**THR1 mediates GCN4 and CDC4 to link morphogenesis with nutrient sensing and the stress response in *Candida albicans***

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**Abstract.** *Candida albicans* (*C. albicans*) CDC4 (*CaCDC4*), encoding the F-box protein for the substrate specificity of the Skp1-cullin-F-box E3 ubiquitin ligase complex, suppresses the yeast-to-filament transition in *C. albicans*. In our previous study, Thr1 was identified as a CaCdc4-associated protein using affinity purification. *THR1* encodes a homoserine kinase, which is involved in the threonine biosynthesis pathway. The present study generated a strain with repressible *CaCDC4* expression and continuous *THR1* expression. Colony and cell morphology analyses, as well as immunoblotting, revealed that the Thr1 protein was detectable under conditions in which the expression of *CaCDC4* was repressed and that the filaments resulting from the repressed expression of *CaCDC4* were suppressed by the constitutive expression of *THR1* in *C. albicans*. Additionally, by using the *CaSAT1*-flipper method, the present study produced null mutants of *THR1*, *GCN4*, and *CaCDC4*. The phenotypic consequences were evaluated by growth curves, spotting assays, microscopic analysis, reverse transcription-polymerase chain reaction and XTT-based biofilm formation ability. The results revealed that fewer cells lacking *THR1* entered the stationary phase but had no apparent morphological alteration. It was observed that the expression of *THR1* was upregulated concurrently with *GCN4* during nutrient depletion and that cells lacking *GCN4* rescued the lethality of cells in the absence of *THR1* in conditions accumulating homoserine in the threonine biosynthesis pathway. Of note, it was found that cells with either *CaCDC4* or *THR1* loss were sensitive to oxidative stress and osmotic stress, with those with *THR1* loss being more sensitive. In addition, it was observed that cells with loss of either *CaCDC4* or *THR1* exhibited the ability to increase biofilm formation, with those lacking *CaCDC4* exhibiting a greater extent of enhancement. It was concluded that *CaCDC4* is important in the coordination of morphogenesis, nutrient sensing, and the stress response through *THR1* in *C. albicans*.

**Introduction**

The opportunistic human fungal pathogen *Candida albicans*, a natural diploid with an atypical sexual cycle (1-4), causes vulvovaginal candidiasis in women (5,6) in addition to oral (7,8) and systemic candidiasis in immunocompromised patients (9-11). Substantial effort has been made to elucidate the molecular mechanism underlying morphogenesis in *C. albicans*; morphogenesis is the ability to switch from the ellipsoid blastospore to various filamentous forms (12-15), and it is known to be coupled with virulence and pathogenesis (16-19). Research progress has revealed surprising complexity in that several positive and negative signaling pathways control morphological transition in *C. albicans* (20-22). It is also known that cyclin-dependent kinase and associated cyclins with their regulators control morphological plasticity in *C. albicans* (23,24). As a result, a fundamental issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains to be fully elucidated. It was revealed in our previous study and those of others that certain key cell cycle genes conserved throughout evolution have no essential role in cell cycle but do affect morphogenesis in *C. albicans* (25-30).

Our previous study and those of others have found that *C. albicans* *CDC4* (*CaCDC4*) gene is a negative regulator of filamentation in *C. albicans* (25,30). *C. albicans* Cdc4 (*CaCdc4*) protein contains specific domains of the WD40-repeat and F-box, the homologous of which are required for interacting with Skp1, one of the components of the Skp1-Cdc53/Cull1-F-box (SCF) protein complex, and the substrate (31), respectively. *CaCDC4* appears to encode a canonical F-box protein of SCF ubiquitin ligase (32), termed...
the SCF<sub>CaCDC4</sub>, our previous study found that the domains of F-box and WD40-repeat in CaCdc4 are essential for filamentation (33). Additionally, the domains of the F-box and WD40-repeats in CaCdc4 appeared to suppress flocculation (33). In addition to filamentation (34-36), flocculation is tightly associated with biofilm formation (37-39). Our previous study found that CaCdc4 is involved in negatively regulating biofilm formation in <i>C. albicans</i> (40). Thr1 protein was identified as a CaCdc4-associated protein by in vitro affinity purification (41). The THRI gene encodes a homoserine kinase, which is required for the phosphorylation of homoserine prior to its conversion into threonine by Thr4 protein in the threonine biosynthesis pathway of <i>Saccharomyces cerevisiae</i> (42,43). In <i>C. albicans</i>, THRI null mutants accumulate the toxic biosynthetic intermediate homoserine (44), are attenuated in terms of virulence, and die rapidly during conditions of threonine starvation and serum incubation (45).

It has been shown that GCN4 gene of the TOR nutrient-sensing pathway regulates several biosynthetic pathways of amino acids in <i>S. cerevisiae</i> (46-49). Two Tor proteins, Tor1 and Tor2, have been identified in <i>S. cerevisiae</i> (50,51), whereas only a single Tor homolog is present in <i>C. albicans</i> (52). However, an evolutionarily conserved paradigm for Tor1 signaling in regulating transcriptional responses to nutrient starvation has been observed in <i>S. cerevisiae</i> and <i>C. albicans</i> (53). In <i>S. cerevisiae</i>, starvation of a single amino acid stimulates the expression of genes on all amino acid biosynthetic pathways in a phenomenon known as general amino acid control (GAAC, or the GCN response) (54). This response is dependent upon the bZIP transcriptional activator, Gcn4 protein (55), which interacts with a specific site (56,57) containing RRRWGASTCA (R=purine, W=T or A, and S=G or C), termed the general control response elements (GCREs) (58-60) present in the promoters of its target genes. The expression of GCN4 is regulated at the translational and transcriptional levels in <i>S. cerevisiae</i> (60,61), whereas the translational regulation of GCN4 mRNA does not occur in <i>C. albicans</i> (62-64). Gcn4 is known to stimulate the transcription of at least 30 amino acid biosynthetic genes, representing no less than 12 pathways, in response to starvation of any one of several amino acids in <i>S. cerevisiae</i> (49,54,56,65,66). In <i>C. albicans</i>, GCN4 is involved in the expression of genes in the biosynthetic pathways of amino acids (63,64,67); regulation of the expression of THRI by GCN4 has been confirmed in <i>S. cerevisiae</i> (68), but not in <i>C. albicans</i>. In addition, mitogen-activated protein kinase (MAPK) and Ras-cAMP signaling pathways have been shown to activate filamentous growth in response to starvation (69-72) morphogenesis in <i>C. albicans</i>. MAPK and Ras-cAMP pathways are dependent on transcription factors Cph1 and Efg1, respectively (73). Of note, amino acid starvation induces Gcn4, which activates the transcription of amino acid biosynthetic genes in an Efg1-independent manner via the GCRE element in their promoters of <i>C. albicans</i> (63). Therefore, Gcn4 appears to stimulate morphogenesis by interacting with the Ras-cAMP pathway in <i>C. albicans</i>.

