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 *Ca*Cdc4-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*.

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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 (*Ca*Cdc4) 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/Cul1-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^{CaCdc4}; our previous study found that the domains of F-box and WD40-repeat in *Ca*Cdc4 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 *C. albicans* (40). Thr1 protein was identified as a *Ca*Cdc4-associated protein by *in vitro* affinity purification (41). The *THR1* 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 *Saccharomyces cerevisiae* (42,43). In *C. albicans, THR1* 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 *S. cerevisiae* (46-49). Two Tor proteins, Tor1 and Tor2, have been identified in *S. cerevisiae* (50,51), whereas only a single Tor homolog is present in *C. albicans* (52). However, an evolutionarily conserved paradigm for Tor1 signaling in regulating transcriptional responses to nutrient starvation has been observed in *S. cerevisiae* and *C. albicans* (53). In *S. cerevisiae*, 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 *S. cerevisiae* (60,61), whereas the translational regulation of *GCN4* mRNA does not occur in *C. albicans* (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 *S. cerevisiae* (49,54,56,65,66). In *C. albicans*, *GCN4* is involved in the expression of genes in the biosynthetic pathways of amino acids (63,64,67); regulation of the expression of *THR1* by *GCN4* has been confirmed in *S. cerevisiae* (68), but has not in *C. albicans*. 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 *C. albicans*. 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 *C. albicans* (63). Therefore, Gcn4 appears to stimulate morphogenesis by interacting with the Ras-cAMP pathway in *C. albicans*.

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 *THR1* in *C. albicans*. To investigate the role of *THR1* in association with *GCN4* and *CaCDC4* in *C. albicans*, single *THR1* and *GCN4* null mutants and a double *THR1 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 *THR1* was upregulated along with *GCN4* under nutrient-limited conditions, and the *gcn4* null mutant cells alleviated the lethality of cells lacking *THR1*. Cells without either *THR1* 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 *THR1* in *C. albicans*.

Materials and methods

General manipulation, media and growth conditions. The *Escherichia coli* strain DH5α was used for regular manipulation of the plasmids. All *C. albicans* strains (Table I) were derived from the clinically isolated wild-type strain SC5314 (74) or the auxotrophic strain BWP17 (*arg4*/*arg4 his1*/*his1 ura3*/*ura3*) (75). The regular media and growth conditions for the strains of *E. coli* and *C. albicans* were as described previously (76). Briefly, *E. coli* 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 *C. albicans* 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% K_2HPO_4 (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 3-amino-1,2,4-triazole (3-AT; cat. no. A8056; Sigma-Aldrich; Merck KGaA) were used. Plasmid DNA was purified using the Gene-Spin@-V2 Miniprep Purification kit (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan). The *E. coli* strain DH5a was transformed with plasmid DNA by $CaCl_2$, as described previously (77), or by electroporation (78). The *C. albicans* strains were transformed using the LiAc/PEG/ssDNA method (79) or electroporation (80). Transformants were selected in YPD containing 200 μg/ml nourseothricin (WERNER BioAgents GmbH, Jena, Germany). The Tet-off system was regulated by adding 40 μg/ml of Doxycycline (Dox; Sigma-Aldrich; Merck KGaA) to the medium.

Strain use and construction. The *C. albicans GCN4* deletion strain *gcn4*Δ/*gcn4*Δ (81) and *C. albicans CDC4* deletion strain *Cacdc4*Δ/*Cacdc4*Δ (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*::P_{TET}-*CaCDC4:FRT*	-
Present study	*CaCDC4* Tet-Off/-|p6HF-*ACT1*p	*CaCDC4* Tet-Off/-	*Cacdc4::FRT*/*Cacdc4*::P_{TET}-*CaCDC4:FRT RPS1*/*rps1*::p6HF-*ACT1*p	-
Present study	*CaCDC4* Tet-Off/-|*CaCDC4*	*CaCDC4* Tet-Off/-	*Cacdc4::FRT*/*Cacdc4*::P_{TET}-*CaCDC4: FRT RPS1*/*rps1*::p6HF-*ACT1*p-*CaCDC4*	-
Present study	*CaCDC4* Tet-Off/-|*THR1*	*CaCDC4* Tet-Off/-	*Cacdc4::FRT*/*Cacdc4*::P_{TET}-*CaCDC4:FRT RPS1*/*rps1*::p6HF-*ACT1*p-*THR1*	-
Chen *et al*, 2013	*gcn4*Δ/*gcn4*Δ	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	*THR1*/*thr1*Δ	*thr1::FRT*/*thr1::FRT*	-
Present study	*thr1*Δ/*thr1*Δ *gcn4*Δ/*gcn4*Δ	*thr1*Δ/*thr1*Δ	*thr1::FRT*/*thr1::FRT gcn4::FRT*/*gcn4::FRT*	-

CaCDC4, *Candida albicans* CDC4.

