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TECHNICAL NOTES

A new rapid and efficient system with dominant selection developed to inactivate and conditionally express genes in *Candida albicans*

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Abstract *Candida albicans* is an important human fungal pathogen but its study has been hampered for being a natural diploid that lacks a complete sexual cycle. Gene knock-out and essential gene repression are used to study gene function in *C. albicans*. To effectively study essential genes in wild-type *C. albicans*, we took advantage of the compatible effects of the antibiotics hygromycin B and nourseothricin, the recyclable *CaSAT1*-flipper and the tetracycline-repressible (Tet-off) system. To allow deleting two alleles simultaneously, we created a cassette with a *C. albicans HygB* resistance gene (*CaHygB*) flanked with the *FLP* recombinase target sites that can be operated alongside the *CaSAT1*-flipper. Additionally, to enable conditionally switching off essential genes, we created a *CaHygB*-based Tet-off cassette that consisted of the *CaTDH3* promoter, which is used for the constitutive expression of the tetracycline-regulated transactivator and a tetracycline response operator. To validate the new systems, all strains were constructed based on the wild-type strain and selected by the two dominant selectable markers, *CaHygB* and *CaSAT1*. The *C. albicans* general transcriptional activator *CaGCN4* and its negative regulator *CaPCL5* genes were targeted for gene deletion, and the essential cyclin-dependent kinase *CaPHO85* gene was placed under the Tet-off system. *Cagcn4*, *Capcl5,* the conditional Tet-off *CaPHO85* mutants, and mutants bearing two out of the three mutations were generated. By subjecting the mutants to various stress conditions, the functional relationship of the genes was revealed. This new system can efficiently delete genes and conditionally switch off essential genes in wild-type *C. albicans* to assess functional interaction between genes.

Keywords *Candida albicans* · *CaHygB* · *CaSAT1*-flipper · Tet-off system · *CaGCN4* · *CaPHO85* · *CaPCL5*

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Introduction

Developing molecular tools that involve plasmids and gene cassettes is an important step for the functional analysis of a gene and its product in cells. Polymerase Chain Reaction (PCR)-amplified gene cassettes and plasmid construction via traditional cloning are two common approaches for gene manipulation in yeast and fungi (Wendland 2003). *Candida albicans*, which is a constituent of the normal mucosal surface microflora of the gastrointestinal and genitourinary tracts in healthy persons, is an important opportunistic fungal pathogen (Pfaller and Diekema 2007). To understand the role of genes in *C. albicans*, gene disruption and conditional regulation of gene expression are two common methods (Xu et al. 2014). For gene deletion or disruption, many tools have been developed, including (1) the use

of auxotrophic genes to delete targets, such as *CaURA3*, *CaHIS1*, *CaARG4*, *Candida dubliniensis HIS1* (*CdHIS1*), *ARG4* (*CdARG4*) and *Candida maltose LEU2* (*CmLEU2*) (Noble and Johnson 2005); and (2) the use of dominant selectable markers such as *CaSAT1*, *CaNAT1*, *CaHygB* and *CaIMH3* (Basso et al. 2010; Beckerman et al. 2001; Reuss et al. 2004; Shen et al. 2005) to delete genes and recycle by the *FLP/FRT* (Wirsching et al. 2000) or Cre/*loxP* (Dennison et al. 2005) recombination system. For the repression of essential genes, a regulatory promoter evokes abnormal effects in cells, such as (1) the repression of gene expression by nutrient source response elements such as the promoters of *CaMAL2*, *CaPCK1* and *CaMET3* (Backen et al. 2000; Care et al. 1999; Gerami-Nejad et al. 2004) or (2) gene regulation by using tetracycline regulatory elements (Nakayama et al. 2000; Roemer et al. 2003).

Tetracycline regulation can be divided into an inducible gene expression system (also called Tet-on) (Lai et al. 2011; Park and Morschhauser 2005) and a repressible gene expression system (Tet-off). The Tet-off system was developed in *C. albicans* and designed for the auxotrophic strain CAI8, which carries the plasmid pCAITHE5 and is capable of the constitutive expression of a Tet-responsive transactivator; further, one target allele is deleted, and the other allele is regulated under the Tet promoter (Nakayama et al. 2000). This system used in *C. albicans* depends on two plasmids: one consists of a transactivator gene that encodes the Tet repressor TetR fused with the activation domain of *Saccharomyces cerevisiae* Hap4p under control of the *CaENO1* promoter, which constitutively expresses TetR, and the other is composed of the regulatory element TetO and the *CaHIS1* marker, which is used for integration at the promoter of the target gene. Because these modules are constructed on different plasmids, the use of this Tet-off system requires an auxotrophic strain. Although gene manipulation with auxotrophic markers is a general approach in the study of yeast, the genomic structure of the constructed strains is not close to that of the wild-type strain because mutations of the genes involved in metabolic pathways are present in the auxotrophic strains that might interfere with analyses or interpretations in nutrient-related studies. Therefore, we designed a set of modules for gene deletion and Tet-off gene regulation with dominant selectable markers.

In this study, we modified the selectable marker *CaHygB*, flanking it with *FRT* sequences, to generate a plasmid (pHB1S) with a cassette that is capable of conferring resistance to hygromycin B in *C. albicans*. We used a PCR-based *CaHygB* cassette and a PCR-based *CaSAT1*-flipper cassette to delete *CaGCN4* and *CaPCL5*. Moreover, we created a Tet-off plasmid, pWTF1, with a cassette consisting of a gene that encodes the Tet-responsive transactivator TetR, driven by the *CaTDH3* promoter that expresses the gene constitutively, a *CaHygB* marker flanked by *FRT* sequences and a Tet-response element fused with a minimal *CaOP4* promoter that allows the binding of TetR to turn genes off in *C. albicans*. We applied the PCR-amplified Tet-off cassette and *CaSAT1*-flipper to construct a conditionally regulated *CaPHO85* mutant. By spotting assay with various conditions of nutrient starvation, filament-induction and temperature stress, we analyzed the responses on growth and filamentation in the *Cagcn4*, the *Capcl5* mutants, the conditional *CaPHO85* mutant, and the mutants with two out of the three mutations. Our results confirmed that this new system incorporating dominant selectable markers is a rapid and efficient approach to construct *C. albicans* strains for functional analysis.

Materials and methods

Strains and growth conditions

The wild-type *C. albicans* SC5314 (Gillum et al. 1984) was used as parental strain for derivative construction, as shown in Table 1. All strains were routinely grown in either YPD (1 % yeast extract, 2 % peptone, 2 % glucose) or synthetic complete medium (SC; 0.67 % yeast nitrogen base without amino acids, 0.2 % amino acid dropout mix and 2 % glucose) and synthetic defined minimal medium (SD; 0.67 % yeast nitrogen base without amino acids and 2 % glucose) and on plates with 2 % agar at 30 °C. Transformants were selected on YPD containing 1 mg/ml hygromycin B (Gold Biotechnology, St. Louis, MO, USA), 200 μg/ml nourseothricin (WERNER BioAgents, Jena, Germany), or both. Amino acid starvation was imposed in SC medium lacking histidine (SC-H) containing 3-aminotriazole (A8056, Sigma-Aldrich, St. Louis, MO, USA) and SC medium lacking tryptophan (SC-W) containing 5-methyl-DL-tryptophan (Gold Biotechnology, St. Louis, MO, USA) at 1, 5 and 10 mM. Inhibition of heat shock protein 90 (Hsp90) was treated with 10 μM of Geldanamycin (LC Laboratories, Woburn, MA, USA). Morphogenesis was induced using Spider (Liu et al. 1994) and Lee's (Lee et al. 1975) medium at 30 and 37 °C. The Tet-off system was regulated by adding 40 μg/ml of Dox to various media.

The *Escherichia coli* strain DH10B was used as a host for plasmid DNA construction and routine plasmid maintenance and amplification. Bacterial cultures were grown in Luria–Bertani medium (LB) or LB supplemented with 50 μg/ml of ampicillin or 34 μg/ml of chloramphenicol as required. Plasmid DNA was purified using the GeneSpin$^{@}$-V2 Miniprep Purification Kit (Protech Technology Enterprise Co., Taipei, Taiwan).

Table 1 *C. albicans* strains used in this study

Strain	Parent	Genotype	Plasmids	Reference
SC5314		Wild-type strain		(Gillum et al. 1984)
WCL301	SC5314	*PHO85/pho85::P_{TET}-PHO85:HygB*	pWTF1	This study
WCL302	WCL301	*pho85::SAT1-FLIP/pho85::P_{TET}-PHO85:HygB*	pWTF1, pSFS2A-PHO85	This study
WCL303	WCL302	*pho85::FRT/pho85::P_{TET}-PHO85:FRT*		This study
WCL304	SC5314	*PHO85/pho85::SAT1-FLIP*	pSFS2A-PHO85	This study
WCL305	WCL304	*PHO85/pho85::FRT*		This study
WCL306	SC5314	*GCN4/gcn4::HygB*	pHB1S	This study
WCL307	WCL306	*gcn4::HygB/gcn4::SAT1-FLIP*	pHB1S, pSFS2AS	This study
WCL308	WCL307	*gcn4::FRT/gcn4::FRT*		This study
WCL309	SC5314	*GCN4/gcn4::SAT1-FLIP*	pSFS2AS	This study
WCL310	WCL309	*GCN4/gcn4::FRT*		This study
WCL311	SC5314	*PCL5/pcl5::HygB*	pHB1S	This study
WCL312	SC5314	*PCL5/pcl5::SAT1-FLIP*	pSFS2AS	This study
WCL313	WCL312	*PCL5/pcl5::FRT*		This study
WCL314	WCL311	*pcl5::HygB/pcl5::SAT1-FLIP*	pHB1S, pSFS2AS	This study
WCL315	WCL114	*pcl5::FRT/pcl5::FRT*		This study
WCL316	WCL303	*pho85::FRT/pho85::P_{TET}-PHO85:FRT GCN4/gcn4::HygB*	pHB1S	This study
WCL317	WCL316	*pho85::FRT/pho85::P_{TET}-PHO85:FRT gcn4::HygB/gcn4::SAT1-FLIP*	pHB1S, pSFS2AS	This study
WCL318	WCL317	*pho85::FRT/pho85::P_{TET}-PHO85:FRT gcn4::FRT/gcn4::FRT*		This study
WCL319	WCL303	*pho85::FRT/pho85::P_{TET}-PHO85:FRT PCL5/pcl5::HygB*	pHB1S	This study
WCL320	WCL319	*pho85::FRT/pho85::P_{TET}-PHO85:FRT pcl5::HygB/pcl5::SAT1-FLIP*	pHB1S, pSFS2AS	This study
WCL321	WCL320	*pho85::FRT/pho85::P_{TET}-PHO85:FRT pcl5::FRT/pcl5::FRT*		This study
WCL322	WCL308	*gcn4::FRT/gcn4::FRT PCL5/pcl5::HygB*	pHB1S	This study
WCL323	WCL322	*gcn4::FRT/gcn4::FRT pcl5::HygB/pcl5::SAT1-FLIP*	pHB1S, pSFS2AS	This study
WCL324	WCL324	*gcn4::FRT/gcn4::FRT pcl5::FRT/pcl5::FRT*		This study

Construction of pSFS2AS, pHB1S and pWTF1

The oligonucleotide primers used for plasmid construction are listed in Table 2. To modify the plasmid pSFS2A (Reuss et al. 2004) containing the *CaSAT1*-flipper cassette to add two priming sites S1 and S2 derived from pFA-based tools (Walther and Wendland 2008), first, the primers S1F and S1R were annealed to form an adaptor with extensions of the restriction enzyme sites of *Kpn*I and *Xho*I and cloned into pSFS2A to become pSFS2A-S1. Next, the pair of primers S2F and S2R was annealed to form another adaptor with extensions of the restriction enzyme sites of *Not*I and *Sac*I and cloned into pSFS2A-S1 to become pSFS2AS (Fig. 1a). To introduce a synthetic hygromycin B gene that is codon-optimized for *C. albicans* (*CaHygB*) into pSFS2A, *CaHygB*, which is under control of the *CaTEF2* promoter and *CaACT1* terminator, was PCR-amplified from pYM70 (Basso et al. 2010) with the primers CaHygB BglII F and CaHygB NsiI R, digested with *Bgl*II and *Nsi*I and cloned into pSFS2A digested with *Bam*HI and *Pst*I to generate pHB1. To replace the *CaSAT1*-flipper cassette of pSFS2AS with the *CaHygB* marker, the cassette was released from pHB1 digested with *Xba*I, which exists in the *FRT* sequences, and cloned into pSFS2AS digested with *Xba*I to obtain pHB1S (Fig. 1a).

