as described previously with some modifications [14]. Upon completion of in-gel trypsin digestion, a 3-μl solution of 98% acetonitrile (ACN)/2% formic acid (FA) was added to the digested solution and the solution was sonicated for 10 min. After recovering the supernatant, 0.5 μl of the supernatant sample was spotted onto a MTP AnchorChip™ 600/384 TF (Bruker-Daltonik GmbH, Bremen, Germany) and air-dried for 10 min, followed by spotting 0.5 μl of 1 mg/ml α-cyano-4-hydroxycinammic acid (CHCA) and air-drying for 10 min prior to analysis with an UltraFlexIII MALDI-TOF/TOF mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany) in the Proteomics Research Core Laboratory, Office of Research and Development, China Medical University, Taichung, Taiwan.

2.5. Database-searching to identify proteins

The monoisotopic masses (m/z) of both parent ions and their corresponding fragment ions, parent ion charge states (z), and ion intensities from the acquired mass spectra were automatically extracted using the script in the analyst software and directly submitted for an automated database search against the NCBInr. 2008. 11.25, CANDIDA using MS-Fit of Protein Prospector (University of California San Francisco). Carbamidomethyl cysteine was set as a static modification and one missing cleavage was allowed. The minimum number of peptides required to match was set to four. The Pfactor of the MOWSE score was set to 0.4. The best possible candidate proteins from the search results were evaluated manually for functional relevance and further confirmed by a yeast two-hybrid assay.

2.6. Immunological detection

Escherichia coli cells expressing *Ca*Cdc4 proteins were grown under the optimal inducing condition and the total protein was extracted by the methods described in the previous section. The proteins were resolved by SDS–PAGE and transferred electrophoretically to PVDF membranes (PerkinElmer, Boston, MA). The membranes were probed with a polyclonal antibody against poly-histidine (LTK Biolaboratories, Taoyuan, Taiwan). Detection was performed using a peroxidase-conjugated goat anti-rabbit IgG (H+L) (Pierce, Rockford, IL). The signal was visualized using the SuperSignal West Pico Chemiluminescence Substrate Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.7. Yeast two-hybrid analysis

Yeast two-hybrid interaction assays were performed as described previously [15]. To generate pGBKT7-*CaCDC4*, which expresses a fusion protein of the GAL4 DNA-binding domain, 2307 bp of *CaCDC4* was PCR-amplified with *C. albicans* genomic DNA and the primers CaCDC4_NcoI_F/CaCDC4_NcoI_R, incorporating a NcoI site (Table 1), followed by digesting with NcoI before ligating into the NcoI digested pGBKT7. *Candida albicans* genes, including *GPH1*, *HMT1*, *ST11*, and *THR1*, were initially PCR-amplified and cloned into pCR2.1-TOPO with the primer pairs CaGPH1_NcoI_F/CaGPH1_BamHI_R, CaHMT1_BglII_F/CaHMT1_BglII_R, CaSTI1_BglII_F/CaSTI1_BglII_R, or CaTHR1_NcoI_F/CaTHR1_BamHI_R (Table 1). To generate pACT2-*GPH1*, which expresses a fusion protein of the GAL4 activation domain, the coding region of *GPH1* was PCR-amplified with pCR2.1-TOPO-based *GPH1* and the primer pair CaGPH1_NcoI_F, incorporating an NcoI site, and CaGPH1_BamHI_R, incorporating a BamHI site (Table 1), to produce a 2703-bp fragment, followed by digestion with NcoI/BamHI before ligating into the NcoI/BamHI-digested pACT2 (Clontech; GenBank Accession No. U29899). Similarly, pACT2-*THR1*, -*HMT1*, and *STI1* were obtained using the coding sequences of the respective genes on pCR2.1-TOPO as the template for PCR amplification with the respective 5′ oligonucleotide incorporating an NcoI site (Table 1) and the 3′ oligonucleotide incorporating a BamHI site (Table 1) for respective 822-, 1020-, and 1770-bp fragments. The interaction assay was conducted by mating *S. cerevisiae Mat*a AH109 carrying pGBKT7-*CaCDC4* (our unpublished data) with *S. cerevisiae Mat*α Y187 carrying either pACT2-*GPH1*, -*THR1*, -*HMT1*, or -*ST11*. The ability of diploid *S. cerevisiae* cells to grow on plates of selective media lacking histidine was used as an indicator of interaction due to the *HIS3* reporter gene being activated. The possibility of cell growth being attributed to a basal level of *HIS3* transcription was excluded by growing the cells on plates with selective media lacking histidine and titrating with 3-aminotriazole (3-AT), an inhibitor of *HIS3* gene product.