In the present study, it was found that Thr1 protein was detected in conditions when the expression of CaCdc4 was repressed, and the filaments resulting from the repressed expression of CaCdc4 were suppressed by the constitutive expression of THRI in <i>C. albicans</i>. To investigate the role of THRI in association with GCN4 and CaCdc4 in <i>C. albicans</i>, single THRI and GCN4 null mutants and a double THRI GCN4 null mutant were generated. The Thr1 null mutant cells appeared to be maintained as the yeast form but entered the stationary phase with fewer numbers of cells and did not form hyphae in response to serum. The expression of THRI was upregulated along with GCN4 under nutrient-limited conditions, and the gcn4 null mutant cells alleviated the lethality of cells lacking THRI. Cells without either THRI or CaCdc4 were sensitive to stress conditions but showed enhancement in biofilm formation. Therefore, CaCdc4 appears to be required for the coordination of morphogenesis, nutrient sensing and the stress response through THRI in <i>C. albicans</i>.

**Materials and methods**

**General manipulation, media and growth conditions.** The <i>Escherichia coli</i> strain DH5α was used for regular manipulation of the plasmids. All <i>C. albicans</i> strains (Table I) were derived from the clinically isolated wild-type strain SC5314 (74) or the auxotrophic strain BW17 (arg4/arg4 his4/his4 his3/ura3/ura3) (75). The regular media and growth conditions for the strains of <i>E. coli</i> and <i>C. albicans</i> were as described previously (76). Briefly, <i>E. coli</i> cultures were grown in Luria-Bertani medium (LB) or LB supplemented with 50 µg/ml of ampicillin or 34 µg/ml of chloramphenicol on plates with 2% agar at 37°C. All <i>C. albicans</i> strains were routinely grown in either 1% yeast extract, 2% peptone and 2% glucose (YPD), or synthetic complete medium (0.67% yeast nitrogen base without amino acids, 0.2% amino acid dropout mix and 2% glucose) and synthetic defined minimal medium (0.67% yeast nitrogen base without amino acids and 2% glucose) on plates with 2% agar at 30°C. The reagents for the media used were all supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Spider medium [1% nutrient broth (cat. no. 234000; BD Biosciences, Franklin Lakes, NJ, USA), 1% mannitol (cat. no. M9647; Sigma-Aldrich; Merck KGaA), and 0.2% K2HPO4 (pH 7.2 after autoclaving)] and 10% fetal bovine serum (cat. no. 10099133; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in YPD were used to induce hyphal growth. To induce the TOR-dependent signaling pathway, rapamycin (Rapa; cat. no. R0395; Sigma-Aldrich; Merck KGaA), and 0.2% K2HPO4 (pH 7.2 after autoclaving) and 10% fetal bovine serum (cat. no. 10099133; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in YPD were used to induce hyphal growth. To induce the TOR-dependent signaling pathway, rapamycin (Rapa; cat. no. R0395; Sigma-Aldrich; Merck KGaA), and 0.2% K2HPO4 (pH 7.2 after autoclaving) and 10% fetal bovine serum (cat. no. 10099133; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in YPD were used to induce hyphal growth. To induce the TOR-dependent signaling pathway, rapamycin (Rapa; cat. no. R0395; Sigma-Aldrich; Merck KGaA), and 0.2% K2HPO4 (pH 7.2 after autoclaving) and 10% fetal bovine serum (cat. no. 10099133; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in YPD were used to induce hyphal growth. To induce the TOR-dependent signaling pathway, rapamycin (Rapa; cat. no. R0395; Sigma-Aldrich; Merck KGaA), and 0.2% K2HPO4 (pH 7.2 after autoclaving) and 10% fetal bovine serum (cat. no. 10099133; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in YPD were used to induce hyphal growth.

**Strain use and construction.** The <i>C. albicans</i> GCN4 deletion strain gen4<sup>Δ</sup>/gcn4<sup>Δ</sup> (81) and <i>C. albicans</i> CDC4 deletion strain Cacdc4<sup>Δ</sup>/CaCdc4Δ (40) were derived from
Table I. *Candida albicans* strains used in the present study and their source.

| Author, year | Name of strain | Parental strain | Genotype | (Refs.) |
|--------------|----------------|-----------------|----------|---------|
| Ernst, 2000  | SC5314         | Wild-type strain | (69)     |         |
| Murad *et al*, 2001 | BWP17 | ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG | (70) |         |
| Present study | *CaCDC4* Tet-Off/- | BWP17 | *Cacdc4::FRT/Cacdc4::PTET* | - |
| Present study | *CaCDC4* Tet-Off/-| | *Cacdc4::FRT/Cacdc4::PTET* | - |
| Present study | *CaCDC4* Tet-Off/-| *CaCDC4* Tet-Off/- | *Cacdc4::FRT/Cacdc4::PTET* | - |
| Present study | *CaCDC4* Tet-Off/-| *CaCDC4* Tet-Off/- | *Cacdc4::FRT/Cacdc4::PTET* | - |
| Present study | *CaCDC4* Tet-Off/-| *CaCDC4* Tet-Off/- | *Cacdc4::FRT/Cacdc4::PTET* | - |
| Chen *et al*, 2013 | gcn4Δ/gcn4Δ | SC5314 | gcn4::FRT/gcn4::FRT | (76) |
| Tseng *et al*, 2015 | *Cacdc4*Δ/*Cacdc4*Δ | SC5314 | *Cacdc4::FRT/Cacdc4::FRT* | (40) |
| Present study | *THR1*/*thr1*Δ | SC5314 | *THR1/*thr1*::FRT* | - |
| Present study | *thr1*Δ/*thr1*Δ | SC5314 | *thr1*::FRT/*thr1*::FRT | - |
| Present study | *thr1*Δ/*thr1*Δ gcn4Δ/gcn4Δ | SC5314 | *thr1*::FRT/*thr1*::FRT gcn4::FRT/gcn4::FRT | - |