To clone the *CaTDH3* promoter, the DNA fragment was amplified by PCR with the primers CaTDH3 F and CaTDH3 R and cloned into yT&A (Yeastern Biotech Co., Taipei, Taiwan) to generate yTA-CaTDH3. Next, the primers CaTDH3p SacII F and CaTDH3p XbaI R were used to amplify the region of the *CaTDH3* promoter and subcloned into pBluescriptII to produce pBSII-CaTDH3p. A DNA fragment encoding reverse tetracycline-response transactivator (rtTA) was digested with *Xba*I and *Pst*I, released from pNIM1 (Park and Morschhauser 2005) and subcloned into pBSII-TDH3 to generate pBSII-TDH3p-rtTA. To change rtTA to tTA with site-directed mutagenesis in the five codons encoding critical amino acids, the plasmid pBSII-TDH3p-tTA was built by mutation of G12S, G19E, P56A, E148D and R179H (Urlinger et al. 2000) with 4 pairs of primers, called Tet-mut, shown in Table 2. To create an intermediate plasmid based on the *CaHygB* marker, two pairs of primers, MCS3 F/MCS3 R and MCS4 F/MCS4 R, were used to form adaptors and cloned into

Table 2 Synthetic oligonucleotides used in this study

Primer	Sequence[a]
CaPHO85 F	GCTTCATCCTCAACTTTTCA
CaPHO85 R	AAGCGATTGTTTCCCTGTT
CaPHO85 UP KpnI F	ATAT***GGTACC***TGGGTAATGGTTGTTAGGAC
CaPHO85 UP XhoI R	AGTA***CTCGAG***ACATACTGAGAAGACGAGCC
CaPHO85 DN NotI F	AAAT***GCGGCCGC***TTCAGGTTGATGGGTACTCC
CaPHO85 DN SacI R	TAAT***GAGCTC***TGTTGCTGGTAAGGGTCACT
S1 F	CGAAGCTTCGTACGCTGCAGGTC
S1 R	TCGAGACCTGCAGCGTACGAAGCTTCGGTAC
S2 F	CTGATATCATCGATGAATTCGAGC
S2 R	GGCCGCTCGAATTCATCGATGATATCAGAGCT
CaGCN4S1F	TATTTAAATTAAATTACATTACATTAATTAGCTTTGTTACCATTATTATTATTAGATAAAGAAGCTTCGTACGCTGCAGGTC
CaGCN4S2R	AATTTTCTAAATTTTTCTTTTTTTAAAAAAATAACGAGAGGTATATATAGTAGTAACTTTTCTGATATCATCGATGAATTCGAG
CaPCL5S1F	AATTATCGTCCTGTTTCCTGCTCCCACTCCCGCTCCTAACTGTTTTCCTTCTATTATACAGAAGCTTCGTACGCTGCAGGTC
CaPCL5S2R	TACCCGAGTAATACTAGCCTATATAAAGTTCATTTTCTGCCAAACAGAATAAGTAAGCTGTCTGATATCATCGATGAATTCGAG
CaPHO85TF1F	CTTTTATCCAGAACTGAGCTTCATCCTCAACTTTTCATTAATATAACTTTTTGAACAATACTTGGACTCTTGAATCCGCGG
CaPHO85TF1R	CAGATAAATCGAAATATTGGAAAAAAAAAATTTCAACATACTGAGAAGACGAGCCGGTCATTGCACCAGCTCCGGTACCACT
CaHygB BglII F	GCTT***AGATCT***CCTGCAGGTATAGTGCTTGCTGTTCGATA
CaHygB NsiI R	CGTT***ATGCAT***ACGCGTCATTTTATGATGGAATGAATGGG
CaSAT1 F	GCTCCTTGGCATACGATTAG
CaMAL2p R	CAGACAGTCGAGTTAGACAG
CaHygB F	TTAGATCAGGTGCTGGTACT
CaTEF1p R	GGTATTTGGCTTTCGGTAT
CaGCN4 F	ATTTATTCACCACAAGGACAC
CaGCN4 R	GGGTAGGCAAGATACATTTTA
CaPCL5 F	GGAAACTCGGATGGTGTCTA
CaPCL5 F	AAGTCTGCCTTATCGTTGTATG
Tet-mut1 F	AGTGATTAACAGCGCATTAGAGTTGCTTAATGAGGTCGGAATCG
Tet-mut1 R	CGATTCCGACCTCATTAAGCAACTCTAATGCGCTGTTAATCACT
Tet-mut2 F	GCCATTGAGATGTTAGATAGGCAC
Tet-mut2 R	TAAGGCGTCGAGCAAAGC
Tet-mut3 F	TCAAGAGCATCAAGTCGC
Tet-mut3 R	TCTTCCAATACGCAACCT
Tet-mut5 F	CACCAAGGTGCAGAGCCAGCC
Tet-mut5 R	ATCAAATAATTCGATAGCTTGTCG
CaPHO85 locus F	CAGATGGCTGGGTTGAGAA
CaGCN4 dn PmeI F	ATG***GTTTAAAC***GTTACTACTATATATACCTCTCGT
CaGCN4 dn SpeI R	GG***ACTAGT***GGGTAGGCAAGATACATTTT
CaPCL5 probe F	CAGCAACAAATTGTGGGTCA
CaPCL5 probe R	GTGGGAGCAGGAAACAGGAC
CaTDH3 F	TTTGGTTGCGTTAGTCCG
CaTDH3 R	CTCTATCCTGGGACATTGGT
CaTDH3p SacII F	ATTA***CCGCGG***GTTGCTCCTCGTCGACAA
CaTDH3p XbaI R	CGGG***TCTAGA***CATTGTTAATTAATTTGATTGTAAAG
MCS3 F	CTAGATTTAAATATGCATTGACCGCGGATTCAAGAGTCCAAGCT

Table 2 continued

Primer	Sequence[a]
MCS3 R	TGGACTCTTGAATCCGCGGTCAATGCATATTTAAAT
MCS4 F	TGCACCAGCTCCGGTACCACTAGTCATGTTTAAACCGG
MCS4 R	TCGACCGGTTTAAACATGACTAGTGGTACCGGAGCTGGTGCAGTAC

[a] Restriction enzyme sites included in primers are highlighted in bold italics

pBSII-SFS2A-SpeI (unpublished data) to generate pBSII-SFS-HygB, including TF1-F and TF1-R annealing sites and the additional restriction enzyme sites *Kpn*I, *Nsi*I, *Pme*I, *Swa*I, *Spe*I and *Sac*II. The DNA fragment consisting of the *CaTDH3* promoter, rtTA coding sequence and the *CaACT1* transcription terminator was digested with *Kpn*I and *Pst*I and cloned into the *Kpn*I/*Nsi*I digested vector pBSII-SFS-HygB to generate pBSII-TDH3p-tTA-ACT1-HygB. The Tet operator fused to a minimal *CaOP4* promoter was released from pWTN2 (unpublished data) by digestion with *Pme*I and *Spe*I and cloned into pBSII-TDH3p-tTA-ACT1-HygB digested by *Pme*I and *Spe*I to obtain pWTF0. Finally, *Pme*I/*Swa*I digested *CaHygB* marker flanked by *FRT* sequences was released from pWTF0 and cloned back into pWTF0 digested with *Pme*I/*Swa*I to produce pWTF1 (Fig. 1a) with *FRT* sequences in the same direction as pSFS2A. To make them available to the research community, the plasmids of pWTF1, pSFS2AS and pHB1S will be deposited with the fungal genetics stock center (http://www.fgsc.net/) upon acceptance of publication.

Candida albicans electroporation and yeast colony PCR

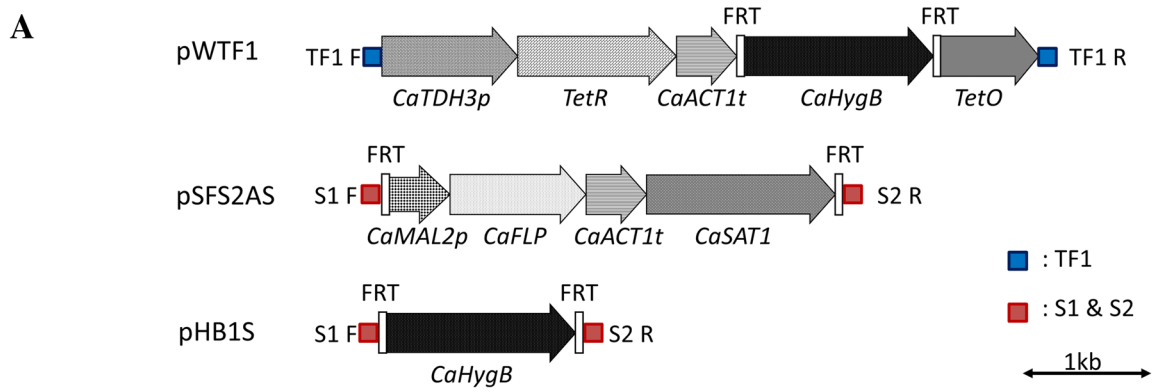
All strains were obtained via electroporation (De Backer et al. 1999). To make competent cells, cells from a colony of approximately 2 mm in diameter were inoculated into 2 ml of YPD and grown at 30 °C overnight. The next day, the culture was diluted into 50 ml of fresh YPD at 1:100 ratio and grown overnight before collection. The cultures were transferred to 50 ml tubes and spun at 4000 r.p.m. for 5 min to collect the pellets. Pellets were resuspended in 10 ml of LiAc/TE buffer (mixture with 1 ml of 1 M lithium acetate, 1 ml of 10X TE buffer consisting of 100 mM Tris–HCl, 10 mM EDTA, pH 7.5 and 8 ml of dH_2O) and incubated at 30 °C with shaking at 200 r.p.m. for 1 h. Subsequently, 250 µl of 1 M dithiothreitol (DTT) was added into the cell suspension of LiAc/TE buffer for an additional 30 min before the next step. After spinning at 4000 r.p.m. for 5 min, the cell pellets were washed with 40 ml of ice-cold dH_2O and collected by spinning. After removing the residual dH_2O, 25 ml of ice-cold dH_2O was added to the cell pellets, and the cells were resuspended. The cell pellets were collected by spinning and resuspended in 10 ml of 1 M ice-cold sorbitol. Subsequently, the pellets were collected, resuspended into 50 µl of 1 M ice-cold sorbitol and kept on ice before electroporation. The cassettes from the plasmids were excised as *Kpn*I/*Sac*II digested fragments and extracted with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Taiwan). One microgram of linear DNA fragments was mixed with 50 µl of competent cells and electroporated in a 0.2-cm cuvette with 1.8 kV via the Gene Pulser Xcell™ system (Bio-Rad Laboratories, Hercules, California, USA). After electroporation, the competent cells were resuspended with 1 ml of 1 M ice-cold sorbitol and transferred to a collection tube. The cells were collected by spinning at 4000 r.p.m. at 4 °C and resuspended in 1 ml of YPD medium before incubation at 30 °C for 4 h. Finally, the cells were plated on YPD plates supplemented with appropriate antibiotics and incubated at 30 °C for 1–2 days. The transformants were selected after screening by colony PCR. Colonies of the indicated strains were treated with 0.1 % NaOH and boiled at 95 °C for 15 min. After boiling, the microtubes with the total mixture were subjected to vortexing at high speed for 5 s and spun down for 10 s as the template. For the PCR constituents, 0.4 µM of primers, 1× Taq buffer, 0.2 mM dNTP and 3 µl of boiling mixture were mixed with dH_2O to a 25-µl total volume. Based on the primers and the amplicon size, the annealing temperature and extension time were set.

Spotting assay

The cells of the strains were grown in YPD medium overnight with or without 40 µg/ml of Dox. Cultures of the indicated strains were diluted to the optical density of 1.0 at OD600 (approximately 2×10^7 cells/ml) and then diluted from 10^7 to 10^2 cells/ml. The diluted cultures of the strains were spotted at the volume of 5 µl on agar plates. Assays on synthetic complete (SC) or synthetic defined (SD) minimal medium were incubated for 2 days at 30 or 37 °C. Induced filamentation with Lee's and Spider medium was incubated for 5–7 days at 30 or 37 °C.