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

Name	Sequence(5'-3')[a]
CaTHR1(2)_XhoI_Full_F	CCG**CTCGAG**ATGACTCAATCAGAAATTTTTTTTT
CaTHR1(2)_SphI_R	ACAT**GCATGC**TGCTAAGACATTTAATTTTTTATTC
THR1(2)_KpnI_US_F	CGG**GGTACC**GCCTGACCCTGATTATAGTT
THR1(2)_XhoI_US_R	CCG**CTCGAG**TGGTTAAATAAAGTTGTAAGCC
THR1(2)_SacII_DS_F	TCC**CCGCGG**GGTCAATATGTTGAATATAAATG
THR1(2)_SacI_DS_R	CTAG**GAGCTC**CGTTAAACTAGCCTAACTTCC
CaCDC4TF1F	ATTATTATTATTATTATTATCGAGAAAGGAACCTGATTTCGTTTTATT TTAACCCATTACCTTGGACTCTTGAATCCGCGGGCTTTGATTCTCAA
CaCDC4TF1R	ATTTAGCCGTCTCCTCGCTCAAAGGATATTTGAATATCTTATCCATG CACCAGCTCCGGTACCACT
CaGCN4S1F	TATTTAAATTAAATTACATTACATTAATTAGCTTTGTTACCATTATTATT ATTAGATAAAGAAGCTTCGTACGCTGCAGGTC
CaGCN4S2R	AATTTTCTAAATTTTTCTTTTTTTAAAAAAATAACGAGAGGTATATAT AGTAGTAACTTTTCTGATATCATCGATGAATTCGAG
CaGCN4-part-SalI-F	ACGC**GTCGAC**GTGAAGTTGTTGTTGCAAC
CaGCN4-part-BamHI-R	CGC**GGATCC**CAAATTGAATACCATTAACTCTT
THR1-KpnI-US-F	CGG**GGTACC**CTGTCACTATTGATCCTAGT
THR1-XhoI-US-R	CCGCTCGAGATTACCTAAATATACTCCAGC
CaACT1-F	TAGAAGAAGTTGCTGCTTTA
CaACT1-R	GCATTTCTTGTTCGAAATCC
CaTHR(1)-BamHI-R	GCG**GGATCC**CTAACTTCCATATTCAACAGTT
Mal-R	AGCGAACGGGGTGTACACAA

[a]Restriction enzyme sites are shown in bold. CaCDC4, *Candida albicans* CDC4; F, forward; R, reverse.

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)_XhoI_Full_F and CaTHR1(2)_SphI_R (Table II) and cloned into the plasmid vector p6HF-*ACT1*p (82) to generate p6HF-*ACT1*p-*THR1* capable of constitutively expressing *THR1*. To construct the *CaCDC4* Tet-Off/-(P_{TET}-*CaCDC4*/*Cacdc4*Δ) strain, the Tet-off system cassette was PCR-amplified from pWTF1 (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 BWP17 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 *Kpn*I/*Sac*I-digested *CaSAT1*-flipper cassette from pSFS2A-*CaCDC4* (40) was transformed into the P_{TET}-*CaCDC4:H*/*CaCDC4* and selected for nourseothricin resistance to obtain P_{TET}-*CaCDC4:H*/*Cacdc4*Δ*S* (*Cacdc4::SAT1-FLIP*/*Cacdc4::*P_{TET}-*CaCDC4:HygB*). Furthermore, the *CaCDC4* P_{TET}-*CaCDC4:H*/*Cacdc4*Δ*S* strain was subjected to maltose-induced *FLP*/*FRT* recombination for recycling of the dominant selectable markers to generate P_{TET}-*Cacdc4*/*Cacdc4*Δ (*Cacdc4::FRT*/*Cacdc4::*P_{TET}-*CaCDC4:FRT*) (Table I), as previously described (81). The *CaCDC4* Tet-Off/-(P_{TET}-*CaCDC4*/*Cacdc4*Δ) 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 *Nco*I-linearized plasmid p6HF-*ACT1*p-*THR1*, in addition to the empty plasmid p6HF-*ACT1*p and p6HF-*ACT1*p-*CaCDC4* (40) targeting and integrating at the *RP10* locus to generate *CaCDC4* Tet-Off/-|*THR1*, *CaCDC4* Tet-Off/-|p6HF-*ACT1*p and *CaCDC4* Tet-Off/-|*CaCDC4*, respectively (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)_XhoI_US_R and THR1(2)_SacII_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 pSFS2A plasmid with a *CaSAT1*-flipper cassette at *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites to generate the pSF2A-*thr1*Δ

plasmid. A cassette released from pSF2A-*thr1*Δ through the use of *Kpn*I/*Sac*II 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*/*thr1*Δ. The pSF2A-*thr1*Δ cassette was introduced into *THR1*/*thr1*Δ and selected for Nou^+, following *CaSAT1* excision by induction in YCB-maltose for *FLP*/*FRT* recombination to generate the *thr1* homozygous null mutant, *thr1*Δ/*thr1*Δ. 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, *thr1*Δ/*thr1*Δ, and selected for $HygB^+$ and Nou^+, respectively, followed by maltose-induced *CaSAT1* pop-out to generate *THR1* and the *GCN4* double deletion mutant, *thr1*Δ/*thr1*Δ *gcn4*Δ/*gcn4*Δ (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 d_2H_2O 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 $MgCl_2$, 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 $MgCl_2$], 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 d_2H_2O 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-XhoI-US-R (Table II), annealing the downstream of the *THR1* coding sequence with a predictive product of 254 bp. The CaGCN4-part-SalI-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 p6HF-*ACT1*p plasmid with the ORF of the gene integrated at *RP10* capable of generating a tagged (6xHis and FLAG) protein using Ni^{2+}-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 Ni^{2+}-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 OD_{600} (~$2x10^7$ cells ml^{-1}) and then serially diluted from 10^7 to 10^2 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 x400 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).