*CaCDC4, Candida albicans* CDC4.
the wild-type strain SC5314 and constructed previously (Table I). To allow the constitutive expression of THR1 in *C. albicans* carrying the expression repressible *CaCDC4*, the coding sequence of the *THR1* gene was polymerase chain reaction (PCR)-amplified from genomic DNA of the *C. albicans* wild-type strain SC5314 (74) with a pair of primers CaTHR1(2)_Xhol_Full_F and CaTHR1(2)_Sphl_R (Table II) and cloned into the plasmid vector p6HF- *ACT1* (40) to generate p6HF- *ACT1*- THR1 capable of constitutively expressing *THR1*. To construct the *CaCDC4* Tet-Off/- (*P_{tet}-CaCDC4/Cacdc4::PTET*) strain, the Tet-off system cassette was PCR-amplified from pWT61 (81) with a pair of primers (Table II) containing a 60-bp sequence corresponding to the upstream of the *CaCDC4* locus and the initial 60 bp of the coding sequence of *CaCDC4*. This was transformed into the auxotrophic strain WP17I and selected for hygromycin B (HygB)-resistance to obtain strain *P_{tet}-CaCDC4::H/Cacdc4* (*CaCDC4/Cacdc4::P_{tet}-CaCDC4::HygB*), in which one of the two *CaCDC4* alleles was replaced with the Tet-off cassette. Subsequently, the *KpnI/SacI*-digested *CaSAT1*-flipper cassette from pSF52A-*CaCDC4* (40) was transformed into the *P_{tet}-CaCDC4::H/Cacdc4* and selected for nourseothricin resistance to obtain *P_{tet}-CaCDC4::H/Cacdc4::S* (*Caacdc4::SAT1-FLIP/Cacdc4::P_{tet}-CaCDC4::HygB*). Furthermore, the *CaCDC4*/*CaCDC4*::*P_{tet}-CaCDC4::H/Cacdc4::S* strain was subjected to maltose-induced FLP/FRT recombination for recycling of the dominant selectable markers to generate *P_{tet}-Caacdc4::FRT* (*Caacdc4::FRT/Cacdc4::P_{tet}-CaCDC4::FRT*) (Table I), as previously described (81). The *CaCDC4* Tet-Off/- (*P_{tet}-CaCDC4/Cacdc4::PTET*) contains the two modified *CaCDC4* alleles, one of which contained deletion of the majority of the *CaCDC4* coding sequence with a copy of *FRT* sequences, and the other had its expression under the control of the Tet-off system. The *CaCDC4* expression-repressible strain *CaCDC4* Tet-Off/- (Table I), for which the expression of *CaCDC4* was repressed in the presence of 40 μg/ml Dox, was used to introduce the *NcoI*-linearized plasmid p6HF- *ACT1*- THR1, in addition to the empty plasmid p6HF- *ACT1* and p6HF- *ACT1*- *CaCDC4* (40) targeting and integrating at the *RPI1* locus to generate *CaCDC4* Tet-Off/- (*P_{tet}-CaCDC4/Cacdc4::PTET*) and *CaCDC4* Tet-Off/- (*P_{tet}-CaCDC4/Cacdc4::PTET*) (Table I). In addition, *THR1* was deleted in the *C. albicans* wild-type strain SC5314 via the *CaSAT1*-flipper method (83). The *SAT1*-flipper method depends on the use of a cassette that contains a dominant nourseothricin resistance marker (*CaSAT1*) for the selection of integrative transformants and a *C. albicans*-adapted FLP gene that permits the subsequent excision of the cassette, which is flanked by FRT sites of the FLP target sequences, from the genome. Briefly, the upstream and downstream regions of *THR1* were amplified with primer pairs THR1(2)_KpnI_US_F/THR1(2)_Xhol_US_R and THR1(2)_SacI_DS_F/THR1(2)_SacI_DS_R, respectively (Table II), and with template DNA of the genomic DNA extracted from SC5314. These were sequentially cloned into the pSF52A plasmid with a *CaSAT1*-flipper cassette at *KpnI/Xhol* and *SacI* sites to generate the pSF2A-thr1Δ

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### Table II. Synthetic oligonucleotide primers used in the present study.