Southern blotting

Genomic DNA from the constructed strains was extracted with a MasterPure DNA kit (Epicentre, Madison, WI, USA). Genomic DNA (10 µg) was digested with the


A
pWTF1
TF1 F
CaTDH3p
TetR
FRT
CaACT1t
FRT
CaHygB
FRT
TetO
TF1 R
pSFS2AS
S1 F
FRT
CaMAL2p
CaFLP
CaACT1t
CaSAT1
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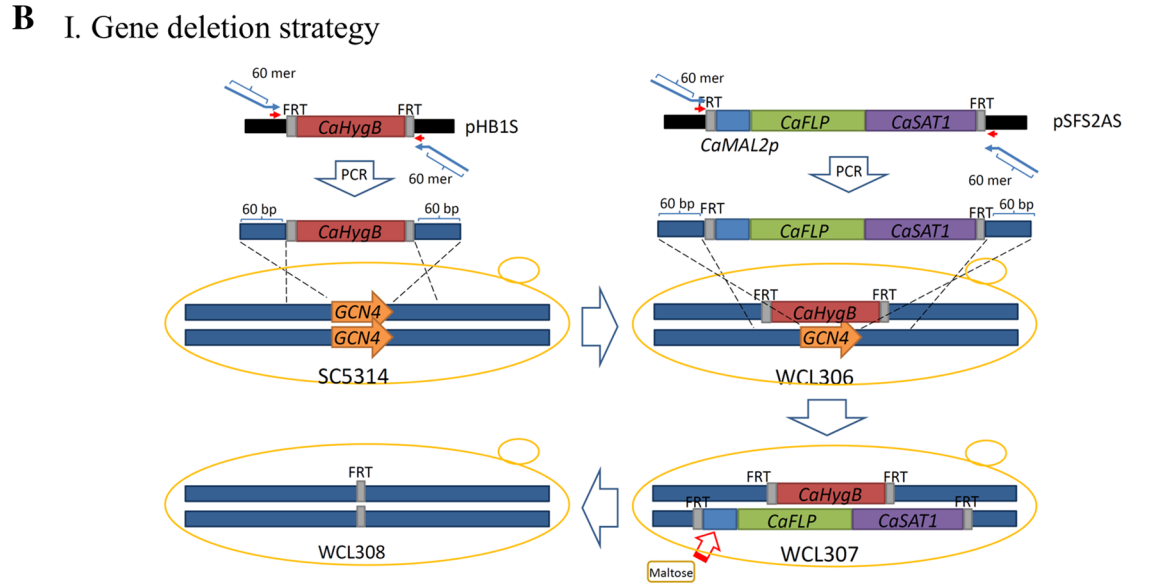
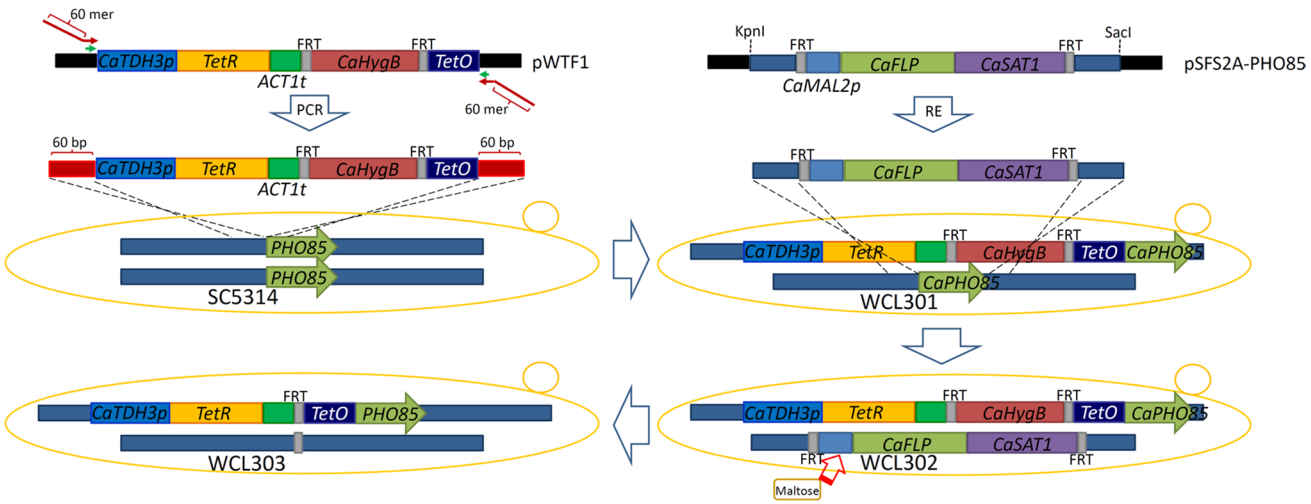
CaHygB
FRT
S2 R
: TF1
: S1 & S2
1kb


B
I. Gene deletion strategy
60 mer
FRT
CaHygB
FRT
pHB1S
PCR
60 mer
60 bp
60 bp
CaHygB
GCN4
GCN4
SC5314
CaMAL2p
CaFLP
CaSAT1
pSFS2AS
WCL306
WCL308
WCL307
Maltose


II. Tet-off gene regulation strategy
60 mer
CaTDH3p
TetR
CaHygB
TetO
ACT1t
pWTF1
PCR
60 bp
PHO85
SC5314
KpnI
SacI
CaFLP
CaSAT1
pSFS2A-PHO85
RE
CaPHO85
WCL301
WCL303
WCL302
Maltose

◀**Fig. 1** Scheme of the dominant selection system. **a** Maps of pWTF1, pSFS2AS and pHB1S. For the Tet-off cassette in plasmid pWTF1, the elements are arranged with TetR representing a Tet-responsive transactivator regulated by the constitutive *CaTDH3* promoter, a *CaACT1* transcriptional terminator, *CaHygB* flanked by *FRT* sequences as *white boxes*, and seven copies of the Tet operator. TF1-F and TF1-R are two annealing sites indicated as *blue squares*. For the *CaSAT1*-flipper cassette in pSFS2AS, the elements are followed by *CaFLP* recombinase driven by the *CaMAL2* promoter with the *CaACT1* transcriptional terminator, a connected *CaSAT1* marker, all flanked by *FRT* sequences. For the *CaHygB* cassette in plasmid pHB1S, the *CaHygB* gene is flanked by *FRT* sites. S1-F and S2-R are two annealing sites indicated as *red squares* for PCR. **b** Flowchart of gene deletion and conditional regulation by the Tet-off system via dominant markers. *I* Gene deletion of *CaGCN4*. PCR-based *CaHygB* and *CaSAT1*-flipper cassettes are transformed into wild-type strain SC5314 sequentially to generate hygromycin B resistant WCL306 and hygromycin B and nourseothricin resistant WCL307, which is a *Cagcn4* null mutant. Maltose-induced *CaFLP* excision of *CaHygB* and *CaSAT1* results in strain WCL308 with both alleles of one *FRT* sequence. *II* Tet-off gene regulation in *CaPHO85*. PCR-generated Tet-off system and *CaSAT1*-flipper cassettes are transformed into wild-type strain SC5314 sequentially to obtain hygromycin B resistant WCL301 and hygromycin B and nourseothricin resistant WCL302 serving as a conditional Tet-off *CaPHO85* mutant. With *CaFLP* excision of *CaHygB* and *CaSAT1*, the modules involved in the Tet-off system remained at the *CaPHO85* locus in WCL303

appropriate restriction enzyme and separated in 0.8 % agarose by electrophoresis. After ethidium bromide staining, all DNA fragments in the gel were transferred onto Biodyne A membrane (Pall Corporation, New York, USA) via TurboBlotter System (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). UV-crosslinked membrane was hybridized with a gene-specific DIG-labelled probe at 42 °C overnight. With anti-DIG antibodies conjugated to alkaline phosphatase, the membrane was visualized via enhanced chemiluminescence with the DIG Luminescent Detection Kit (Roche Life Science, Indianapolis, USA). Images were captured using an ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences).

Protein extraction and western blotting

Cells from cultures after Dox induction were pelleted via centrifugation at 5000 r.p.m. and washed with PBS twice. After washing, the pellets were resuspended in 200 µl ice-cold lysis buffer (50 mM Tris–HCl, pH 5.0, 150 mM NaCl, 50 mM NaF, 0.5 % Triton X-100, 0.1 % Tween20, 0.5 % NP40, 10 % glycerol), and fresh 2 mM Na_3VO_4, 2 mM PMSF, 10 mM β-mercaptoethanol and protease inhibitor cocktail (P8215, Sigma) were added. All of the resuspension was transferred into a 2-ml twist-cap microtube with 100 µl of pre-packaged acid-washed glass beads (G8772, Sigma) and mechanically disrupted by bead-beating in a MagNA lyser (Roche, Germany) at 5000 r.p.m. for 30 s four times, with a 1-min break on ice in between. The concentration of the whole protein extract was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad, USA). For separation of total extract, 25 µg of each total extract sample was loaded into 10 or 12 % SDS-PAGE and run in gel at constant 100 Volts for 90 min. Following separation, all of the proteins in SDS-PAGE were transferred to a methanol-treated PVDF membrane (Pall Corporation, USA) with a protein transfer apparatus at constant 100 V for 90 min. After transfer, the PVDF membrane was blocked in washing buffer (137 mM sodium chloride, 20 mM Tris, 0.1 % Tween-20, pH 7.6) with 5 % skim milk for 1 h, washed three times in washing buffer, each for 5 min, before detection with the respective antibody. *Ca*Pho85p was probed with anti-Cdc2/p34 (PSTAIRE) antibodies (sc-53, Santa Cruz Biotechnology, Inc.) and *Ca*Act1p was probed with anti-β-actin antibodies (GTX109639, GeneTex, Inc.)

Nucleotide sequence accession numbers

The sequences of plasmids pHB1S, pSFS2AS and pWTF1 have been submitted to the GenBank with accession numbers KR936168, KR936169 and KT275258, respectively.

Results

Construction of a dominant selection system

Previous studies have shown that the *CaSAT1*-flipper is a useful tool for gene disruption in the molecular manipulation of *C. albicans* (Reuss et al. 2004). Though the benefit of *FLP/FRT* recombination is that it recycles the *CaSAT1* marker, to disrupt two alleles of the target gene, two sequential runs still need to be conducted. Because the functionality of the adopted *CaHygB* marker for resistance to hygromycin B has been confirmed in *C. albicans* and the resistance provided by *CaHygB* and by *CaSAT1* to hygromycin B and nourseothricin are distinct but compatible in *C. albicans* (Basso et al. 2010), we took advantage of the above considerations and sought to develop a rapid and efficient way of making strains with gene knockouts or with conditional gene expression when essential genes are encountered. To facilitate the steps of the disruption procedure, synthetic *CaHygB*-based cassettes were constructed and combined with *FLP/FRT* recombination via the *CaSAT1*-flipper. First, the *CaHygB* cassette in plasmid pHB1S was created by replacing the *CaSAT1*-flipper with the *CaHygB* marker flanked by the *FRT* direct repeat sequences, along with the S1-F and S2-R priming sites from the pFA-based gene deletion modules (Walther and Wendland 2008) (Fig. 1a). Next, because no desired transformants can be obtained from deletions of essential genes

that are involved in cell growth and survival, the tetracycline-regulatable system was introduced to conditionally turn off gene expression in *C. albicans*. The *CaHygB*-based Tet-off cassette in the plasmid pWTF1 consists of two major components of the Tet-off system (Fig. 1a), including a tetracycline-responsive transactivator (tTA) under the constitutive *CaTDH3* promoter and a minimal *CaOP4* promoter fused with a tetracycline-response element (*TetO*), and a *CaHygB* marker flanked with *FRT* sequences that stands between these two functional components of the Tet-off system. TF1-F and TF1-R annealing sites are used for PCR amplification of the Tet-off cassette. Additionally, the *CaSAT1*-flipper cassette in plasmid pSFS2A was inserted with S1-F and S2-R annealing sites to become pSFS2AS for PCR amplification (Fig. 1a).