3. Results and discussion

3.1. Optimal expression of recombinant CaCdc4 in E. coli

We have previously identified *Ca*Cdc4 as a hyphal suppressor in *C. albicans*. To determine if *Ca*Cdc4 plays this role as an ubiquitin E3 ligase of the SCF complex and to identify substrates or potential regulators of *Ca*Cdc4, we adopted an *in vitro* affinity purification approach using a recombinant *Ca*Cdc4 protein purified from *E. coli* as the bait. The approach was based on the fact that the ubiquitination activity of the ubiquitin E3 ligase is functional when all of its components are co-expressed in *E. coli* cells [16]. We anticipated that the *E. coli*-expressed and purified recombinant *Ca*Cdc4 has this function, and hence has an affinity for its associated proteins. In addition, using recombinant *Ca*Cdc4 purified from *E. coli* as a bait to probe for *Ca*Cdc4-associated proteins from *C. albicans* cell lysate eliminates the possibility of the *Ca*Cdc4 substrates being degraded as occurs when *Ca*Cdc4 is expressed in *C. albicans* cells. Under the IPTG-induced condition, *Ca*Cdc4 tagged with 6×histidine at the C-terminus was optimally expressed and purified from *E. coli*, despite a greater abundance of *Ca*Cdc4 in the pellet than in the supernatant (Fig. 1), and used in the affinity purification of its associated proteins *in vitro*.

3.2. In vitro affinity purification of the CaCdc4-associated proteins

To ensure *Ca*Cdc4-associated proteins, particularly the potential targets, would be revealed, we used proteins extracted from *C. albicans Cacdc4* homozygous null mutant (Tseng and Shieh, unpublished data) cell lysate as a source of prey proteins. In fact, we have previously adopted an approach that allows patterns of affinity-purify proteins from *C. albicans* cell lysate of either hyphal or yeast form. The approach is capable of using doxycycline to induce the expression of *Ca*Cdc4 that is C-terminally tagged with 6×histidine and FLAG. It appeared that the doxycycline-inducible system was able to induce the expression of *Ca*Cdc4 in a defined window of time and produce a massive amount of *Ca*Cdc4 for affinity purification. Nevertheless, this over-expressed and presumably hyperactive *Ca*Cdc4 might lead to its own degradation (Lai and Shieh, unpublished data). As a result, such an approach proved to be ineffective for the purification of *Ca*Cdc4 *in vivo*.

The *Ca*Cdc4 purified from bacteria was used in the affinity column purification of proteins from *Cacdc4*−/− *C. albicans* cell lysates. After being washed and eluted, *Ca*Cdc4 and its associated proteins were resolved by SDS–PAGE. The presence of bait *Ca*Cdc4 protein during the wash–elution process was monitored by Western blotting (Fig. 2). The resolved protein samples were also visualized by silver staining (Fig. 2) and the prey proteins selected from the elution steps were those that were not present or were less prominent, although not entirely excluded, in the wash steps. A total of 10 prey proteins (Fig. 2) that appeared consistently in at least two experiments were subjected to further in-gel tryptic digestion and mass spectrometry.

3.3. Determination of the identity of CaCdc4-associated proteins and the implication of their functional relevance to CaCdc4

To determine the identity of proteins detected by affinity purification of *Ca*Cdc4, each of the visualized proteins from SDS–PAGE was recovered and in-gel digested with trypsin to generate peptides for mass spectrometry analysis. After obtaining the specific mass spectra (Fig. 3) of the purified proteins from the bands migrating at positions around 110, 100, 72, 45, 40, 35, 30, 28, 25, or 20 kDa (see Fig. 2), the data were used to search against the NCBInr. 2008. 11.25, CANDIDA using the MS-Fit of Protein Prospector. The 10 examined bands were categorized into 10 groups