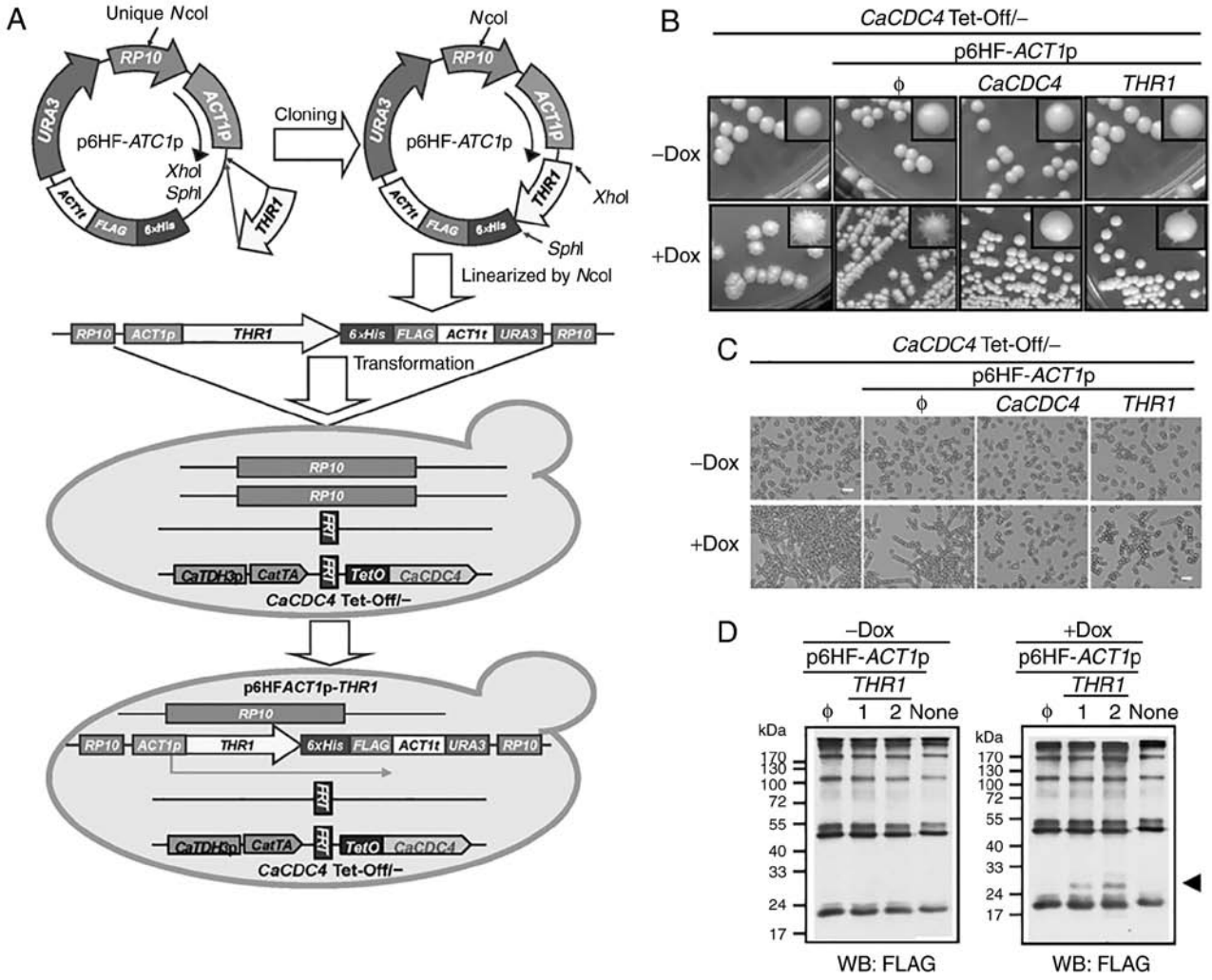

Figure 1. Constitutive expression of either type of *THR1* suppresses the filamentous mode of growth when the expression of *CaCDC4* is repressed. (A) A diagram to illustrate the strains used. (B) The cells were plated on YPD plates, each of which produced an enlarged colony monograph (magnification, x160) of the original image (magnification, x40). (C) The cells were grown in SC, with or without 40 μg/ml Dox. Scale bar=10 μm. (D) Concurrent presence of CaCdc4 and Thr1 proteins. Cells of the strains were grown in SC with or without 40 μg/ml Dox and subjected to western blot analysis. Anti-FLAG antibody was used as Thr1 is tagged with FLAG. Lanes 1 and 2 represent different isolates of strains with p6HF-*ACT1*p-*THR1*. The triangle indicates the migrated position of the Thr1 protein. *Ca*Cdc4, *Candida albicans CDC4*; SC, synthetic complete medium; ϕ, empty plasmid p6HF-*ACT1*p; Dox, doxycycline.

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 *Ca*Cdc4-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 (Fig. 1B) 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 *Ca*Cdc4 and is regulated by ubiquitination for degradation. To assess the possible regulation of *Ca*Cdc4 and Thr1, cells of the same strains were grown in minimal media with or without Dox, and the proteins were extracted