| Name                        | Sequence(5′-3′)               |
|-----------------------------|------------------------------|
| CaTHR1(2)_Xhol_Full_F       | CCGCTCGAGATGACTCAATCAGAAGATTTTTTTT |
| CaTHR1(2)_Sphl_R            | ACATGCGATGCGTCAAGATTTTTTTATC   |
| THR1(2)_KpnI_US_F           | CGGGGTACCGCCTGACCTGATTTAGTT    |
| THR1(2)_Xhol_US_R           | CCCGCCTCGAGTGTAAATAGGTTAGGCC   |
| THR1(2)_SacI_DS_F           | TCCCCCGGGGTTCAATGTGAAATATAATG  |
| THR1(2)_SacI_DS_R           | CTAGGAGGCTCGGTTAAATGCTCAATCTCC |
| CaCDC4TF1F                  | ATTAATCAATTATATATTATAGCGAAAACTTTAT |  
| CaCDC4TF1R                  | TATTAGCCATCCTCTCGCTCAAGGATATTGATTCTCATG |
| CaGCN4S1F                   | AGTTAGACCTTTCTGATATATCTGATGAAAGTGGAGGATTTATATC |
| CaGCN4S2R                   | AAGTTAGCACTTTGTTTTTTTTTTTTTTTTTTTTTTTTT |
| CaGCN4-part-SalI-F          | ACAGCGTCAAGCGTGAATTGTTGTCGAC   |
| CaGCN4-part-BamHI-R         | CGGCGATCCAAAATGTTACATATACCTCTT |
| THR1-KpnI-US-F              | CGGGGTACCGTCATATTGATCGACAG    |
| THR1-Xhol-US-R              | CCCGCCTCGAGATACCTAATATACCTCAG  |
| CaACT1-F                    | TAGAGAAGATTGCTGTTTTTA          |
| CaACT1-R                    | GCATTTCTTGTTCGAAATTCC         |
| CaTHR1(1)-BamHI-R           | GCGGGAATCCATACCTCATATACACAGTT |
| Mal-R                       | AGCGAACGGGGTGTCACAAAA          |

*Restriction enzyme sites are shown in bold. CaCDC4, *Candida albicans* CDC4; F, forward; R, reverse.*
plasmid. A cassette released from pSF2A-thr1Δ through the use of KpnI/SacII was introduced into SC5314 and selected for a nourseothricin-positive response (Nou*), following the excision of the CaSAT1-containing cassette by induction in YCB-maltose to generate the thr1 heterozygous null mutant, THR1/thr1Δ. The pSF2A-thr1Δ cassette was introduced into THR1/thr1Δ and selected for Nou*, following CaSAT1 excision by induction in YCB-maltose for FLP/FRT recombination to generate the thr1 homozygous null mutant, thr1Δ/thr1Δ. To generate the THR1 and GCN4 double deletion strain, the CaHygB and CaSAT1-flipper cassettes (81) were PCR-amplified with a pair of primers CaGCN4S1F and CaGCN4S2R (Table II), each of which contained 60 bp of the sequence corresponding to the upstream and downstream sequences of the CaGCN4 locus. The amplified cassettes were then sequentially transformed into the thr1 homozygous null mutant, thr1Δ/thr1Δ, and selected for HygB* and Nou*, respectively, followed by malto-induced CaSAT1 pop-out to generate THR1 and the GCN4 double deletion mutant, thr1Δ/thr1Δ gcn4ΔA/gcn4ΔA (Table I).

**Nucleic acid extraction and PCR analysis.** The **C. albicans** cells were grown to mid-log phase, and genomic DNA was isolated using a MasterPure™ Yeast DNA Purification kit (Epicentre, Madison, WI, USA) following the manufacturer's protocol. The total RNA derived from cells cultured to mid-log phase was extracted using a MasterPure™ Yeast RNA Purification kit (Epicentre; Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocol. Subsequently, 5 µg of total RNA was used to generate cDNA using a SuperScript III Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Briefly, a total of 13 µl mix containing 1 µl oligo(dT)20 (50 µM), 5 µl of total RNA (1 µg/µl), 1 µl dNTP mix (10 mM), and 6 µl of DEPC-treated d2H2 O was heated to 65°C for 5 min and incubated on ice for 3 min to denature and keep the 2’ structure of RNA. Next, a total of 20 µl mix containing the 13 µl-mix, 4 µl 5X first-strand buffer [250 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 15 mM MgCl2, and 500 µl of 100 mM DTT], 1 µl DTT (0.1 M), and 1 µl SuperScript III RT (200 U/µl) was produced and incubated at 50°C for 60 min to generate first-strand cDNA, followed by incubation at 72°C for 15 min to terminate the reaction. A total of 25 µl mix containing 1 µl cDNA (from first-strand cDNA reaction), 2.5 µl 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 37.5 mM MgCl2], 2.5 µl dNTP mix (2 mM), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.3 µl Taq DNA polymerase (5 U/µl), and 17.7 µl d2H2 O was also produced. The mix was heated to 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 85 sec, then a further extension for 7 min at 72°C was performed. The cDNA was then subjected to PCR with a pair of THR1-specific primers, THR1-KpnI-US-F and THR1-Xhol-US-R (Table II), annealing the downstream of the THR1 coding sequence with a predictive product of 254 bp. The CaGCN4-part-Sall-F and CaGCN4-part-BamHI-R primers (Table II) annealing the GCN4 coding sequence were used to generate a predicted product of 380 bp. The CaActin-F and CaActin-R primers (Table II) were used to generate a **C. albicans** ACT1-specific product, which was used as a loading control. To verify the THR1 deletion strain, the THR1-KpnI-US-F, CaTHR1(1)-BamHI-R, and Mal-R primers were used as they specifically generate products with predictive sizes that are associated with changes in the THR1 locus.

**Protein extraction and western blot analysis.** The total protein was extracted from the **C. albicans** cells as described previously (81). The protein was partially purified from cells containing the p6HFACTIp plasmid with the ORF of the gene integrated at RP10 capable of generating a tagged (6xHis and FLAG) protein using Ni2+-NTA-agarose beads (Qiagen, Inc., Valencia, CA, USA) as previously described (84). The concentration of the whole protein extracts was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For separation of total extract, 25 µg of each total extract was subjected to Ni2+-NTA-agarose beads purification, loaded into each lane of the gel and resolved using 10% SDS-PAGE and transferred electrophoretically onto PVDF membrane (Pall Life Sciences, Port Washington, NY USA). The PVDF membrane was blocked at room temperature for 1 h in blocking buffer [TBST (137 mM sodium chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6) with 5% nonfat milk]. The blot was washed thrice for 5 min each in TBST. Subsequently, the blot was probed with anti-FLAG antibody (cat. no. #F7425, Sigma-Aldrich; Merck KGaA; 1:2,000) in the blocking buffer at 4°C overnight. The blot was then washed thrice for 15 min each with TBST prior to the addition of the secondary anti-mouse Immunoglobulin G-peroxidase conjugated (cat. no. A9044; Sigma-Aldrich; Merck KGaA; 1:10,000) for 1 h at room temperature. Finally, the blot was washed thrice with TBST for 15 min prior to visualization using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce; Thermo Fisher Scientific, Inc.). The proteins detected were recorded with a Luminescent Image Analyzer (FUJIFILM LAS-1000; Fujifilm, Tokyo, Japan) and analyzed using ImageGauge 3.46 and L Process v 1.96 (Fujifilm).