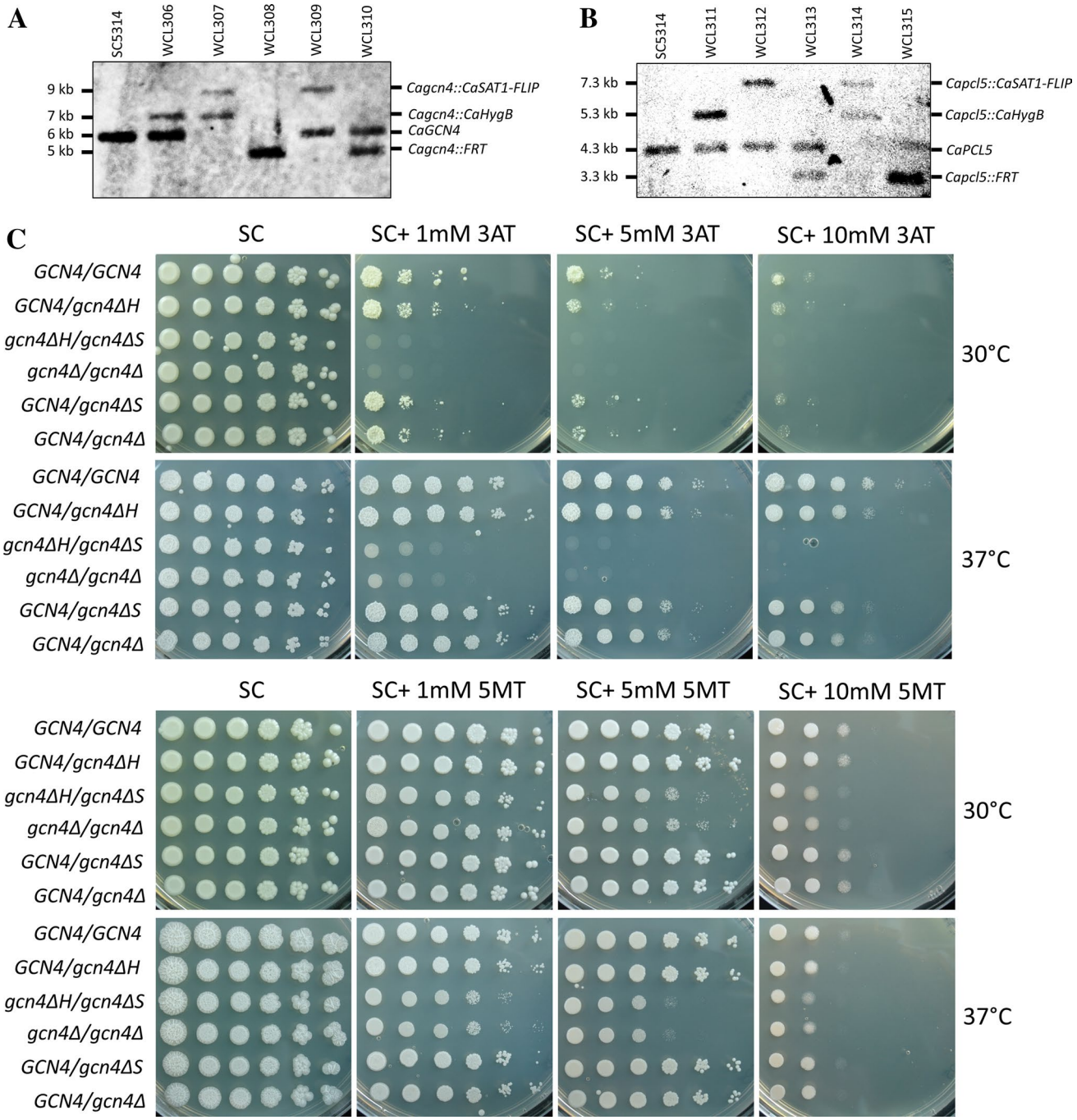
Strategies of gene deletion and conditional gene expression

In the gene deletion strategy (Fig. 1b, I), we took *CaGCN4* as a test gene. The *CaHygB* and *CaSAT1*-flipper cassettes were PCR-amplified with a pair of primers, each of which contains 60 bp of sequence corresponding to the up- and down-stream sequence of *CaGCN4* locus. The amplified cassettes were then sequentially transformed into wild-type SC5314. After transformation with the *CaHygB* cassette, WCL306 (*GCN4/gcn4ΔH*, "*H*" is the abbreviation of *CaHygB)* transformants were selected by hygromycin B. After subsequent transformation of the *CaSAT1* flipper cassette into WCL306, WCL307 (*gcn4ΔS/gcn4ΔH*, "*S*" is the abbreviation of *CaSAT1*) was selected using hygromycin B and nourseothricin together. To obtain the *Cagcn4* mutant WCL308 (*gcn4Δ/gcn4Δ*), WCL307 was maltose-induced for *FLP/FRT* recombination in which the dominant selectable markers, *CaHygB* and *CaSAT1*, could be recycled. Additionally, we used *CaPHO85* as a gene to verify the Tet-off gene regulation strategy (Fig. 1b, II). The Tet-off system cassette that was PCR-amplified from pWTF1, with a pair of primers containing 60 bp of sequence corresponding to upstream of the *CaPHO85* locus and the initial 60 bp of the coding sequence of *CaPHO85*, was transformed into wild-type SC5314 and selected with hygromycin B to obtain WCL301 (P_{TET}-*PHO85:H/PHO85*). Next, *Kpn*I/*Sac*I digested *CaSAT1*-flipper cassette from pSFS2A-PHO85 was transformed into WCL301 to obtain WCL302 (P_{TET}-*PHO85:H/pho85ΔS*) by hygromycin B and nourseothricin selection. Further, the strain WCL302 was maltose-induced *FLP/FRT* recombination for recycle of the dominant selectable markers to generate WCL303 (P_{TET}-*PHO85/pho85Δ*). After popping out these markers by maltose induction, the treated cells were spread onto YPD plates containing 20 μg/ml of nourseothricin to select the sensitive cells, which formed small colonies, from the resistant cells, which formed larger colonies, similar to the case with the *CaSAT1*-flipper. These small colonies were picked up and spotted onto YPD plates containing either hygromycin B or nourseothricin to examine their sensitivity to these antibiotics. These two markers, *CaHygB* and *CaSAT1*, were absent in the final mutants, in which the two modified alleles containing one copy of the *FRT* sequences and the regulatory elements of the Tet-off system constituted the difference from the genome of the wild-type strain.

Fig. 2 Validation of *Cagcn4* and *Capcl5* mutants created by PCR-based *CaHygB* and *CaSAT1*-flipper cassettes. **a** Southern blotting analysis of *CaGCN4* loci. *Afl*II-digested chromosomal DNA from wild-type strain SC5314 and its derivatives WCL306-310 was probed with a *CaGCN4* specific probe. All *Afl*II-digested chromosomal DNA fragments are indicated as wild-type SC5314 (*CaGCN4/CaGCN4*; *GCN4/GCN4*); WCL306 (*CaGCN4/Cagcn4::CaHygB*; *GCN4/gcn4ΔH*); WCL307 (*Cagcn4::CaHygB/Cagcn4::CaSAT1-FLIP*; *gcn4ΔH/gcn4ΔS*); WCL308 (*Cagcn4::FRT/Cagcn4::FRT*; *gcn4Δ/gcn4Δ*); WCL309 (*CaGCN4/Cagcn4::CaSAT1-FLIP*; *GCN4/gcn4ΔS*); WCL310 (*CaGCN4/Cagcn4::FRT*; *GCN4/gcn4Δ*). H: *CaHygB*, S: *CaSAT1*-flipper. **b** Southern blotting analysis of *CaPCL5* loci. *Afl*II-digested chromosomal DNA from wild-type strain SC5314 and *Capcl5* mutants WCL311-315 was hybridized with a specific *CaPCL5* probe. All hybridizing fragments are indicated as wild-type SC5314 (*CaPCL5/CaPCL5*; *PCL5/PCL5*); WCL311 (*CaPCL5/Capcl5::CaHygB*; *PCL5/pcl5ΔH*); WCL312 (*CaPCL5/Capcl5::CaSAT1-FLIP*; *PCL5/pcl5ΔS*); WCL313 (*CaPCL5/Capcl5::FRT; PCL5/pcl5Δ*); WCL314 (*Capcl5::CaHygB/Capcl5::CaSAT1-FLIP*; *pcl5ΔH/pcl5ΔS*); WCL315 (*Capcl5::FRT/Capcl5::FRT*; *pcl5Δ/pcl5Δ*). H: *CaHygB*, S: *CaSAT1*-flipper. **c** Response to amino acid starvation in *Cagcn4* null mutants. Cells of the indicated strains were grown to stationary phase and spotted in serial tenfold dilutions from 10^7 to 10^2 cells/ml on SC medium; SC-His medium containing 1, 5, or 10 mM 3-AT; or SC-Trp medium containing 1, 5, or 10 mM 5-MT at 30 or 37 °C for 2 days. 3-AT, 3-aminotriazole, serves as an analogue of a histidine biosynthesis precursor and a competitive inhibitor of His3p. 5-MT, 5-methyl-tryptophan, functions as an inhibitor of tryptophan synthesis. **d** Colony morphology of wild-type SC5314 and *Cagcn4* mutants. The images were extracted from (**c**). **e** Response to amino acid starvation in *Capcl5* null mutants. Cells of the indicated strains were diluted and spotted on SC or SC-His medium containing 1, 5, or 10 mM 3-AT for 2 days. **f** Colony morphology of wild-type SC5314 and *Capcl5* mutants. The images were extracted from (**e**)

Deletion of *CaGCN4* and *CaPCL5* from the wild-type strain

The metabolism of amino acids is a pivotal process in eukaryotic cells. *CaGCN4* encodes a transcriptional activator that plays a key role in general amino acid control (GAAC) (Tripathi et al. 2002). *CaPCL5* encodes a cyclin, *Ca*Pcl5p, that is transcriptionally regulated by *Ca*Gcn4p (Gildor et al. 2005). *Ca*Gcn4p and *Ca*Pcl5p are in turn regulated by the phosphorylation of *Ca*Pcl5p by the cyclin-dependent kinase *Ca*Pho85p, which leads to ubiquitylation-dependent degradation (Simon et al. 2013).



The use of auxotrophic selection, such as the endogenous markers *CaARG4*, *CaHIS1* and *CaURA3* or the non-albicans markers *CmLEU2* and *CdHIS1*, is limited to auxotrophic strains such as CAI4, BWP17, SN95 and SN152 (Noble and Johnson 2005). Importantly, these markers and strains would be unsuitable for the analysis of *CaGCN4* and *CaPCL5* in nutritional metabolism in *C. albicans*. For these reasons, the *CaHygB* and *CaSAT1*-flipper cassettes were used to construct *Cagcn4* and *Capcl5* mutants. To construct heterozygous and homozygous *Cagcn4* null mutants with the dominant selection system, the *CaHygB* and *CaSAT1*-flipper cassettes were PCR amplified from the templates pHB1S and pSFS2AS with a pair of long primers, including S1-F and S2-R annealing sites and 60 bp of sequence corresponding to the *CaGCN4* locus for deleting the coding region, and they were transformed into wild-type SC5314. WCL306 (*GCN4/gcn4ΔH*), a hygromycin B-resistant strain, and WCL309 (*GCN4/gcn4ΔS*), a nourseothricin-resistant strain, were selected on YPD containing hygromycin B and nourseothricin, respectively. Southern