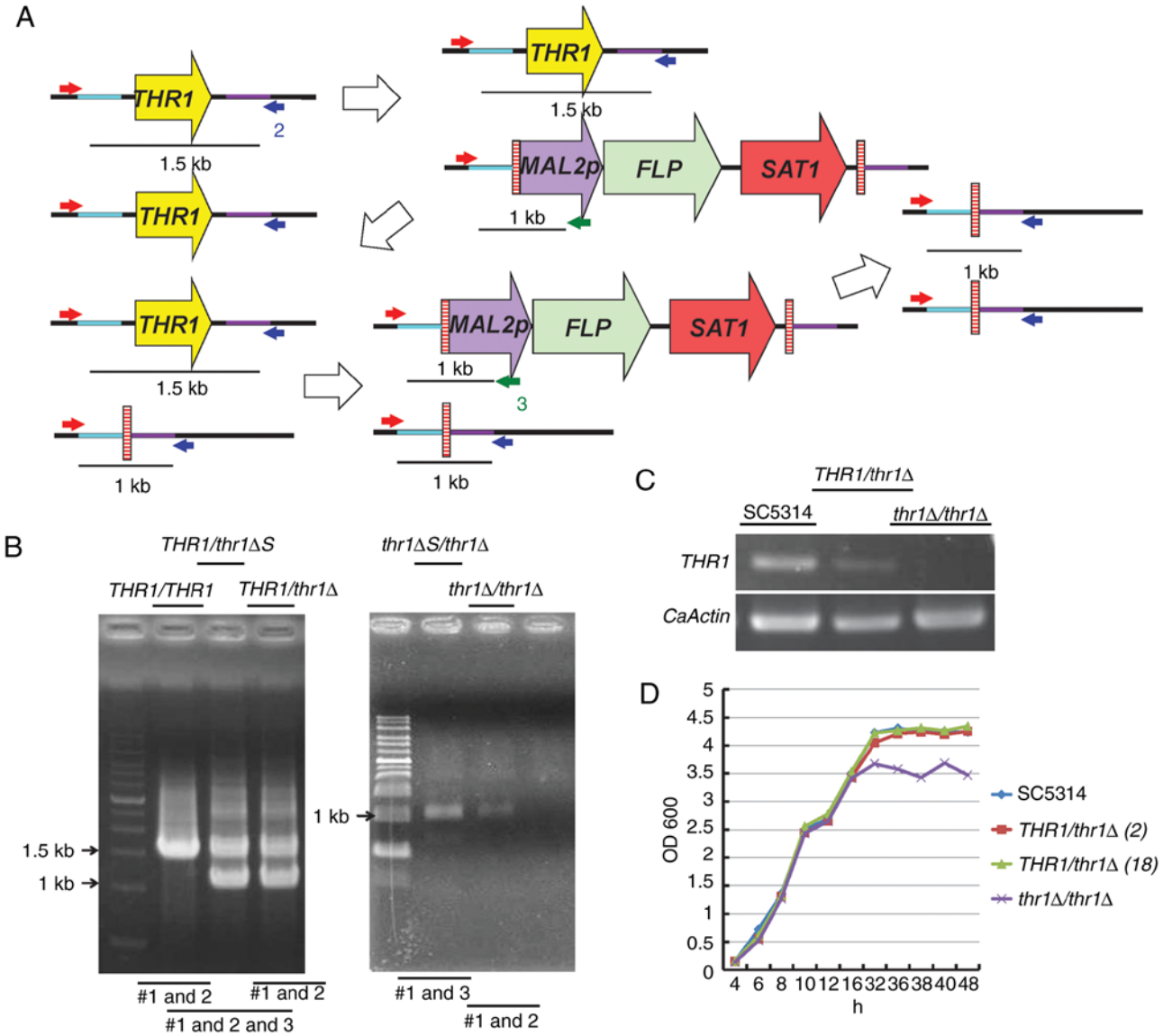

Figure 2. Construction and growth curve establishment of *C. albicans THR1* homozygous null mutants. (A) Diagram of the *THR1* locus and the primers used for detection. Red arrows donate primer THR1(2)_KpnI_US_F. Blue arrows donate primer THR1(2)_SacI_DS_R. Green arrows donate primer Mal-R. (B) Assessment of size change of the *THR1* locus by PCR with specific primers. *thr1*ΔS and *thr1*Δ donate *THR1* deleted with or without the *CaSAT1* cassette, respectively. The expected sizes of products are indicated. (C) RT-PCR evaluation of different *THR1* null mutants. (D) Growth curves established in YPD with *THR1* homozygous null mutants and the wild-type strain SC5314. Two independent isolates (2 and 18) of *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.

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*CDC4 resulted in a reduction in the protein level of Thr1. The results suggested that the *Ca*Cdc4 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*Δ/*thr1*Δ). 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 (*THR1*/*THR1*) and the *THR1* heterozygous null mutant (*THR1*/*thr1*Δ), but not in the homozygous null mutant (*thr1*Δ/*thr1*Δ) (Fig. 2C), which was as expected. Cells of the *thr1*Δ/*thr1*Δ mutant, together with the *THR1* heterozygous null mutant (*THR1*/*thr1*Δ) and the wild-type SC5314 (*THR1*/*THR1*), were grown in YPD for 48 h to establish the growth curves. As shown in Fig. 2D, the *thr1*Δ/*thr1*Δ entered a stationary phase with fewer cells than the other strains, suggesting that *THR1* maintains threonine biosynthesis for normal growth in *C. albicans*.