**Spotting assay.** The spotting assays were performed as previously described (81). Briefly, cells of the **C. albicans** strains were grown in YPD medium (Sigma-Aldrich; Merck KGaA) with 180 rpm shaking at 30°C to the mid-log phase. The cultured strains were diluted to an optical density (OD) of 1.0 at OD600 (~<x>10^2</x> cells ml^-1) and then serially diluted from 10^7 to 10^1 cells ml^-1. The diluted cultures were spotted on agar plates at a volume of 5 µl and left to grow a colony.

**Biofilm assay.** To assess the ability to form biofilm between **C. albicans** cells lacking THR1 or CaCDC4, cells of the strains were used to establish biofilms on nonpyrogenic polystyrene, and the XTT reduction capabilities of the biofilm cells were determined as previously described (40).

**Cellular image capture and recording.** The cells in liquid culture were visualized and recorded with a Nikon 50i microscope at 400 magnification. Images of the colonies were captured with a MEIJI stereoscopic microscope EMZ5 at x40 magnification. The monographs were digitized and processed using Adobe Photoshop software version 7.0.1 (Adobe Systems, Inc., San Jose, CA, USA).
Statistical analysis. The quantification of the biofilm formation assay was conducted in three independent experiments, performed in triplicate. Statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA), by one-way analysis of variance, followed by Tukey's post hoc analysis. The results are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Filamentous growth due to the repressed expression of CaCDC4 in C. albicans can be partially induced by the expression of THR1. Our previous study identified the Thr1 protein as a CaCdc4-interactor (41). To understand the functional association between CaCDC4 and THR1, a C. albicans strain CaCDC4 Tet-Off/capable of repressing the expression of CaCDC4 in the presence of Dox (Tet-Off) (data not shown) and constitutively expressing THR1, together with those expressing and not expressing CaCDC4, were constructed (Fig. 1A). To assess the effect of the expression of THR1 on the filamentous growth of cells with the expression of CaCDC4 repressed, the cells of these strains described, together with their parental strain, were plated onto rich (YPD) media or were grown in synthetic complete media (Fig. 1C) with or without 40 µg/ml Dox. As expected, the constitutive expression of CaCDC4, but not the empty plasmid, completely suppressed the filamentous mode of growth when the expression of CaCDC4 was repressed. The constitutive expression of THR1 partially induced the filamentous mode of growth when the expression of CaCDC4 was repressed. These results suggest that THR1 is functionally associated with CaCDC4 with regard to the control of morphogenesis and that THR1 positively modulates hyphal formation.

As THR1 was shown to positively modulate hyphal development (Fig. 1B and C), it was hypothesized that Thr1, similar to Sol1 (25), is the target of CaCdc4 and is regulated by ubiquitination for degradation. To assess the possible regulation of CaCdc4 and Thr1, cells of the same strains were grown in minimal media with or without Dox, and the proteins were extracted...
and subjected to western blot analysis (Fig. 1D). The repressed expression of CaCDC4 led to an increase of the protein level of Thr1 but the de-repressed expression of Ca\textit{cdc}4 resulted in a reduction in the protein level of Thr1. The results suggested that the CaCdc4 negatively regulates the protein level of Thr1 and that Thr1 positively controls filamentation.

Cells with loss of THR1 reach a stationary phase earlier than those of the wild-type. Thr1 is a homoserine kinase in S. cerevisiae and presumably also in C. albicans, which is responsible for the biosynthesis of threonine. The present study aimed to ascertain whether cells that lack THR1 show impaired growth. To determine whether THR1 is involved in growth, the CaSAT1-flipper method (83) was used to construct the THR1 homozygous null mutant (thr1\textDelta/thr1\textDelta). PCR-based analyses were used to verify the mutants. As shown in Fig. 2A and B, genomic DNAs extracted from each of the strains with specific primers generated PCR products with the expected sizes. Therefore, it was confirmed that the constructed mutants were correct. By RT-PCR analyses, the expression of THR1 was only observed in the wild-type SC5314 (\textit{THR1}/\textit{THR1}) and the \textit{THR1} heterozygous null mutant (\textit{THR1}/thr1\textDelta), but not in the homozygous null mutant (thr1\textDelta/thr1\textDelta) (Fig. 2C), which was as expected. Cells of the thr1\textDelta/thr1\textDelta mutant, together with the \textit{THR1} heterozygous null mutant (\textit{THR1}/thr1\textDelta) and the wild-type SC5314 (\textit{THR1}/\textit{THR1}), were grown in YPD for 48 h to establish the growth curves. As shown in Fig. 2D, the thr1\textDelta/thr1\textDelta entered a stationary phase with fewer cells than the other strains, suggesting that \textit{THR1} maintains threonine biosynthesis for normal growth in \textit{C. albicans}.

Figure 2. Construction and growth curve establishment of \textit{C. albicans} THR1 homozygous null mutants. (A) Diagram of the \textit{THR1} locus and the primers used for detection. Red arrows donate primer THR1(2)\_KpnI\_US\_F. Blue arrows donate primer THR1(2)\_SacI\_DS\_R. Green arrows donate primer Mal-R. (B) Assessment of size change of the \textit{THR1} locus by PCR with specific primers. thr1\textDeltaS and thr1\textDelta donate \textit{THR1} deleted with or without the CaSAT1 cassette, respectively. The expected sizes of products are indicated. (C) RT-PCR evaluation of different \textit{THR1} null mutants. (D) Growth curves established in YPD with \textit{THR1} homozygous null mutants and the wild-type strain SC5314. Two independent isolates (2 and 18) of \textit{THR1} heterozygous null mutants were used. #1, THR1(2)\_KpnI\_US\_F primer; #2, THR1(2)\_SacI\_DS\_R primer; #3, Mal-R primer; OD, optical density; RT-PCR, reverse transcription-polymerase chain reaction.
The present study assessed whether mutant (thr1 Δ) in hyphal development. Cells of the thr1 Δ heterozygous null mutants were used. thr1ΔS represents the presence of the SAT1 marker and can be induced by growth with maltose to become thr1Δ. Scale bar=10 µm.