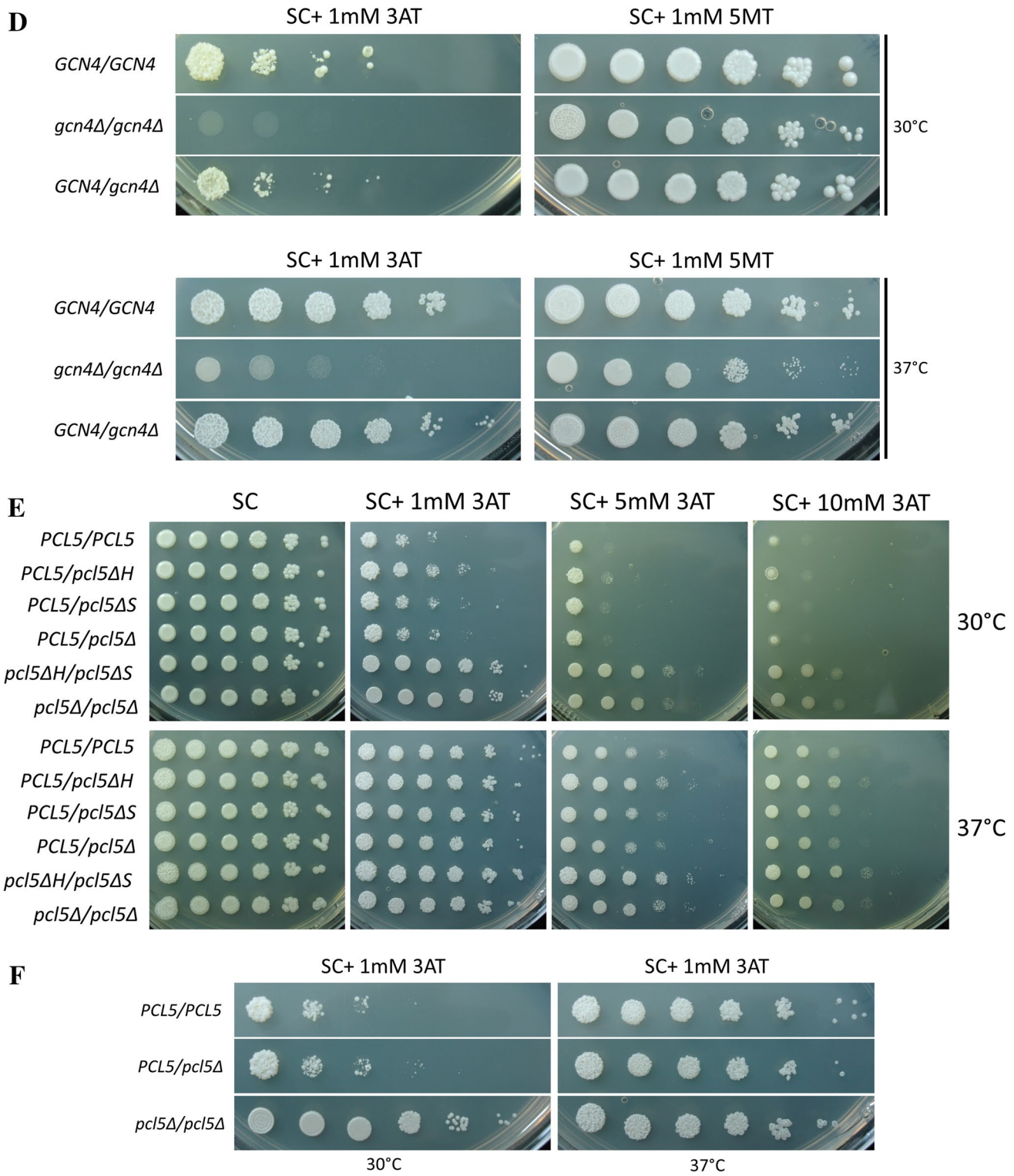


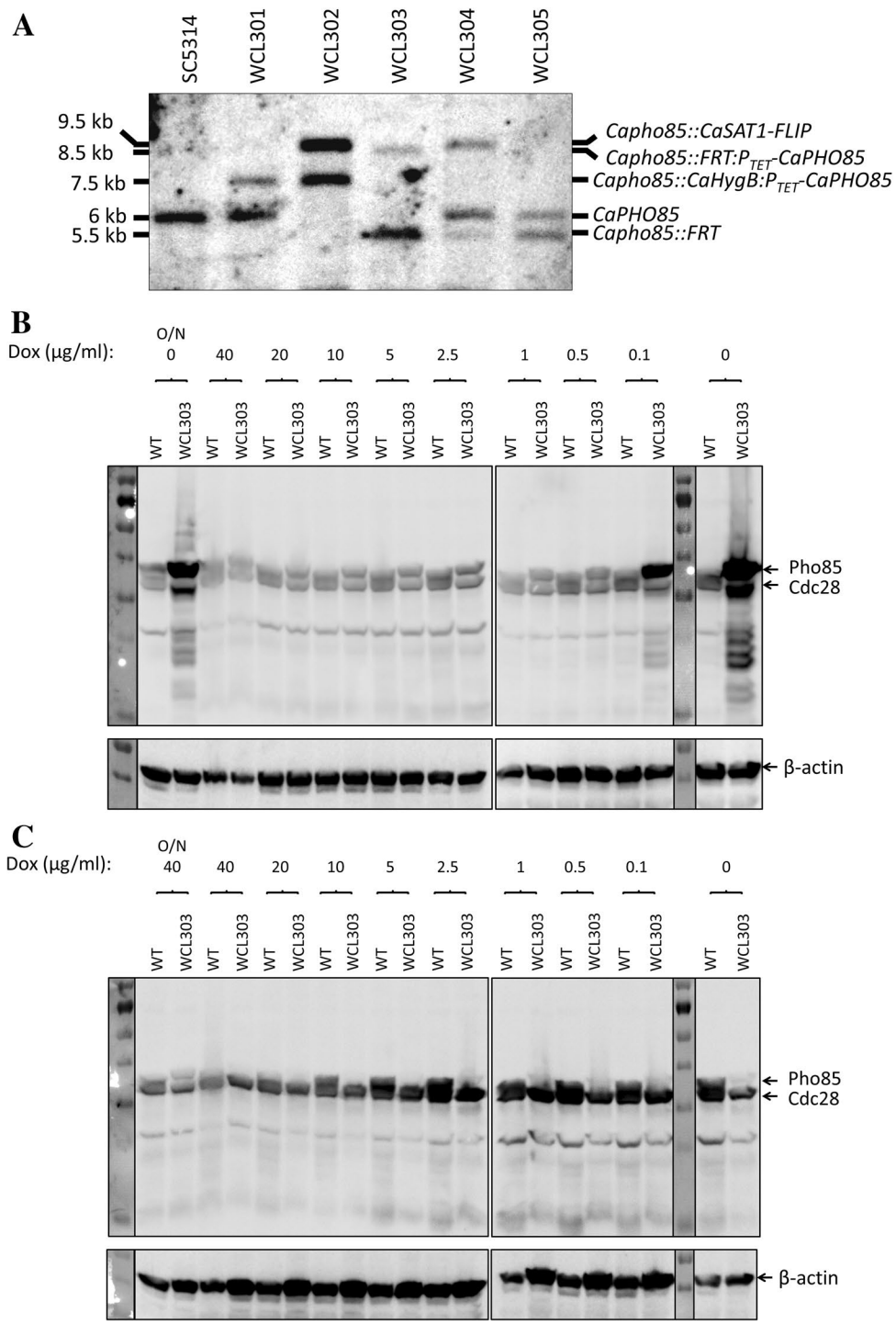
Fig. 2 continued

blotting analysis was used to confirm the genomic structure of *CaGCN4* with the *CaHygB* and *CaSAT1*-flipper cassettes inserted. In wild-type SC5314, both *CaGCN4* alleles were located on a 6-kb *Afl*II-digested fragment that hybridized with a probe homologous to the region of nucleotide position +976 to +1573 with primers CaGCN4 dn PmeI F and CaGCN4 dn SpeI R. The presence of 7 and 9 kb *Afl*II-digested fragments on Southern blotting

revealed the replacement of one *CaGCN4* allele with the *CaHygB* and *CaSAT1*-flipper cassettes in strains WCL306 (*GCN4/gcn4ΔH*) and WCL309 (*GCN4/gcn4ΔS*), respectively (Fig. 2a). To delete the second allele of *CaGCN4*, the *CaSAT1*-flipper cassette was transformed into WCL306, from which WCL307 (*gcn4ΔH/gcn4ΔS*) was obtained by selection with hygromycin B and nourseothricin, keeping both the *CaHygB* and *CaSAT1* markers at the target loci. Integration of the *CaHygB* and *CaSAT1* flipper cassettes at the alleles of *CaGCN4* resulted in the generation of 7 and 9 kb *Afl*II-digested fragments from the 6-kb fragment in the wild-type strain (Fig. 2a). To induce *FLP*/*FRT*-mediated intrachromosomal recombination to generate homozygous and heterozygous *Cagcn4* mutants without any dominant selectable markers, single colonies of the strains WCL307 (*gcn4ΔH/gcn4ΔS*) and WCL309 (*GCN4/gcn4ΔS*) were grown in YPM (maltose) to induce the *CaMAL2* promoter-driven expression of *CaFLP* recombinase. The culture of the resulting strains was streaked on YPD agar plates with a low concentration (20 μg/ml) of nourseothricin, and the nourseothricin-sensitive colonies grew more slowly than did the resistant ones. Strains WCL308 (*gcn4Δ/gcn4Δ*) and WCL310 (*GCN4/gcn4Δ*) were derived from strains WCL307 (*gcn4ΔH/gcn4ΔS*) and WCL309 (*GCN4/gcn4ΔS*) via excision of the dominant markers. In Southern hybridization, both disrupted alleles of *CaGCN4* in WCL308 (*gcn4Δ/gcn4Δ*) presented as a 5-kb *Afl*II-digested fragment, whereas 5 and 6 kb *Afl*II-digested fragments showed the disrupted and wild-type alleles of *CaGCN4* in WCL310 (*GCN4/gcn4Δ*) (Fig. 2a). Following the construction of the *Cagcn4* mutants, heterozygous and homozygous *Capcl5* mutants were also constructed. Southern blotting was used to validate the genomic structure of the indicated strains with a *CaPCL5* specific probe amplified by primers CaPCL5 probe F and CaPCL5 probe R. Both alleles of *CaPCL5* digested with *Afl*II probed as 4.3 kb fragments, whereas the alleles of *CaPCL5* with *CaHygB* and *CaSAT1* flipper cassette insertions presented as 5.3 and 7.3 kb *Afl*II-digested fragments on the blots. After popping out the dominant markers via *FLP*/*FRT* recombination, a 3.3-kb fragment with one copy of the *FRT* sequence existed in WCL313 (*PCL5/pcl5Δ*) as a heterozygous mutant and WCL315 (*pcl5Δ/pcl5Δ*) as a homozygous mutant (Fig. 2b). To confirm that the *Cagcn4* and *Capcl5* mutants have the ability to resist hygromycin B and nourseothricin, the relevant strains were streaked onto plates containing hygromycin B, nourseothricin, or both. The *Cagcn4* mutant derivatives WCL306 (*GCN4/gcn4ΔH*) and WCL307 (*gcn4ΔH/gcn4ΔS*) and the *Capcl5* mutant derivatives WCL311 (*PCL5/pcl5ΔH*) and WCL314 (*pcl5ΔH/pcl5ΔS*) were able to grow on plates with hygromycin B (Figure S1). The *Cagcn4* and *Capcl5* homozygous null mutants WCL307 (*gcn4ΔH/gcn4ΔS*) and WCL314 (*pcl5ΔH/pcl5ΔS*) and the heterozygous mutants WCL 309 (*GCN4/gcn4ΔS*) and WCL312 (*PCL5/pcl5ΔS*) were able to grow on plates with nourseothricin (Figure S1). Only WCL307 and WCL314 with two inserted markers were able to grow on plates with both hygromycin B and nourseothricin (Figure S1). The dominant markers were excised by *FLP*/*FRT* recombination to obtain *Cagcn4* heterozygous and homozygous mutants, WCL310 (*GCN4/gcn4Δ*) and WCL308 (*gcn4Δ/gcn4Δ*), and *Capcl5* heterozygous and homozygous mutants, WCL313 (*PCL5/pcl5Δ*) and WCL315 (*pcl5Δ/pcl5Δ*). These mutants were incapable of growing on plates with any antibiotics other than the YPD plates (Figure S1). Through Southern blotting and genotyping assays, the deletion strategy used in the wild-type strain SC5314 was validated.

Responses to nutrient starvation in *Cagcn4* and *Capcl5* mutants

The chemical agents of 3-aminotriazole (3-AT), 5-methyltryptophan (5-MT) and sulfometuron methyl (SM) are generally used as inducers of nutrient starvation in yeast (Dever 1997). To examine the sensitivity to nutrient starvation in *C. albicans* resulting from the deletion of *CaGCN4* and *CaPCL5*, cultured cells were serially diluted and spotted onto SC agar plates with or without 3-AT or 5-MT at concentrations of 1, 5 and 10 mM at 30 and 37 °C. The *Cagcn4* heterozygous and homozygous mutants were sensitive to 3-AT and 5-MT compared with wild-type SC5314 (Fig. 2c), which is consistent with previous observations (Gildor et al. 2005; Tripathi et al. 2002). Interestingly, the *Cagcn4* homozygous mutants, including WCL307 (*gcn4ΔH/gcn4ΔS*) and WCL308 (*gcn4Δ/gcn4Δ*), were more sensitive to 3-AT than was the *Cagcn4* heterozygous mutant. With respect to colony morphology, colonies of both the wild-type and the *Cagcn4* heterozygous mutants displayed a wrinkled style, with the wild-type exhibiting more extensive wrinkling (Fig. 2d). Conversely, the colonies of the *Cagcn4* homozygous mutants sustained the flatted form at 37 °C. Additionally, an increased concentration of 3-AT resulted in a growth defect of the *Cagcn4* mutants (Fig. 2c). Because *CaPCL5* negatively regulates *CaGCN4*, the *Capcl5* homo- and heterozygous mutants were also tested in the same conditions as above. The resistance of *Capcl5* homozygous mutants WCL314 (*pcl5ΔH/pcl5ΔS*) and WCL315 (*pcl5Δ/pcl5Δ*) to nutrient starvation by 3-AT was stronger than that of the wild-type (Fig. 2e). However, the colony morphology of *Capcl5* homozygous mutant was less wrinkled than that of the wild-type (Fig. 3f). These results are consistent with previous studies regarding the functionality of *CaGCN4* and *CaPCL5* (Gildor et al. 2005; Tripathi et al. 2002).