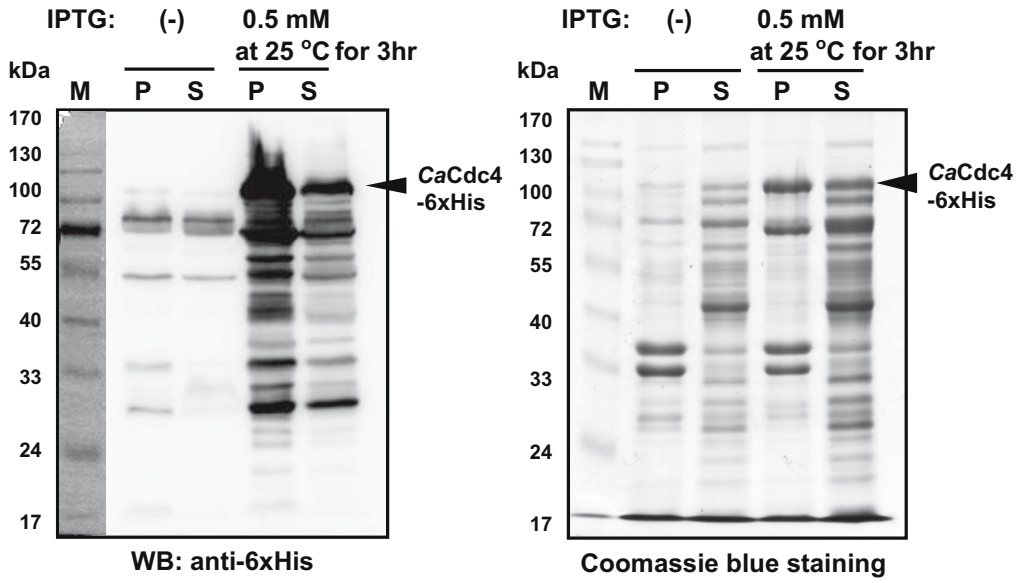

Fig. 1. Optimal expression and purification of *Ca*Cdc4 from *E. coli*. The C-terminal 6xHis-tagged *Ca*Cdc4 was produced in *E. coli BL21* cells containing the plasmid *pET-29b* (+)-*CaCDC4* grown at 25 °C for 3 h under 0.5 mM IPTG induction. Induction of the *Ca*Cdc4 protein (indicated by arrows) was revealed both by Western blotting and Coomassie blue staining. The generated *Ca*Cdc4 was present both in the pellet (P) and in the supernatant (S) of the *E. coli* cell lysates.