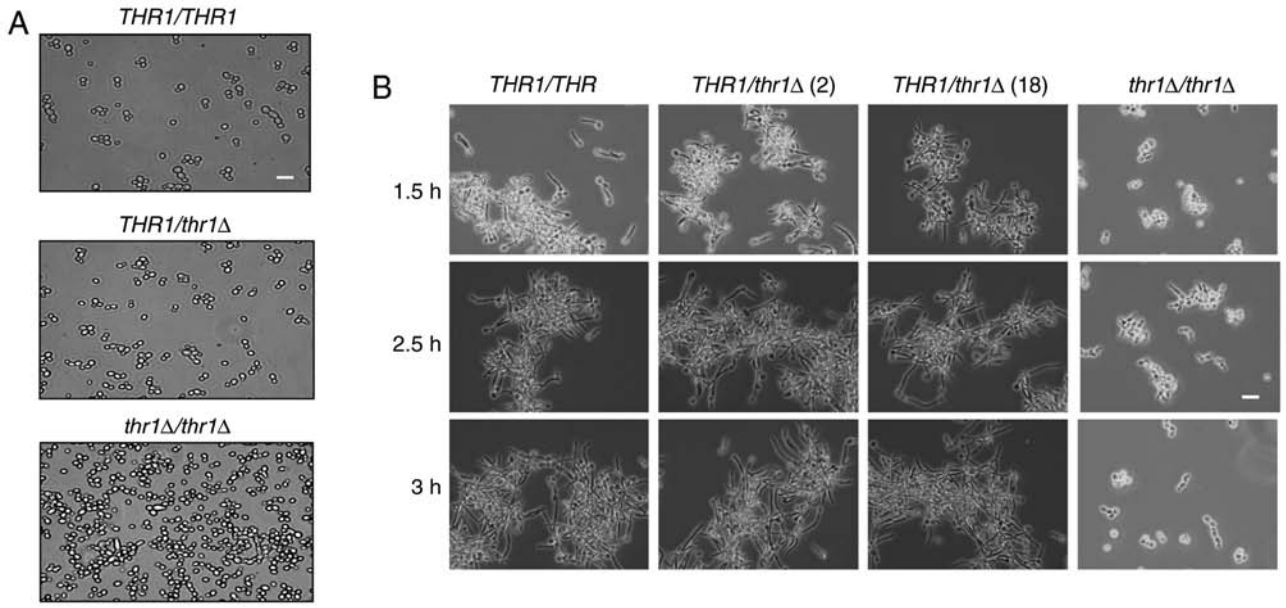

Figure 3. *THR1* does not directly contribute the yeast-to-hypha transition but is required for hyphal growth under serum-induced conditions. Cells of the strains in the mid-log phase were grown in (A) YPD or (B) YPD with 10% fetal bovine serum at 37˚C for 3 h. Two independent isolates (2 and 18) of the *THR1* heterozygous null mutants were used. *thr1*ΔS represents the presence of the *SAT1* marker and can be induce by growth with maltose to become *thr1*Δ. 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 (*thr1*Δ/*thr1*Δ), together with the *THR1* heterozygous null mutant (*THR1*/*thr1*Δ) 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 (*thr1*Δ/*thr1*Δ), together with the *THR1* heterozygous null mutant (*THR1*/*thr1*Δ) 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 'TGACTCA', 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 (*gcn4*Δ/*gcn4*Δ) (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

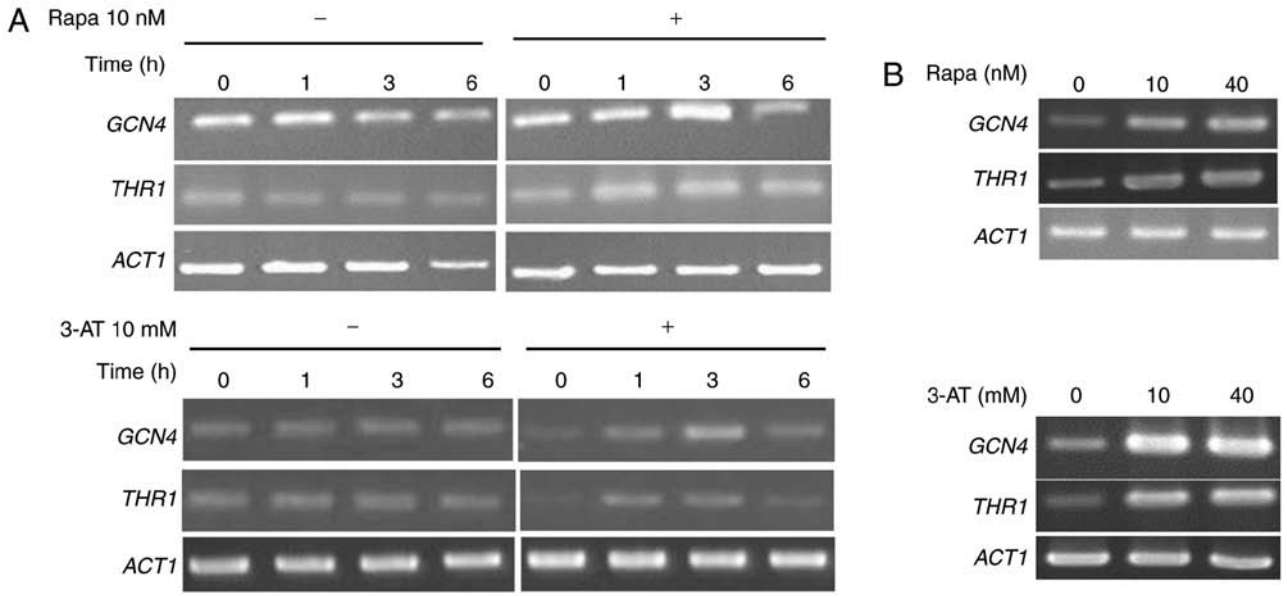

Figure 4. Nutrient limitation induces the expression of *GCN4* and *THR1*. (A) RT-PCR analyses of the mRNA levels of *GCN4* and *THR1* were performed following 0, 1, 3 and 6 h of growth in YPD with or without 10 nM Rapa (upper panel) or 10 mM 3-AT (lower panel). (B) RT-PCR analyses of mRNA levels of *GCN4* and *THR1* were performed following 3 h of growth in YPD in the presence of absence of 10 and 40 nM Rapa (upper panel) or 10 and 40 mM 3-AT (lower panel). The mRNA level of *ACT1* was used as a loading control. Rapa, rapamycin; 3-AT, 3-amino-1,2,4-triazole; RT-PCR, reverse transcription-polymerase chain reaction.