Construction of a tetracycline-regulated *CaPHO85* expression mutant

In our preliminary test, *CaPHO85* was unable to be disrupted by the dominant selection system, though both *CaHygB* and *CaSAT1* markers were used in strain construction, demonstrating that it is an essential gene. For this reason, the tetracycline-regulated gene expression (Tet-off) system with dominant selection was used as an alternative way to switch off gene expression in wild-type SC5314. To replace the endogenous promoter with the Tet-off system, the Tet-off cassette with the *CaHygB* marker consisting of the gene encoding tTA and *TetO* was PCR-amplified and transformed to target the *CaPHO85* locus of *C. albicans*.

◀**Fig. 3** Validation of conditional Tet-off *CaPHO85* mutant created by PCR-based pWTF1 and pSFS2AS. **a** Southern blotting analysis of *CaPHO85* loci in *C. albicans*. *Nco*I-digested chromosomal DNA fragments from wild-type SC5314 and conditional Tet-off *CaPHO85* mutant derivatives WCL301-305 hybridized with a specific probe are indicated as a 6-kb fragment specific to SC5314 (*CaPHO85/CaPHO85*; *PHO85/PHO85*); two fragments of 6 and 7.5 kb specific to WCL301 (*CaPHO85/Capho85::*P_{TET}*-CaPHO85:CaHygB*; *PHO85/*P_{TET}*-PHO85:H*); two fragments of 7.5 and 9.5 kb specific to WCL302 (*Capho85::*P_{TET}*-CaPHO85:CaHygB/Capho85::CaSAT1-FLIP*; P_{TET}*-PHO85:H/pho85ΔS*); two fragments of 8.5 and 5.5 kb specific to WCL303 (*Capho85::*P_{TET}*-CaPHO85:FRT/Capho85::FRT*; P_{TET}*-PHO85/pho85Δ*); two fragments of 6 and 9.5 kb specific to WCL304 (*CaPHO85/Capho85::CaSAT1-FLIP*; *PHO85/pho85ΔS*); two fragments of 6 and 5.5 kb specific to WCL305 (*CaPHO85/Capho85::FRT*; *PHO85/pho85Δ*). H: *CaHygB*; S: *CaSAT1*-flipper. **b** The level of *Ca*Pho85p expression is repressed by the Tet-off system in a dose-dependent manner. Cells of the strains SC5314 and WCL303 (the conditional Tet-off *CaPHO85* mutant) were grown overnight in YPD medium and diluted into fresh YPD medium with 40, 20, 10, 5, 2.5, 1, 0.5 or 0.1 μg/ml of Dox for 3 h of incubation at 30 °C along with the control without Dox. Lysate was extracted from the strains by beating the cells with glass beads after Dox repression. Total protein of the lysates was quantified and separated by 12 % SDS-PAGE, and western blotting analysis was used to detect the amount of *Ca*Pho85p via anti-PSTAIRE antibody. Anti-β-actin antibody was used as the loading control. **c** The expression of *Ca*Pho85p is shut down by the Tet-off system. Cells of the strains SC5314 and WCL303 were grown overnight in YPD medium containing 40 μg/ml Dox and diluted with the same dilution series of Dox for 3 h of incubation at 30 °C for comparing the effect of dose on repression. Anti-PSTAIRE antibody was used to detect *Ca*Pho85p and anti-β-actin antibody was used as the loading control. **d** Phenotype of the conditional Tet-off *CaPHO85* mutant under *CaPHO85* repression by the Tet promoter under inhibition of Hsp90p with Geldanamycin (GdA). Cells of the indicated strains were grown overnight with 40 μg/ml Dox and serially diluted for spotting on agar with and without Dox at 30 and 37 °C for 2 days. SD, synthetic minimal medium containing ammonium sulphate as the nitrogen base with glucose. **e** Images of colony morphology of the wild-type and the conditional Tet-off *CaPHO85* mutants were extracted and enlarged from (**d**), framed in *black* (SD and SD + Dox) and *red* (SD + GdA and SD + GdA + Dox). **f** Phenotype of the conditional Tet-off *CaPHO85* mutant repressed *CaPHO85* in a variety of nutrients. Cells of the indicated strains were spotted on the SGal and SProD medium with or without Dox at 30 and 37 °C for 2 days. SGal, synthetic minimal medium with galactose in place of glucose; SProD, synthetic minimal medium with proline in place of ammonium sulphate. **g** Images of colony morphology of the wild-type and the conditional Tet-off *CaPHO85* mutant were extracted and enlarged from (**f**), framed in *yellow* (SGal and SGal + Dox) and *blue* (SProD and SProD + Dox). **h** Decreased filamentation resulting from *CaPHO85* repression by the Tet-off system. Cells of the indicated strains were grown overnight in YPD medium containing 40 μg/ml Dox and spotted in tenfold serial dilutions of the culture from OD600 of 1 on Spider and Lee's pH 4.5 or 6.8 medium at 30 or 37 °C to induce filaments to form wrinkled colonies. **i** Comparison of colony morphology with image enlargement among these colonies selected from (**h**), framed in *red* and *blue*

Subsequently, hygB-resistant WCL301 (P_{TET}*-PHO85:H/PHO85*) transformants were selected on plates containing hygromycin B and confirmed by colony PCR and Southern blotting analysis used a probe with primers CaPHO85 DN NotI F and CaPHO85 DN SacI R. As shown in the Southern blots (Fig. 3a), a 6-kb *Nco*I fragment was obtained in wild-type SC5314 and WCL301 (P_{TET}*-PHO85:H/PHO85*), which indicates the presence of the original form of the *CaPHO85* locus. After transformation with the Tet-off cassette, a 7.5-kb *Nco*I DNA fragment was present in strain WCL301 strain as the Tet-off cassette had been integrated at the *CaPHO85* locus (Fig. 3a). To further disrupt the other allele of *CaPHO85*, *Kpn*I/*Sac*I digested *CaSAT1*-flipper cassette released from pSFS2A-CaPHO85 was transformed into strain WCL301 to disrupt the other allele of *CaPHO85*, and strain WCL302 (P_{TET}*-PHO85:H/pho85ΔS*) was obtained. The presence of both 7.5 and 9.5 kb *Nco*I-digested DNA fragments from WCL302 indicated that one allele of *CaPHO85* was regulated by the Tet-off system with *CaHygB* and that the other was disrupted with the *CaSAT1*-flipper (Fig. 3a). By *FLP*/*FRT* mediated recombination, the two dominant markers *CaHygB* and *CaSAT1* were excised from strain WCL302 to generate WCL303 (P_{TET}*-PHO85/pho85Δ*), in which the genome is the same as the wild-type except at the *CaPHO85* locus. As observed in the blots, the presence of both 8.5 and 5.5 kb *Nco*I-digested DNA fragments indicated a Tet-off system inserted allele of *CaPHO85* and a disrupted allele, respectively (Fig. 3a). Moreover, to construct a heterozygous *Capho85* mutant, the *CaSAT1*-flipper cassette was used to delete one *CaPHO85* allele to generate strain WCL304 (*PHO85/pho85ΔS*). Next, the release of *CaSAT1* was achieved by means of maltose induction to obtain WCL305 (*PHO85/pho85Δ*). In Southern blots, the presence of 9.5 and 5.5 kb *Nco*I-digested DNA fragments was an indication of the inserted allele and a deleted allele with one copy of *FRT* (Fig. 3a). Therefore, by constructing a series of *Capho85* mutants, a dominant selectable Tet-off system was established.

Responses of the strain with conditionally repressed *Ca*Pho85p

After constructing the conditional Tet-off *CaPHO85* mutant WCL303 (P_{TET}*-PHO85/pho85Δ*), it was grown in parallel with wild type strain SC5314 in YPD medium with a series of concentrations of Dox to reveal the level of *Ca*Pho85p regulation by the Tet-off system. Total protein extracted from strains WCL303 and SC5314 after Dox treatment was quantified and subjected to Western blot analysis with anti-Cdc2/p34 antibodies, which specifically recognize PSTAIRE motif of the cyclin-dependent kinases (Cdk) *Ca*Cdc28p and *Ca*Pho85p (Nishizawa et al. 1999). In general, the cells of the strains being assayed were grown in either YPD or SD medium before any treatment. Following this concept, the cells of WCL303 and SC5314 were grown overnight in YPD medium without Dox, and on the