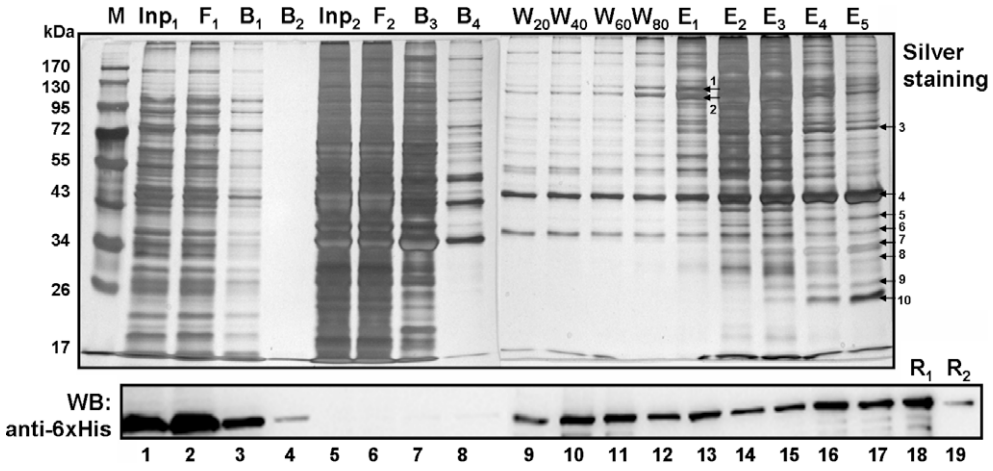

Fig. 2. *In vitro* purification of *Ca*Cdc4-associated proteins. Proteins from *C. albicans Cacdc4*−/− cell lysate were applied to a HIS-Select nickel affinity gel column bound with purified 6×His-tagged *Ca*Cdc4. *Ca*Cdc4-associated proteins purified by *Ca*Cdc4 affinity-chromatography were resolved by SDS–PAGE and the presence of *Ca*Cdc4 was verified by Western blotting (bottom panel). Potential *Ca*Cdc4-associated proteins were eluted and visualized by silver staining (top panel), 10 of which (as indicated by arrows) were subjected to MALDI-TOF analysis. M: size marker; Inp_1: cell lysate from *E. coli* with 6×histidine-tagged *Ca*Cdc4; F_1: flow through after Inp_1; B_1 and B_2: binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9); Inp_2: cell lysate from the *C. albicans Cacdc4*−/− mutant; F_2: flow through after Inp_2; B_3 and B_4: the same as B_1; W_{20}–W_{80}: wash buffer composed of 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9 with imidazole at a concentration of 20 mM (W_{20}), 40 mM (W_{40}), 60 mM (W_{60}), or 80 mM (W_{80}), respectively; E_1–E_5: 0.5 M NaCl, 20 mM Tris–HCl, and 1 M imidazole, pH 7.9; R_1: 10 μl of resin was recovered after B_2; R_2: 10 μl of resin was recovered after B_4.

numbered 1–10 (Fig. 2), some of which contained more than one potential protein (data not shown). Based on both the highest mass spectrum identity score among the proteins within each group and the possible functional relevance to *Ca*Cdc4 matching our interests, 10 prey proteins were initially listed (Table 2). We were particularly interested in the proteins encoded by *GPH1* from group 2, *STI1* from group 5, *HMT1* from group 7, and *THR1* from group 8. *Candida albicans GPH1*, encoding a putative glycogen phosphorylase that is regulated by Tup1, is involved in the hyphal-specific regulation of gene expression [17] and interacts with Ssk1 to provide a regulatory function in cell wall biosynthesis [18]. *Candida albicans* Sti1 is a hyphae-specific protein [19] and is up-regulated during biofilm formation [20]. *Candida albicans HMT1* encodes a major type I protein arginine methyltransferases that is involved in the nuclear export of Npl3, an mRNA binding protein [21], whose *S. cerevisiae* ortholog is required to export mRNA from the nucleus to the cytoplasm and is involved in regulating the budding pattern [22]. *Candida albicans THR1*, encoding a putative homoserine kinase, is not only transcriptionally regulated by Tup1 [17] but also regulated by Gcn4 [23] and is known to co-ordinate morphogenetic and metabolic responses to amino acid starvation [24,25]. These proteins were further verified as *Ca*Cdc4-interacting proteins by yeast two-hybrid assays.

3.4. Validation with yeast two-hybrid reveals two novel proteins encoded by GPH1 and THR1 that directly interact with CaCdc4

To validate that the affinity-purified proteins are indeed *Ca*Cdc4-interacting proteins, yeast two-hybrid assays were performed. Cells of *S. cerevisiae Mat*a AH109 transformed with plasmid