Figure 3. THR1 does not directly contribute the yeast-to-hypha transition but is required for hyphal growth under serum-induced conditions. Cells of the strains in the mid-log phase were grown in (A) YPD or (B) YPD with 10% fetal bovine serum at 37°C for 3 h. Two independent isolates (2 and 18) of the THR1 heterozygous null mutants were used. thr1ΔS represents the presence of the SAT1 marker and can be induce by growth with maltose to become thr1Δ. Scale bar=10 µm.

THR1 positively regulates filamentous development indirectly. As constitutively expressing THR1 partially induced the filamentous development caused by the repression of CaCDC4 in C. albicans, this suggests that THR1 may positively control hyphal formation in C. albicans (Fig. 1). Therefore, the present study assessed whether THR1 is directly involved in hyphal development. Cells of the THR1 homozygous null mutant (thr1Δ/thr1Δ), together with the THR1 heterozygous null mutant (THR1/thr1Δ) and the wild-type SC5314 (THR1/THR1), were grown exponentially in YPD and were subjected to microscopic analysis. As shown in Fig. 3A, cells of the mutants grew as yeast forms, as in the wild-type, with none of the mutants exhibiting the hyphal mode of growth. These results indicated that THR1 is not directly involved in the yeast-to-hypha transition in C. albicans. Although the THR1 homozygous null mutant of C. albicans was not observed to be directly involved in the yeast-to-hypha transition, the present study examined the effect of the hyphal induction condition on the mutant. Cells of the THR1 homozygous null mutant (thr1Δ/thr1Δ), together with the THR1 heterozygous null mutant (THR1/thr1Δ) and the wild-type SC5314 (THR1/THR1), were grown exponentially in YPD supplemented with 10% fetal bovine serum at 37°C and were subjected to microscopic analysis. The cells of the THR1 heterozygous null mutant and the wild-type SC5314 proliferated and induced with the hypha normally, whereas those of the THR1 homozygous null mutant were inhibited in their growth and showed impaired in hyphal formation (Fig. 3B). The filament was also induced using Spider medium (85) at 30 and 37°C, with similar results as that of serum (data not shown). These results suggested that C. albicans THR1 is indirectly involved in the resistance of serum and the yeast-to-hypha transition.

Expression of THR1 and GCN4 are concurrently induced upon nutrient depletion in C. albicans. In the budding yeast S. cerevisiae, THR1 encodes the homoserine kinase required for threonine biosynthesis, one of the biosynthetic pathways of amino acids, several of which have been known to be regulated by the transcription factor Gcn4 (63), under the control of the target of rapamycin (TOR) signaling pathway (46,47). To ascertain whether activation of the TOR pathway in C. albicans induces the expression of THR1 via GCN4, the expression of those two genes in cells under limited nutrient conditions was examined. Wild-type SC5314 cells were grown to mid-log phase, followed by treatment with either Rapa or 3-AT, a competitive inhibitor of the product of the HIS3 gene that is known to activate the TOR pathway (63). The cells were collected and subjected to RT-PCR analysis (Fig. 4). As shown in Fig. 4A, the cells treated with either Rapa or 3-AT showed activated expression of GCN4 and THR1 maximally at 3 h but weakened activation at 6 h. This response appeared to be dose-dependent as shown in Fig. 4B. Additionally, it was found that, although the 1-kb upstream region of one THR1 allele contains ‘TGACTGA’, that of the other THR1 allele contains ‘TGACTGA’ and ‘TGATTCA’ (data not shown), which is the known GCRE element as the target site for Gcn4 (59). In the present study, no expression of THR1 was detected in cells of the GCN4 homozygous null mutant (gcna4Δ/gcna4Δ) (data not shown) (81), suggesting the dependency of the expression of THR1 on GCN4. These results suggested that GCN4 may be the direct transcription factor activating the expression of THR1, and THR1 is likely transactivated by Gcn4 through the TOR nutrient-sensing pathway.

C. albicans cells lacking THR1 show impaired growth on nutrient limitation, or when deprived of amino acid supply, but can be rescued by the absence of GCN4. As the stress sensitivity of the thr1 homozygous mutant has been reported previously (44,45), the present study examined whether the thr1 homozygous mutant is also sensitive to nutrient limitation.
or altered conditions in terms of amino acid supply. It was first verified that the thr1 homozygous mutant showed impaired growth. Homoserine markedly enhanced the toxicity (Fig. 5A), which is consistent with a previous observation that the accumulation of homoserine in C. albicans cells lacking THR1 or the addition of homoserine to the culture of C. albicans leads to the death of cells (44). The growth response of the thr1 homozygous mutant in the TOR pathway-induced condition was then examined. As shown in Fig. 5B, cells lacking THR1 were significantly impaired in their ability to grow in either the rich YPD medium or the minimal SC medium. The growth of the cells weakened further in the presence of Rapa and 3-AT, with 3-AT exhibiting a more potent effect. However, this growth inhibition was partially reversed with simultaneous deletion of GCN4, suggesting that the growth defect due to the absence of THR1, most likely via the accumulation of homoserine in the threonine biosynthesis pathway (Fig. 5B), was rescued by the deletion of GCN4. This may be a result of inhibiting the expression of the genes upstream of THR1 in the threonine biosynthesis pathway or directing them to other biosynthetic pathways at or downstream from THR1. As expected, although the addition of aspartate had no effect on the inhibitory growth in cells lacking THR1 or those lacking both THR1 and GCN4 in the minimal synthetic defined (SD) medium without amino acids (Fig. 5C upper panel), the addition of threonine rescued the growth defect with or without aspartate (Fig. 5C lower panel). These results confirmed that the relief of homoserine accumulation frees the cells from the effect of toxicity.