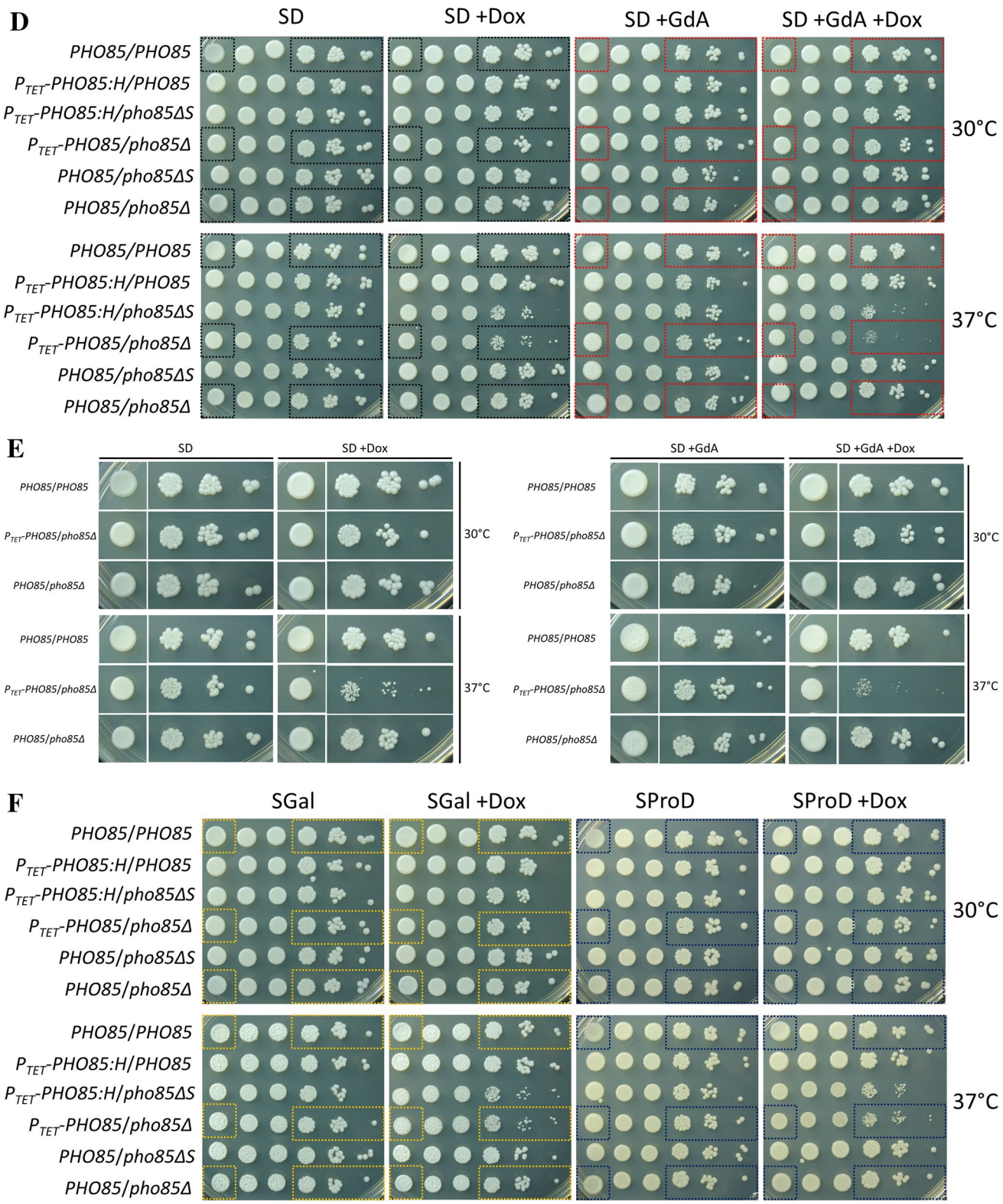


Fig. 3 continued

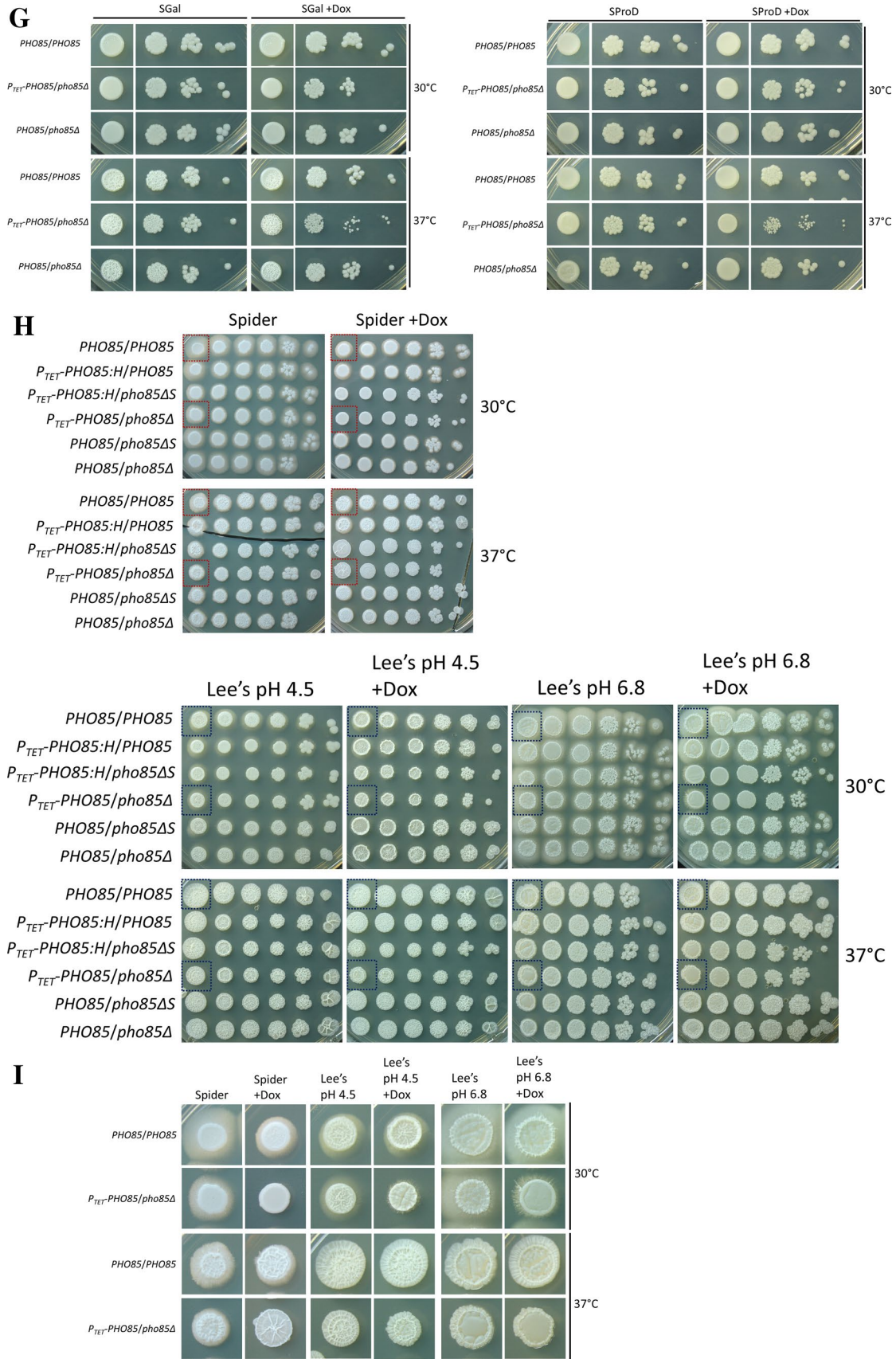


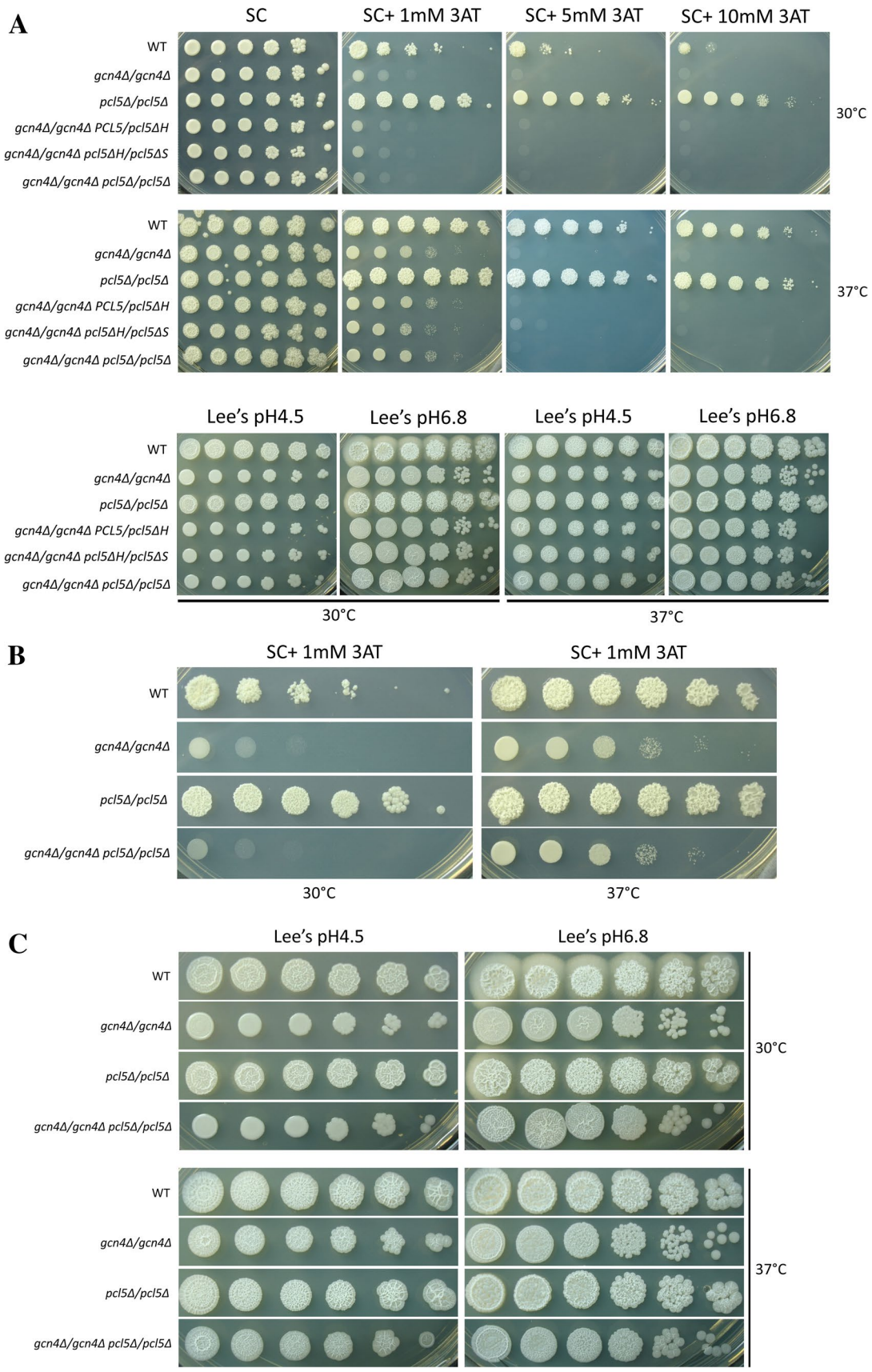
Fig. 3 continued

next day, the cultures were diluted at a 1:10 ratio into fresh YPD medium with 0, 0.1, 0.5, 1, 2.5, 5, 10, 20, or 40 μg/ml of Dox for 3 h to collect pellets of repressed and unrepressed cells. Lysates from these indicated samples were separated with SDS-PAGE, and the levels of *Ca*Pho85p were analysed by Western blotting. A reduced amount of *Ca*Pho85p was detected in strain WCL303 (P_{TET}-*PHO85/pho85Δ*) under the higher dose of Dox (Fig. 3b). Compared with the decrease of *Ca*Pho85p in strain WCL303 (P_{TET}-*PHO85/pho85Δ*), *Ca*Pho85p in the wild-type strain remained as usual (Fig. 3b). Two major bands correspond to *Ca*Cdc28p and *Ca*Pho85p in the Western blots because these two Cdks are alike in molecular weight (Fig. 3b, c, arrows indicated), even though minor signals get picked up, presumably due to the non-specific proteins being cross-reacted to the anti-Cdc2/p34 antibodies. Furthermore, to completely shut off the level of *Ca*Pho85p, the cells of WCL303 (P_{TET}-*PHO85/pho85Δ*) and SC5314 were grown overnight in YPD medium with 40 μg/ml of Dox, and the cultures were diluted into fresh YPD medium as above to collect pellets on the next day. While *Ca*Pho85p was noticeable in the wild-type strain, it was barely detectable in strain WCL303 (P_{TET}-*PHO85/pho85Δ*) with addition of Dox (Fig. 3c).

It has been shown that a Tet-repressible *CaPHO85* mutant grown in the presence of Dox decreases in growth due to the depletion of *Ca*Pho85p (Shapiro et al. 2012). To test the effect on growth, *CaPHO85* derivative strains under *CaPHO85*-repressed conditions were serially diluted and spotted on synthetic minimal agar plates with or without Dox, followed by incubation at 30 and 37 °C for the 3 days. When *CaPHO85* was repressed in the presence of Dox, the cells of strains WCL302 (P_{TET}-*PHO85:H/pho85ΔS*) and WCL303 (P_{TET}-*PHO85/pho85Δ*) grew significantly slower than did those of the wild-type SC5314 or the heterozygous mutants WCL304 (*PHO85/pho85*ΔS) and WCL305 (*PHO85/pho85Δ*), thus formed smaller colonies, especially at 37 °C (Fig. 3d, e). These results are consistent with previous observations in which small colonies formed by the conditional Tet-off *CaPHO85* mutants resulted from slow growth when the expression of *CaPHO85* is repressed (Shapiro et al. 2012). Additionally, it is known that *Ca*Hsp90p being compromised by the inhibitor geldanamycin (GdA) and temperature stress up to 42 °C induces filamentation through the transcription factor *Ca*Hms1p regulated by *Ca*Pho85p-*Ca*Pcl1p (Shapiro et al. 2012). On the colony morphology level, the circular colonies transformed into wrinkled form due to temperature stress, but how GdA affects the morphology of the colonies remained unclear. Based on the above functional relationship between *Ca*Pho85p and *Ca*Hsp90p, the strains were subjected to GdA treatment in the spotting assay to assess the effect of GdA. Regardless of the presence or absence of Dox, the ability to form colony in cells of all strains with GdA at 30 °C was indistinguishable (Fig. 3d, e). However, unlike other strains that formed colonies normally, the conditional Tet-off *CaPHO85* mutants WCL302 (P_{TET}-*PHO85:H/pho85ΔS*) and WCL303 (P_{TET}-*PHO85/pho85Δ*) were almost incapable of forming colonies in the presence of Dox and GdA at 37 °C (Fig. 3e). These results suggest the requirement of *Ca*Pho85p for the survival of *C. albicans* under stress of high temperature, particularly when *Ca*Hsp90p is compromised.

It has been shown that *S. cerevisiae* with defective *PHO85* ceases to grow on carbon sources other than glucose, including galactose, sucrose, maltose, lactose, raffinose and ethanol (Lee et al. 2000). To test the involvement of *CaPHO85* in responses to carbon sources and, possibly, to nitrogen sources, we inoculated strains WCL 301–305 and wild-type SC5314 into YPD with Dox overnight and then serially diluted them onto agar plates with galactose as the sole carbon source or proline as the sole nitrogen source. The slow growth of those strains with repressed *CaPHO85* could be clearly observed on agar at 37 °C compared with