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 H_2O_2 or menadione. Cells with loss of either *CaCDC4* or *THR1* alone were impaired in growth (Fig. 6A), with minimal negative effects in 2 mM H_2O_2 (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

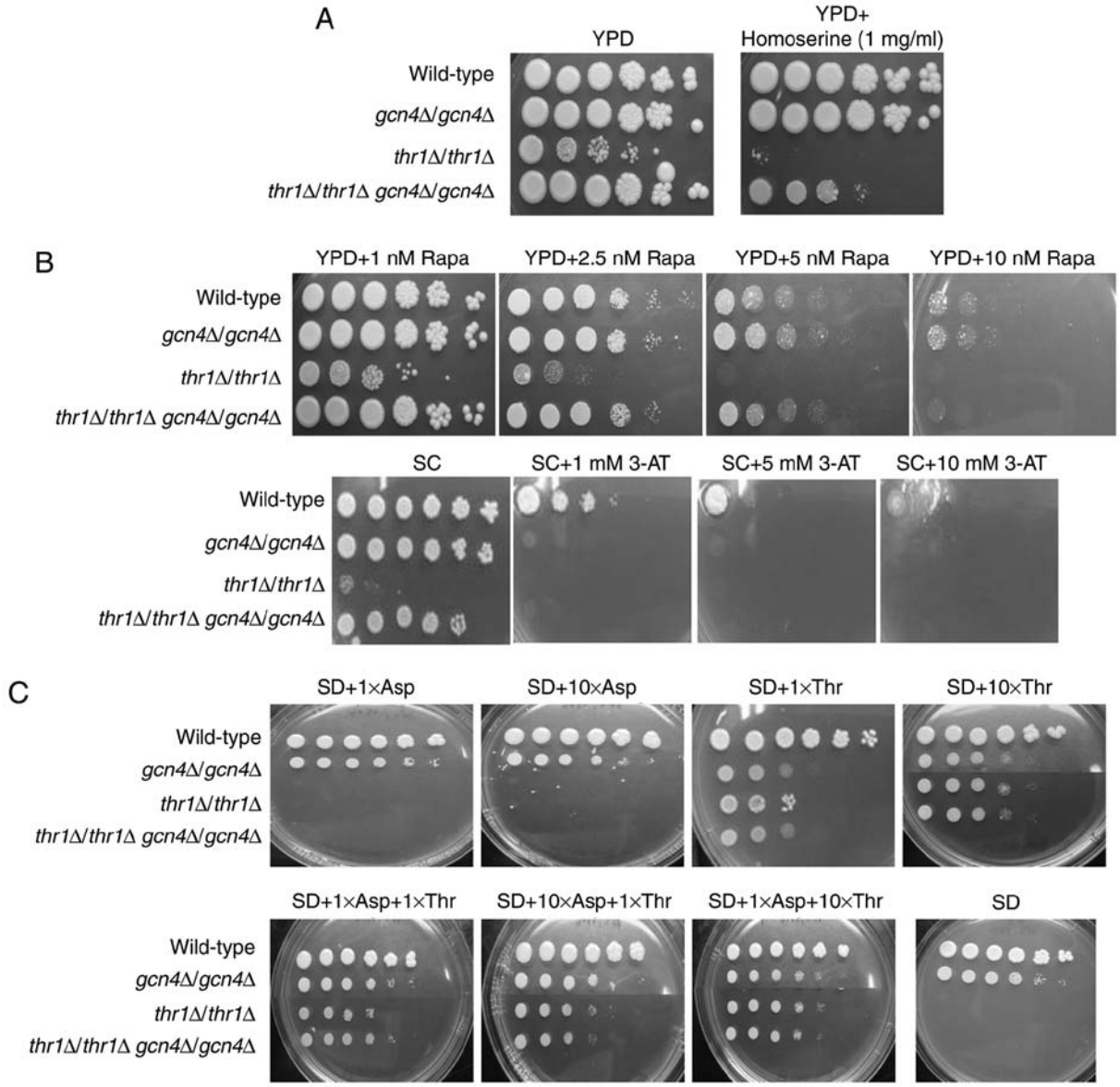

Figure 5. Cells without *GCN4* rescue those without *THR1* that are sensitive to activation of the TOR pathway by Rapa or 3-AT. The growth of *C. albicans* strains assayed using a spotting assay on limited nutrient conditions. Wild-type, null mutants of *gcn4, thr1, gcn4 thr1* were grown on (A) YPD with or without homoserine at 30˚C for 2 days, with (B) on YPD with or without indicated Rapa at 30˚C for 2 days or SC with or without indicated 3-AT plates at 30˚C for 3 days, or (C) on SD plates at 30˚C for 3 days with or without different concentration of Asp, Thr, or their combination: 1x Asp, 10x Asp, 1x Thr, 10x Thr, 1x Asp + 1x Thr, and 10x Asp + 10x Thr. 1x, 80 ng ml^{-1}; 10x, 800 ng ml^{-1}; SC, synthetic complete medium; SD, synthetic defined medium lacking amino acids; Rapa, rapamycin; 3-AT, 3-amino-1,2,4-triazole; Asp, aspartate; Thr, threonine.