C. albicans THR1 and CaCDC4 are involved in adaptation to stressful conditions. As previous reports have shown that cells that loss of THR1 is sensitive to a variety of stresses (44) and specifically oxidative stress (data not shown), the present study aimed to ascertain whether CaCDC4 was similar. Cells of the homozygous null CaCdc4 and Thr1 were subjected to a spotting assay on a plate containing H2O2 or menadione. Cells with loss of either CaCDC4 or THR1 alone were impaired in growth (Fig. 6A), with minimal negative effects in 2 mM H2O2 (Fig. 6B). However, cells with loss either CaCDC4 or THR1 were sensitive to menadione, with those lacking THR1 exhibiting higher sensitivity. To determine whether THR1 and CaCDC4 are involved in other stress responses, cells were cultured in media containing 0.7 M NaCl. As shown in Fig. 6B, cells lacking THR1 were more sensitive to high osmolarity than those lacking CaCDC4. These results suggested that both CaCDC4 and THR1 are required for growth under oxidative and osmotic stresses, although THR1 more so, and both may have a general role in stress adaptation.

Cells with loss of either CaCDC4 or THR1 exhibit enhanced biofilm formation, with CaCDC4 loss showing greater enhancement. As CaCDC4 negatively regulates biofilm formation, the present study aimed to ascertain whether THR1 has a similar effect. The homozygous null Cacdc4 and Thr1 cells were subjected to a biofilm formation XTT assay. As shown in Fig. 7, cells with loss of either CaCDC4 or THR1 exhibited the ability to augment biofilm formation, with those lacking CaCDC4 showing a greater degree of enhancement. These results suggested that, although both CaCDC4 and THR1 negatively regulate biofilm formation, CaCDC4 has a major effect.

It is known that C. albicans GCN4, which is dependent on TOR1 control (53), negatively regulates filamentous growth (86) and positively regulates biofilm formation (87), and that C. albicans high osmolarity glycerol (HOG1) suppresses filamentous development (88) and requires stress resistance (89,90). These reports, and the fact that decreased TOR signaling leading to reduced Hog1 basal activity sustains...
hyphal development in *C. albicans* (91), links the two signaling pathways of HOG stress and TOR nutrient sensing. The results of the present study suggest that the functional interactions among *CaCDC4*, *THR1* and *GCN4*, in which *THR1* mediates *GCN4* and *CaCDC4*, links morphogenesis and the nutrient sensing/stress response in *C. albicans*.

**Discussion**

In the present study, the *CaCdc4*-associated protein Thr1, which had been identified previously (41), was further characterized. The functional interaction of the Thr1-encoded gene *THR1*, *CaCDC4*, and the potential *THR1*-associated gene *GCN4* were assessed. As the domains of the F-box and WD40-repeat are present in *CaCdc4*, *CaCDC4* is likely to encode a typical F-box protein of SCF ubiquitin ligase (32) known as SCF<sup>CaCdc4</sup>. These domains are critical for filamentous growth (33), demonstrating that *CaCdc4* is a negative regulator of filamentation (25,30) and is likely to regulate its targets via SCF<sup>CaCdc4</sup> ubiquitin ligase-dependent degradation. It was found that the filamentous growth caused by the repressed expression of *CaCDC4* in *C. albicans* was partially suppressed by the constitutive expression of *THR1* (Fig. 1B and C). The reason for this can be explained by the avoidance of Thr1 being completely degraded by the SCF<sup>CaCdc4</sup> ubiquitin ligase. Similar to the degradation of Sol1 in *CaCdc4*-depleted *C. albicans* cells (25), the present study observed the increased level of Thr1 when the TetO-driven *CaCDC4* was de-repressed in *C. albicans* (Fig. 1D). Although F-box proteins have been shown to act independently of the SCF complex, through

![Figure 5](image_url)
Figure 6. Cells lacking either CaCDC4 or THR1 are sensitive to oxidative and osmotic stress. Cells were diluted in 10^6 cells ml^-1, and 10-fold dilutions were spotted in 5 µl aliquots on (A) YPD plates containing (B) 2 mM H₂O₂ or 0.1 mM menadione, or (C) 0.7 M NaCl. The plates were incubated at 30°C for up to 2 days. CaCDC4, Candida albicans CDC4.
binding interactions or with intrinsic enzymatic activities (92), thrl represents a typical SCF\textsuperscript{CaCdc4} target, negatively regulated by CaCdc4 that depends on the ubiquitin-proteasome pathway. Of note, threonine is known to be the critical residue for O-linked mannosylation (93). Whether the constitutive expression of \textit{THR1} increases the levels of mannoproteins that are associated with cell wall structure and leads to the suppression of filamentous growth in the \textit{CaCDC4}-deficient condition requires further investigation.

The subset of amino acid biosynthetic pathways including the threonine biosynthetic pathway are absent in humans (94) but are conserved in fungi, and several are required for virulence and survival \textit{in vivo} (45,95-97). Therefore, various fungal amino acid biosynthetic enzymes and their encoded genes, including the \textit{C. albicans} homoserine kinase-encoded gene \textit{THRL} (44,45), have become potential antifungal targets to be exploited. The \textit{thr1Δ/thr1Δ} mutant was created in the present study, and it was demonstrated that \textit{THRL} is nonessential, as previously reported (44,45). However, it was found that \textit{C. albicans} cells lacking \textit{THRL} entered the stationary phase at a lower density than the wild-type cells in non-starved conditions (Fig. 2D), which agrees with a previous observation (45) and is likely the result of low threonine concentration in the serum (98) as a threonine-starved condition.