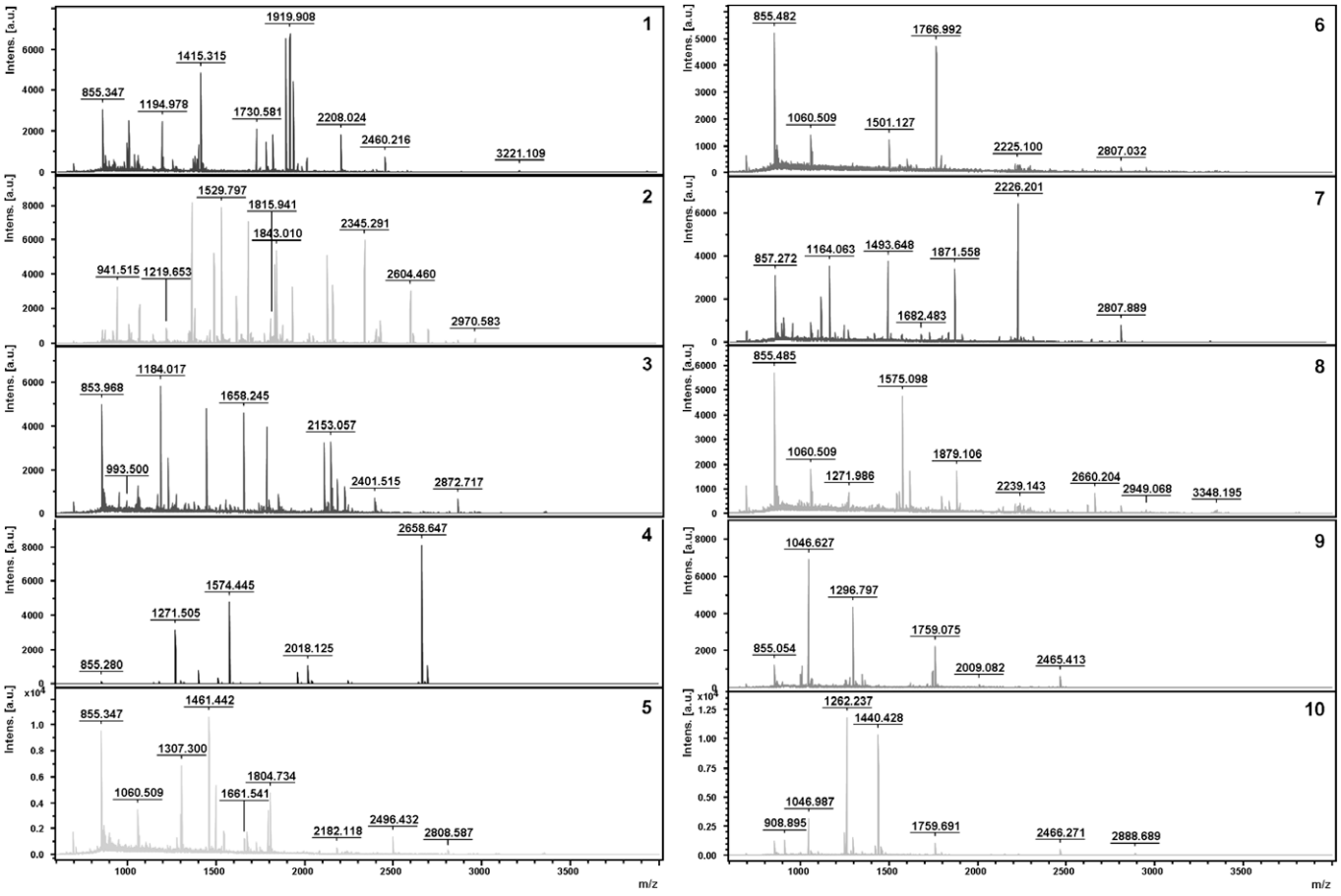

Fig. 3. MALDI-TOF spectra of 10 prey proteins. The *Ca*Cdc4-associated proteins were separated by 10% SDS–PAGE and visualized by silver staining. The bands of interest were excised and in-gel digested with trypsin to generate a spectra profile by mass spectrometry as described in Section 2. The recovered bands were designated as 1–10.

Table 2
Candida albicans Cdc4 associated proteins identified by MALDI-TOF MS.

Band No.[a]	Protein name	CGD systematic name[b]	Score[c]	Seq Cov[d] (%)
1	Potential jumonji-like transcription factor	orf19.5651 (*JHD2*)	64.8	15.4
2	Hypothetical protein CaO19_7021	orf19.7021 (*GPH1*)	1.26E+16	39.2
3	Hypothetical protein CaO19.604	orf19.604 (*PHH1*)	655	8.6
4	Hypothetical protein CaO19.13973	orf19.6652 (*DPB8*)	111	18
5	Hypothetical protein CaO19.3191	orf19.3192 (*STI1*)	4.06	9
6	Likely mitochondrial ribosomal protein MRPL40p	orf19.484 (*MRPL40*)	7.03	13
7	Hypothetical protein CaO19.10801	orf19.3291 (*HMT1*)	486	17.1
8	Likely homoserine kinase	orf19.923 (*THR1*)	47.5	13.2
9	Hypothetical protein CaO19.3689	orf19.3689	62.3	24.4
10	Hypothetical protein CaO19.8055	orf19.425	40.7	20.8

[a] Numbering of the protein bands detected in Fig. 2.
[b] Genes shown in the brackets are those with known homologues of *S. cerevisiae*.
[c] MS-Fit search score of identified proteins.
[d] Sequence coverage (Seq Cov) of the matched peptides in protein.