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 *Ca*Cdc4-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 *Ca*Cdc4, *CaCDC4* is likely to encode a typical F-box protein of SCF ubiquitin ligase (32) known as SCF^{CaCdc4}. These domains are critical for filamentous growth (33), demonstrating that *Ca*Cdc4 is a negative regulator of filamentation (25,30) and is likely to regulate its targets via SCF^{CaCdc4} 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^{CaCdc4} ubiquitin ligase. Similar to the degradation of Sol1 in *Ca*Cdc4-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

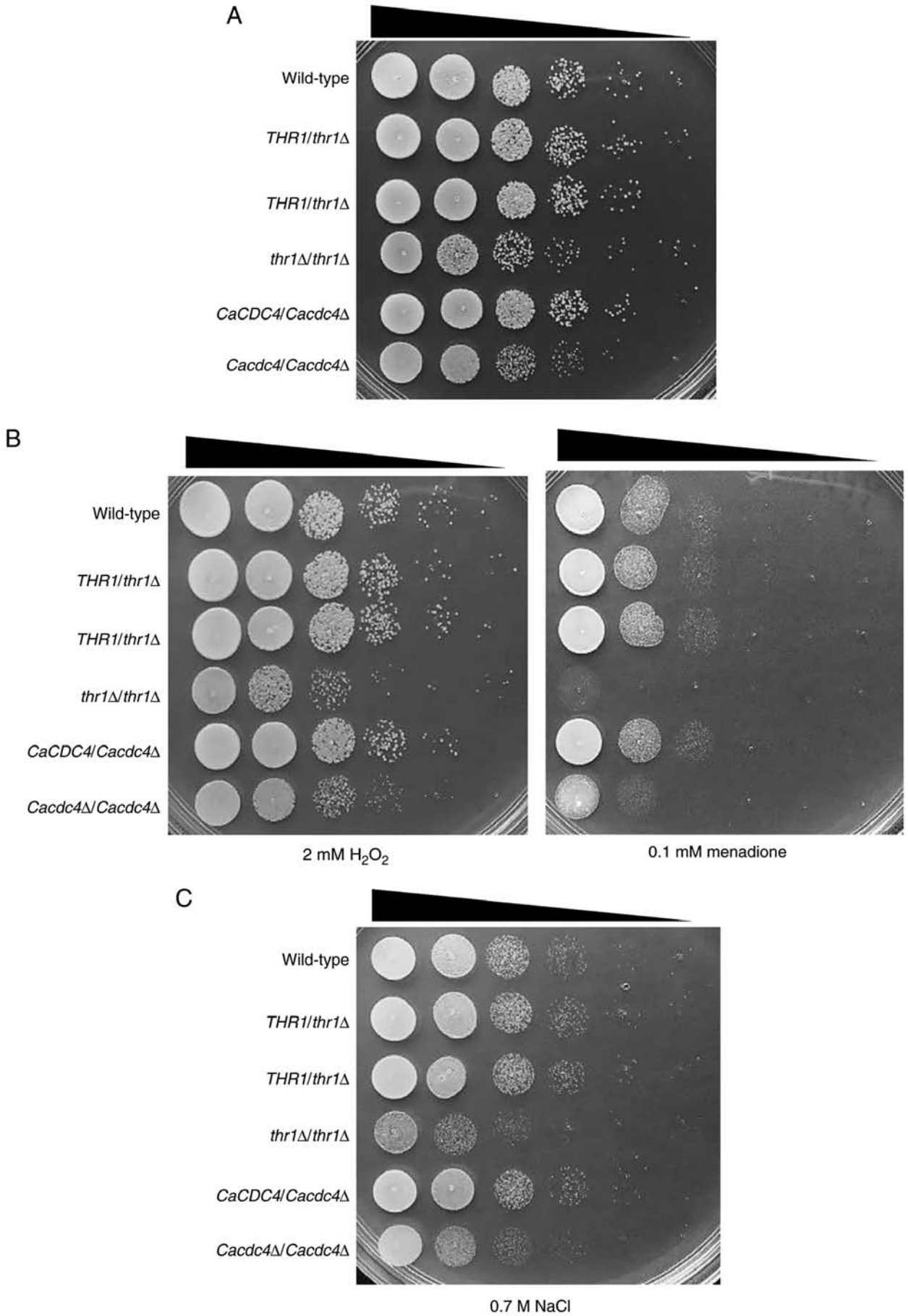

Figure 6. Cells lacking either *CaCDC4* or *THR1* are sensitive to oxidative and osmotic stress. Cells were diluted in 10^6 cells ml^{-1}, and 10-fold dilutions were spotted in 5 μl aliquots on (A) YPD plates containing (B) 2 mM H_2O_2 or 0.1 mM menadione, or (C) 0.7 M NaCl. The plates were incubated at 30˚C for up to 2 days. *CaCDC4*, *Candida albicans CDC4*.