The expression of several genes involved in the biosynthesis of a variety of amino acids through the TOR signaling pathway in a \textit{GCN4}-dependent manner has been well characterized in \textit{S. cerevisiae} (46,47), and similar regulation exists in \textit{C. albicans} (52,53,63,81,99). However, the nature of the regulation of the expression of \textit{THRL} during threonine biosynthesis in \textit{C. albicans} remains unclear. The present study verified that \textit{THRL} is under the control of the TOR pathway and is dependent on \textit{GCN4}, due to the induced expression of \textit{THRL} and \textit{GCN4} by Rapa and 3-AT, respectively. The growth impairment of the \textit{thr1Δ/thr1Δ} mutant in either the rich YPD or minimal SC media was revealed and was more marked on the YPD plate with Rapa. This agrees with the previous observation that the accumulation of homoserine in the \textit{C. albicans} \textit{thr1Δ/thr1Δ} mutant is toxic (44). However, the growth impairment of the \textit{thr1Δ/thr1Δ} mutant was relieved by introduction of the \textit{GCN4} deletion (\textit{thr1Δ/thr1Δ gcn4Δ/gcn4Δ}) under the conditions described above. These results indicate that \textit{THRL} in \textit{C. albicans} is under the control of the \textit{TOR-GCN4} pathway, and \textit{GCN4} may also control the expression of threonine biosynthesis genes upstream of \textit{THRL} in \textit{C. albicans}, which has been demonstrated in \textit{S. cerevisiae} (100,101), as the loss of \textit{GCN4} reduces the expression of those genes, decreasing the accumulation of homoserine. Threonine appeared to rescue cells lacking \textit{THRL} in a dose-dependent manner, presumably

Figure 7. \textit{CaCDC4} and \textit{THRL} suppress biofilm formation. (A) Cells of the WT SC5314, heterozygous \textit{CaCDC4} null mutant (\textit{CaCDC4}/\textit{CaCdc4Δ}) and homozygous \textit{CaCDC4} null mutant (\textit{CaCdc4Δ}/\textit{CaCdc4Δ}) and the (B) WT SC5314, heterozygous \textit{THRL} null mutant (\textit{THRL}/\textit{thr1Δ}), and homozygous \textit{THRL} null mutant (\textit{thr1Δ/thr1Δ}) were subjected to an in vitro XTT reduction assay for biofilm formation. \textit{***}P<0.001, as indicated. ns, not significant; \textit{CaCDC4}, \textit{Candida albicans CDC4}; WT, wild-type.
due to the inhibition of homoserine accumulation. Homoserine is converted from aspartate consecutively by Hom3, Hom2, and Hom6, followed by the sequential actions of Thr1 and Thr4 to generate threonine. Therefore, the toxicity resulting from homoserine accumulation in cells lacking THR1 was enhanced in the presence of aspartate, but threonine was able to alleviate the toxicity. The feedback inhibition of Hom3 by threonine may be present in C. albicans as in S. cerevisiae (102). Additionally, the C. albicans THR1-deleted strain showed sensitivity to 5-fluorocytosine under specific conditions, but not to other antifungal agents, including amphotericin B, fluconazole, ketoconazole, and caspofungin (45), indicating intertwining between the threonine and nucleotide biosynthetic pathways in which only specific conditions exert an effect. Further investigation of the interplay between the threonine and nucleotide biosynthetic pathways is required to examine the therapeutic potential.

The present study confirmed that cells lacking either CaCDC4 or THR1 were susceptible to stressful conditions, including oxidative and osmotic agents, although those lacking THR1 were more susceptible. These results indicate the possibility of THR1 and CaCDC4 interacting with the HOG pathway. In S. cerevisiae, it has been shown that mutations of CDC4 suppress cell death due to the Hog1-induced reactive oxygen species (ROS) accumulation (103), which is the opposite to that observed in C. albicans cells lacking CaCDC4. Although the Sir2-induced suppression of Hog1-induced ROS accumulation is dependent on the transcription of Msn2 and Msn4 in S. cerevisiae, Msn2- and Msn4-like transcription factors have no clear roles in the stress responses of C. albicans (104). This result underlines the rewiring of the transcriptional regulatory circuits in C. albicans (62,105). The fact that cells lacking CaCDC4 and THR1 enhanced biofilm formation may be necessary for their survival, as they are susceptible to stressful conditions and nutrient limitation.

It is known that C. albicans Gcn4 activates the GCERLUC reporter in an Efg1-dependent manner (63). Therefore, C. albicans Gcn4 may activate morphogenesis by interacting with Efg1, which is required for filamentation (73) and the downstream component of the Ras-cAMP pathway. The C. albicans CDC4 homozygous null mutant is known to enhance the expression of hyphal-specific ECE1 and HWPI genes and stabilize the filament inducer Sol1 (25) and Thr1 (Fig. 1D). ECE1 and HWPI appear to be regulated by Efg1 (106,107). Therefore, Thr1 is negatively regulated by CaCdc4 and may positively control filamentation through ECE1 and HWPI in either an Efg1-dependent or -independent manner. Additionally, the transcription factor Efg1 has been identified as a downstream target of the cAMP regulatory circuit (108,109). Therefore, the Ras-cAMP pathway and Gcn4 activate filamentation through ECE1 and HWPI in an Efg1-dependent manner. It appears that while CaCDC4 and GCN4 can modulate threonine biosynthesis and morphogenesis mediated by THR1, GCN4 and the Ras-cAMP pathway can regulate morphogenesis through Efg1. Finally, as CaCDC4 suppressed filamentation, it was hypothesized that the morphological alteration of C. albicans is a result of its response to environmental cues in which the availability of the required molecules in cells is reprogrammed so that the cellular structures can be reorganized. Therefore, it is logical that common targets are shared by the morphological transition, stress response and nutrient limitation.

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Authors’ contributions

YTL and JCS conceived and designed the study and supervised the project. YWS and YYF established and verified the strains, and performed various phenotypic analyses. HCH and SMW performed critical phenotypic analyses and provided reagents. TLT contributed to the establishment of the initial strains and analyses. THL designed the study and provided consultation of data analyses. All authors analyzed the data, discussed the results and commented on the manuscript. JCS wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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