pGBKT7-*CaCDC4* and capable of expressing a fusion protein of the GAL4 DNA-binding domain were mated with cells of *S. cerevisiae* *Mat*α Y187 transformed with one of the following plasmids: pACT2-*GPH1*, -*THR1*, -*HMT1*, or -*STI1*. The interaction between *Ca*Cdc4 and the potential associated proteins was demonstrated by their ability to grow on agar plates lacking histidine. As shown in Fig. 4A, in the presence of pGBKT7-*CaCDC4*, only the strains of diploid *S. cerevisiae* carrying pACT2-*GPH1* or pACT2-*THR1* were able to form colonies on agar plates lacking histidine, demonstrating that *Ca*Cdc4 interacts with either Gph1 or Thr1 but not with Hmt1 or Sti1. However, we cannot entirely exclude the possibility that Sti1 and Hmt1 are indirectly associated with *Ca*Cdc4 via other proteins that were affinity-purified with *Ca*Cdc4 but not examined by yeast two-hybrid. To eliminate the possibility of cells growing due to a basal level of *HIS3* transcription, serially-diluted cells were spotted onto agar plates without histidine but with various concentrations of 3-AT, and the ability to form colonies was assessed. It was apparent that the interaction of either Gph1 or Thr1 with *Ca*Cdc4 was moderately strong, as transcriptionally activating *HIS3* in diploid *S. cerevisiae* cells co-expressing *Ca*Cdc4 and Gph1/Thr1 resulted in colonies on the plate with 1.25 mM 3-AT that were comparable to those on a plate without 3-AT, even though growth-

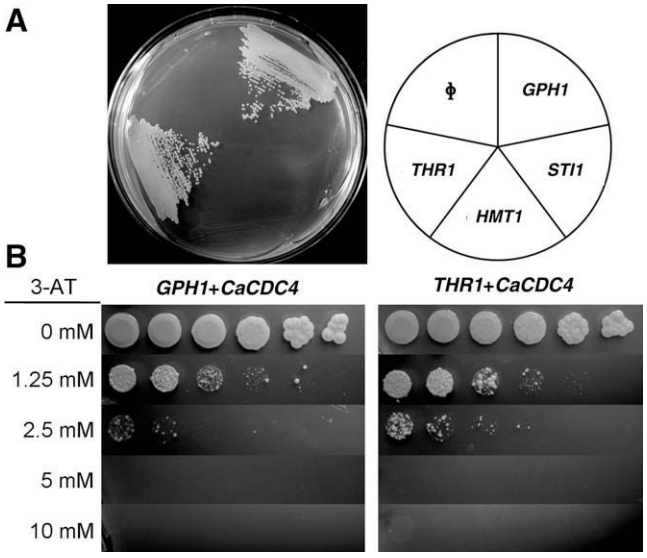

Fig. 4. Interaction of *Ca*Cdc4 with Gph1 or Thr1 using yeast two-hybrid assays with the *HIS3* reporter. (A) Strains of diploid *S. cerevisiae* carrying pGBKT7-*CaCDC4* and the indicated pACT2-based plasmid were streaked onto agar plates with selective media. Diploid *S. cerevisiae* carrying the empty vectors pGKT7 and pACT2 is shown as ϕ. (B) Five-fold serially-diluted cells with a starting concentration of 10^5 cells from (A) were spotted onto agar plate with selected medium and indicated concentration of 3-AT.

inhibition was observed on plates with 3-AT concentrations equal to or greater than 2.5 mM (Fig. 4B). This result indicates that the interaction between Gph1/Thr1 and *Ca*Cdc4 that activates the *HIS3* reporter is indeed genuine and is unlikely to be due to a basal level of *HIS3* transcription.

4. Conclusions

We conclude that two novel proteins encoded by *GPH1* and *THR1* physically interact and functionally associate with *Ca*Cdc4, an F-box protein of the E3 ubiquitin ligase family, in *C. albicans* by using *in vitro* purification in conjunction with mass spectrometry and yeast two-hybrid assays. Establishing the mechanism of how Gph1 and Thr1 regulate *Ca*Cdc4 or how Gph1 and Thr1 are regulated by *Ca*Cdc4 will be critical for further understanding the role *CaCDC4* plays in morphogenesis.