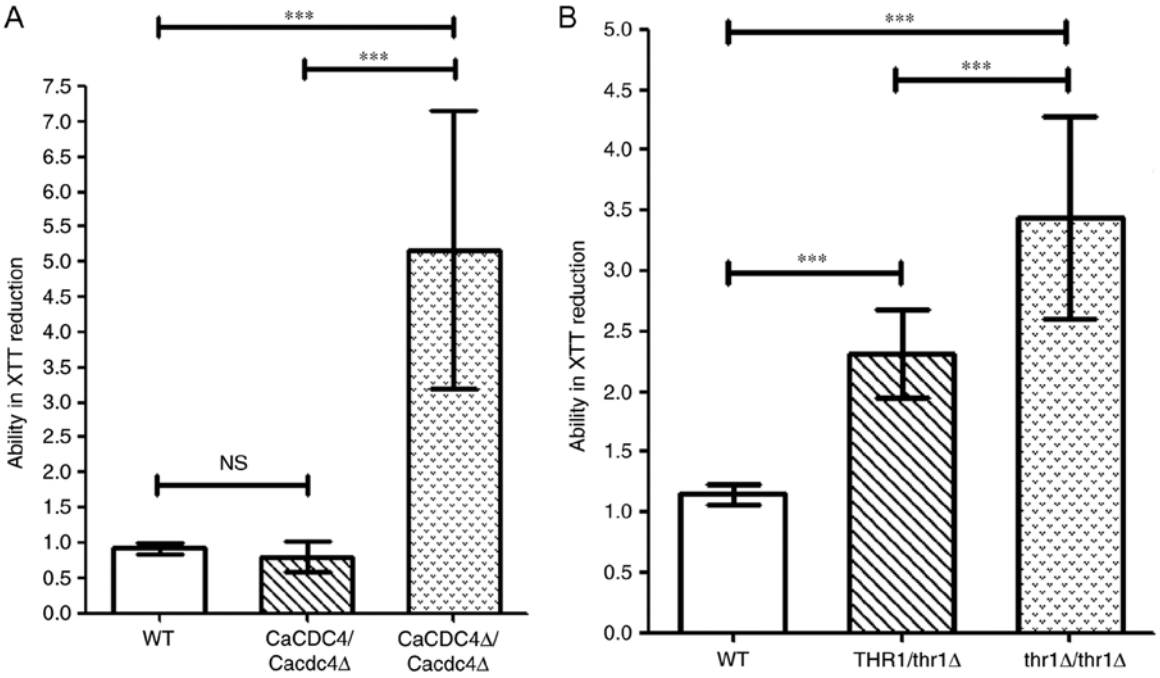

Figure 7. *CaCDC4* and *THR1* suppress biofilm formation. (A) Cells of the WT SC5314, heterozygous *CaCDC4* null mutant (*CaCDC4*/*Cacdc4*Δ), and homozygous *CaCDC4* null mutant (*Cacdc4*Δ/*Cacdc4*Δ) and the (B) WT SC5314, heterozygous *THR1* null mutant (*THR1*/*thr1*Δ), and homozygous *THR1* null mutant (*thr1*Δ/*thr1*Δ) were subjected to an *in vitro* XTT reduction assay for biofilm formation. ***P<0.001, as indicated. ns, not significant; *CaCDC4*, *Candida albicans CDC4*; WT, wild-type.

binding interactions or with intrinsic enzymatic activities (92), thr1 represents a typical SCF^{CaCdc4} target, negatively regulated by *Ca*Cdc4 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 *THR1* increases the levels of mannoproteins that are associated with cell wall structure and leads to the suppression of filamentous growth in the *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 *in vivo* (45,95-97). Therefore, various fungal amino acid biosynthetic enzymes and their encoded genes, including the *C. albicans* homoserine kinase-encoded gene *THR1* (44,45), have become potential antifungal targets to be exploited. The *thr1*Δ/*thr1*Δ mutant was created in the present study, and it was demonstrated that *THR1* is nonessential, as previously reported (44,45). However, it was found that *C. albicans* cells lacking *THR1* entered the stationary phase at a lower density than the wild-type cells in non-starved conditions (Fig. 2D), which has not been characterized previously. It is known that the stationary phase is a non-proliferating state in which microorganisms respond to starvation by ceasing growth. Therefore, fewer cells of the *thr1*Δ/*thr1*Δ mutant entering the stationary phase may reflect combined deleterious phenotypes, potentially due to impaired threonine biosynthesis, which is more than just a consequence of auxotrophy.

As *CaCDC4* is a negative regulator of filamentation (25,30), and *Ca*Cdc4 positively regulates Thr1, the present study aimed to ascertain whether *C. albicans THR1* negatively controls filamentation. Although no filamentous development was observed in the *thr1*Δ/*thr1*Δ mutant, the mutant almost lost its ability to form the filament in the presence of serum at 37˚C as the filament-induced condition (Fig. 3B). Additionally, the *thr1*Δ/*thr1*Δ mutant appeared to impair the ability to proliferate (Fig. 3B), 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 *GN4*-dependent manner has been well characterized in *S. cerevisiae* (46,47), and similar regulation exists in *C. albicans* (52,53,63,81,99). However, the nature of the regulation of the expression of *THR1* during threonine biosynthesis in *C. albicans* remains unclear. The present study verified that *THR1* is under the control of the TOR pathway and is dependent on *GCN4*, due to the induced expression of *THR1* and *GCN4* by Rapa and 3-AT, respectively. The growth impairment of the *thr1*Δ/*thr1*Δ 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 *C. albicans thr1*Δ/*thr1*Δ mutant is toxic (44). However, the growth impairment of the *thr1*Δ/*thr1*Δ mutant was relieved by introduction of the *GCN4* deletion (*thr1*Δ/*thr1*Δ *gcn4*Δ/*gcn4*Δ) under the conditions described above. These results indicate that *THR1* in *C. albicans* is under the control of the *TOR-GCN4* pathway, and *GCN4* may also control the expression of threonine biosynthesis genes upstream of *THR1* in *C. albicans*, which has been demonstrated in *S. cerevisiae* (100,101), as the loss of *GCN4* reduces the expression of those genes, decreasing the accumulation of homoserine. Threonine appeared to rescue cells lacking *THR1* in a dose-dependent manner, presumably

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 GCRE-RrLUC 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 *HWP1* genes and stabilize the filament inducer Sol1 (25) and Thr1 (Fig. 1D). *ECE1* and *HWP1* appear to be regulated by Efg1 (106,107). Therefore, Thr1 is negatively regulated by *Ca*Cdc4 and may positively control filamentation through *ECE1* and *HWP1* 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 *HWP1* 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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