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References

[1] S. Biswas, P. Van Dijck, A. Datta, Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*, Microbiol. Mol. Biol. Rev. 71 (2007) 348–376.
[2] M. Whiteway, C. Bachewich, Morphogenesis in *Candida albicans* (*), Annu. Rev. Microbiol. 61 (2007) 529–553.
[3] J. Berman, Morphogenesis and cell cycle progression in *Candida albicans*, Curr. Opin. Microbiol. 9 (2006) 595–601.
[4] A. Atir-Lande, T. Gildor, D. Kornitzer, Role for the SCFCDC4 ubiquitin ligase in *Candida albicans* morphogenesis, Mol. Biol. Cell 16 (2005) 2772–2785.
[5] D.K. Butler, O. All, J. Goffena, T. Loveless, T. Wilson, K.A. Toenjes, The GRR1 gene of *Candida albicans* is involved in the negative control of pseudohyphal morphogenesis, Fungal Genet. Biol. 43 (2006) 573–582.
[6] W.J. Li, Y.M. Wang, X.D. Zheng, Q.M. Shi, T.T. Zhang, C. Bai, D. Li, J.L. Sang, Y. Wang, The F-box protein Grr1 regulates the stability of Ccn1, Cln3 and Hof1 and cell morphogenesis in *Candida albicans*, Mol. Microbiol. 62 (2006) 212–226.
[7] J.C. Shieh, A. White, Y.C. Cheng, J. Rosamond, Identification and functional characterization of *Candida albicans* CDC4, J. Biomed. Sci. (2005) 1–12.
[8] J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1972.
[9] R.B. Wilson, D. Davis, A.P. Mitchell, Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions, J. Bacteriol. 181 (1999) 1868–1874.
[10] F. Sherman, G.R. Fink, J. Hick, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1986.
[11] J.C. Shieh, H. Martin, J.B. Millar, Evidence for a novel MAPKKK-independent pathway controlling the stress activated Sty1/Spc1 MAP kinase in fission yeast, J. Cell Sci. 111 (Pt 18) (1998) 2799–2807.
[12] J.C. Shieh, Y.C. Cheng, M.C. Su, M. Moore, Y. Choo, A. Klug, Tailor-made zinc-finger transcription factors activate FLO11 gene expression with phenotypic consequences in the yeast *Saccharomyces cerevisiae*, PLoS ONE 2 (2007) e746.
[13] H. Blum, H. Beier, H.J. Gross, Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, Electrophoresis 8 (1987) 93–99.
[14] M. Marchetti-Deschmann, J. Kemptner, C. Reichel, G. Allmaier, Comparing standard and microwave assisted staining protocols for SDS–PAGE of glycoproteins followed by subsequent PMF with MALDI MS, J. Proteomics 72 (2009) 628–639.
[15] M.G. Wilkinson, M. Samuels, T. Takeda, W.M. Toone, J.C. Shieh, T. Toda, J.B. Millar, N. Jones, The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast, Genes Dev. 10 (1996) 2289–2301.
[16] L. Su, N. Lineberry, Y. Huh, L. Soares, C.G. Fathman, A novel E3 ubiquitin ligase substrate screen identifies Rho guanine dissociation inhibitor as a substrate of gene related to anergy in lymphocytes, J. Immunol. 177 (2006) 7559–7566.
[17] A.M. Murad, C. d'Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, A.J. Brown, Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1, Mol. Microbiol. 42 (2001) 981–993.
[18] N. Chauhan, D. Inglis, E. Roman, J. Pla, D. Li, J.A. Calera, R. Calderone, *Candida albicans* response regulator gene SSK1 regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress, Eukaryot. Cell 2 (2003) 1018–1024.
[19] R. Hernandez, C. Nombela, R. Diez-Orejas, C. Gil, Two-dimensional reference map of *Candida albicans* hyphal forms, Proteomics 4 (2004) 374–382.
[20] C.J. Seneviratne, Y. Wang, L. Jin, Y. Abiko, L.P. Samaranayake, *Candida albicans* biofilm formation is associated with increased anti-oxidative capacities, Proteomics 8 (2008) 2936–2947.
[21] A.E. McBride, C. Zurita-Lopez, A. Regis, E. Blum, A. Conboy, S. Elf, S. Clarke, Protein arginine methylation in *Candida albicans*: role in nuclear transport, Eukaryot. Cell 6 (2007) 1119–1129.
[22] L. Ni, M. Snyder, A genomic study of the bipolar bud site selection pattern in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 2147–2170.
[23] H. Tournu, G. Tripathi, G. Bertram, S. Macaskill, A. Mavor, L. Walker, F.C. Odds, N.A. Gow, A.J. Brown, Global role of the protein kinase Gcn2 in the human pathogen *Candida albicans*, Eukaryot. Cell 4 (2005) 1687–1696.
[24] G. Tripathi, C. Wiltshire, S. Macaskill, H. Tournu, S. Budge, A.J. Brown, Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*, EMBO J. 21 (2002) 5448–5456.
[25] T. Gildor, R. Shemer, A. Atir-Lande, D. Kornitzer, Coevolution of cyclin Pcl5 and its substrate Gcn4, Eukaryot. Cell 4 (2005) 310–318.