Fig. 4 Epistatic relationships of *CaPHO85*, *CaPCL5* and *CaGCN4* ▶ in nutrient starvation. **a** Epistasis of *Cagcn4* over *Capcl5* under nutrient starvation by 3-AT and amino acid-rich conditions with Lee's medium. Cells of strains WCL308 (*gcn4Δ/gcn4Δ*), WCL315 (*pcl5Δ/pcl5Δ*), WCL324 (*gcn4Δ/gcn4Δ pcl5Δ/pcl5Δ*) and its derivatives WCL322 (*gcn4Δ/gcn4Δ PCL5/pcl5ΔH*) and WCL323 (*gcn4Δ/gcn4Δ pcl5ΔH/pcl5ΔS*) were grown overnight and spotted on SC-his medium with 3-AT in contrast to SC at 30 or 37 °C for 2 days and Lee's medium with pH 4.5 or 6.8 at 30 or 37 °C for 6 days. **b** Images of colony morphology of the indicated strains under nutrient starvation by 1 mM 3-AT were extracted from (**a**). **c** *CaGCN4* serving as an epistatic regulator of filamentous growth over *CaPLC5*. Cells of *Cagcn4* and *Capcl5* mutants, together with *Cagcn4 Capcl5* double mutant were spotted on Lee's medium with pH 4.5 or 6.8 at 30 or 37 °C for 6 days. Images of colony morphology were extracted from (**a**). **d** The relationship of *CaPHO85* and *CaGCN4* under nutrient starvation by 3-AT. Cells of strains WCL303 (P_{TET}-*PHO85/pho85Δ*), WCL308 (*gcn4Δ/gcn4Δ*), WCL318 (P_{TET}-*PHO85/pho85Δ gcn4Δ/gcn4Δ*) and its derivatives WCL316 (P_{TET}-*PHO85/pho85Δ GCN4/gcn4ΔH*) and WCL317 (P_{TET}-*PHO85/pho85Δ gcn4ΔH/gcn4ΔS*) were grown overnight with Dox and spotted on SC medium containing 1, 5, or 10 mM 3-AT with or without Dox at 30 or 37 °C for 2 days. **e** Sensitivity of the conditional Tet-off *CaPHO85* mutant with deletion of *CaGCN4* to 3-AT. Images of colony morphology of the indicated strains under nutrient starvation by 1 mM 3-AT were extracted and enlarged from (**d**). **f** Cooperation of *CaPHO85* and *CaPCL5* in resistance to 3-AT and sensitivity to high temperature. Cells of strains WCL303 (P_{TET}-*PHO85/pho85Δ*), WCL315 (*pcl5Δ/pcl5Δ*), WCL321 (P_{TET}-*PHO85/pho85Δ pcl5Δ/pcl5Δ*) and its derivatives WCL319 (P_{TET}-*PHO85/pho85Δ PCL5/pcl5ΔH*) and WCL320 (P_{TET}-*PHO85/pho85Δ pcl5ΔH/pcl5ΔS*) were inoculated into YPD with Dox overnight and spotted onto SC plus 1, 5, or 10 mM 3-AT with or without Dox at 30 or 37 °C for 2 days. **g** Weaker wrinkly colonies developed by the conditional Tet-off *CaPHO85* mutant with depletion of *CaPCL5* under nutrient starvation by 3-AT. Images of colony morphology of the indicated strains under nutrient starvation by 1 mM 3-AT were extracted and enlarged from (**f**)


A
SC
SC+ 1mM 3AT
SC+ 5mM 3AT
SC+ 10mM 3AT
WT
gcn4Δ/gcn4Δ
pcl5Δ/pcl5Δ
gcn4Δ/gcn4Δ PCL5/pcl5ΔH
gcn4Δ/gcn4Δ pcl5ΔH/pcl5ΔS
gcn4Δ/gcn4Δ pcl5Δ/pcl5Δ
30°C
37°C
Lee's pH4.5
Lee's pH6.8
B
C

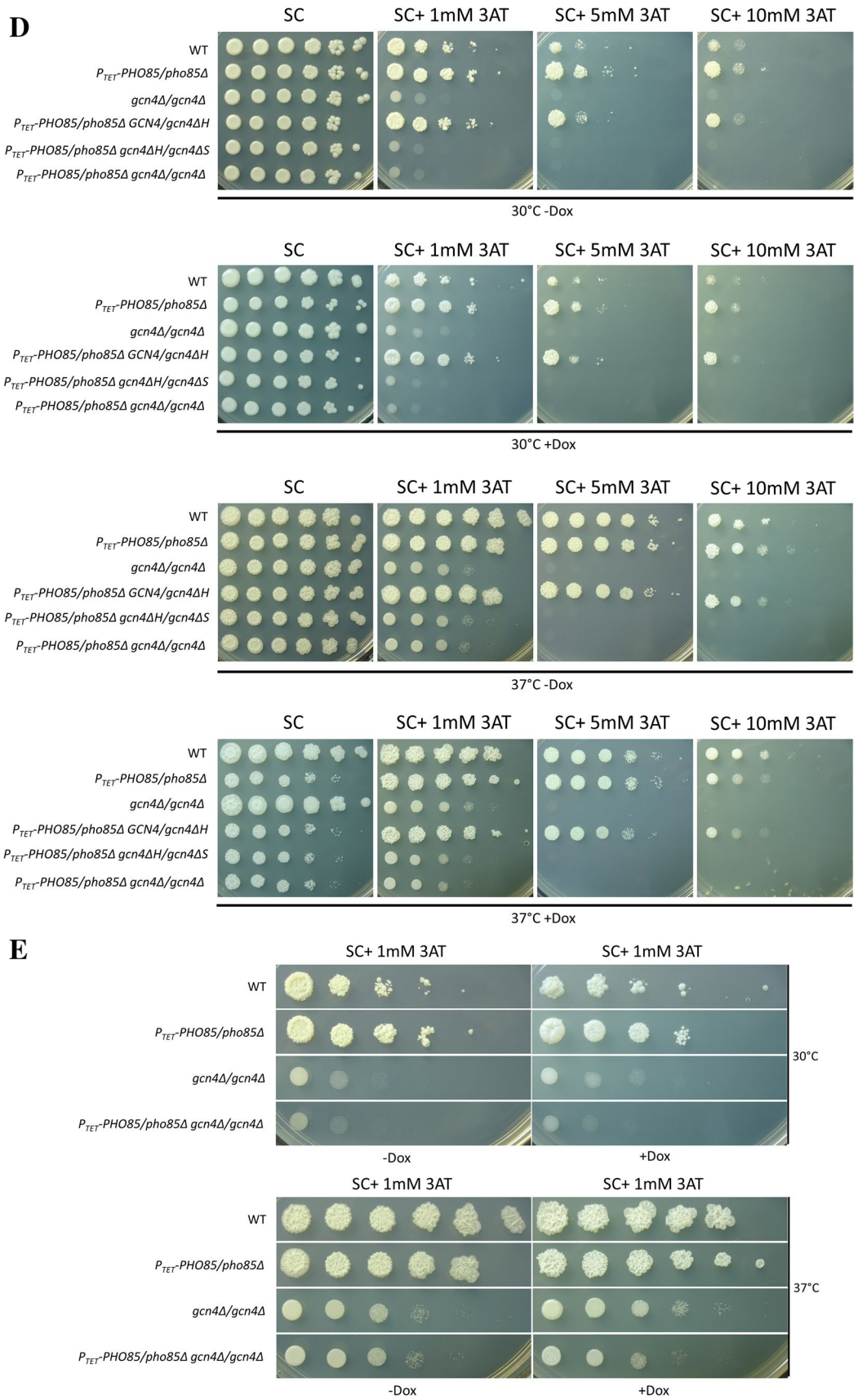


Fig. 4 continued

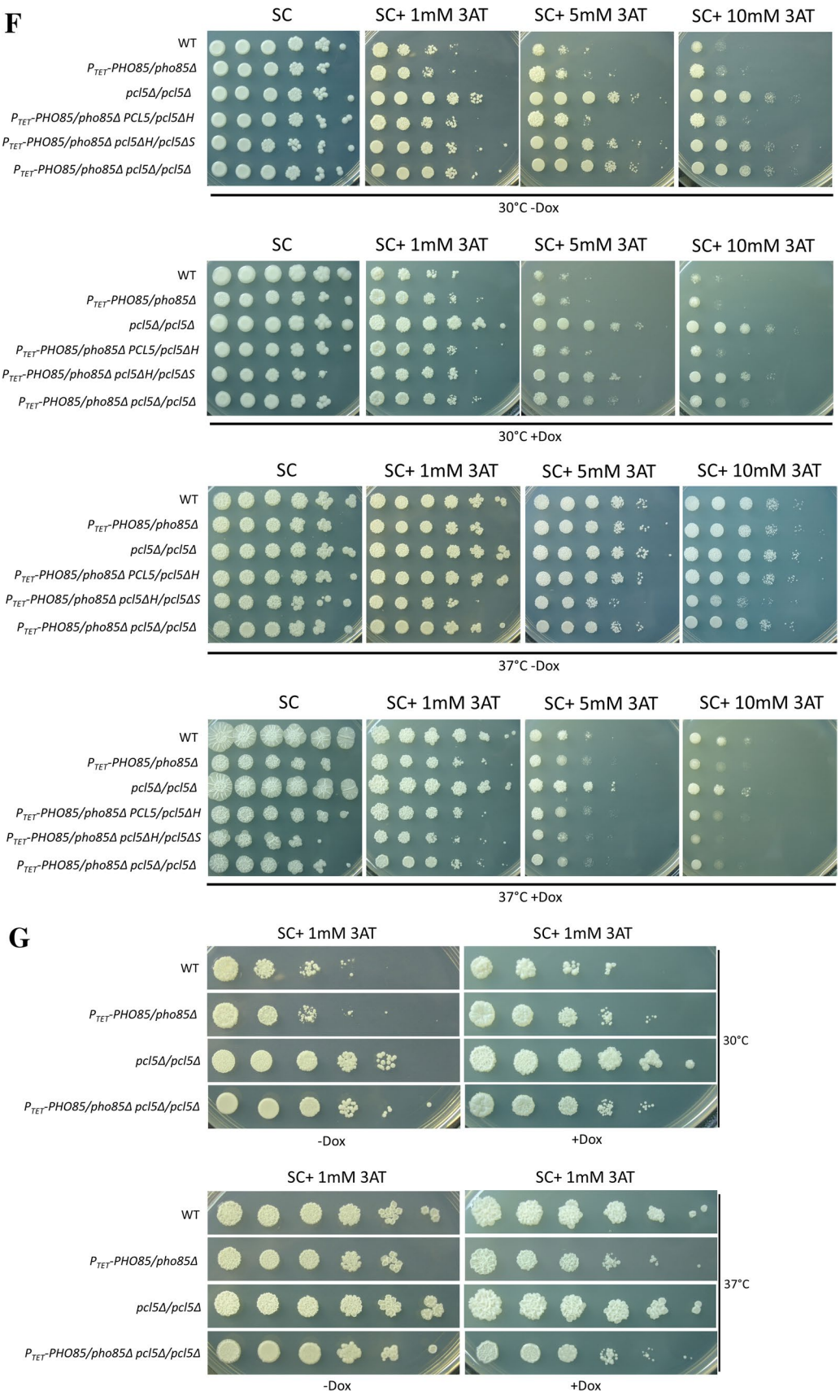


Fig. 4 continued

that of the other strains (Fig. 3f, g). In addition, to monitor the colony phenotypes of the conditional Tet-off *CaPHO85* mutant in response to the filament-induced conditions, the strains WCL301-305 and wild-type SC5314 were serially diluted and spotted on the Spider adjusted to pH 7.2 and Lee's medium adjusted to pH 6.8 and 4.5 with or without Dox. The strains WCL302 (P_{TET}-*PHO85:H/pho85ΔS*) and WCL303 (P_{TET}-*PHO85/pho85Δ*) were unable to form filaments at 30 °C and became smoother colonies with far less filaments on Spider medium at 37 °C in the presence of Dox in contrast to the phenotype of the wild-type SC5314 (Fig. 3h, i). Similarly, reduced filamentous development of the conditional Tet-off *CaPHO85* mutants were observed on the Lee's medium at 30 °C with Dox (Fig. 3h, i). Additionally, compared with the wild-type SC5314 and the heterozygous mutants WCL304 (*PHO85/pho85ΔS*) and WCL305 (*PHO85/pho85Δ*), the two conditional Tet-off *CaPHO85* mutants appeared to develop smaller colonies (Fig. 3i). Taken together, we concluded that the repression of *CaPHO85* in *C. albicans* led to decrease in proliferation, susceptibility to GdA (Hsp90 inhibitor) and unusual nutrients at 37 °C, and diminished filamentation in the filament-induced conditions.

Relationship among *CaPHO85*, *CaPCL5* and *CaGCN4* in nutrient sensing

As described above, we have established a system with dominant selection to switch off target gene expression in *C. albicans*, including gene deletion and conditional regulation. Previous studies in *S. cerevisiae* have indicated that the cyclin-dependent kinase Pho85p interacts with its cyclin Pcl5p to phosphorylate transactivator Gcn4p, which results in ubiquitylation via SCF^{Cdc4p} for degradation by the proteasome (Meimoun et al. 2000). Based on these known cellular processes, we used the dominant system to construct a *Cagcn4 Capcl5* double null mutant and the conditional Tet-off *CaPHO85* mutants deleted for either *CaPCL5* or *CaGCN4* to determine the relationships in nutrient sensing.

To examine the responses of the strains WCL308 (*gcn4Δ/gcn4Δ*), WCL315 (*pcl5Δ/pcl5Δ*) and WCL324 (*gcn4Δ/gcn4Δ pcl5Δ/pcl5Δ*) to nutrient starvation, overnight cultures of the strains were serially diluted and spotted onto SC agar plates with and without 3-AT and Lee's medium. Additionally, the strains WCL322 derived from WCL308 with one allele of *CaPCL5* deleted by insertion of *CaHygB* cassette and WCL322 derivative WCL323 with two alleles of *CaPCL5* deleted by insertion of either a *CaHygB* or a *CaSAT1*-flipper cassette were tested in parallel. The observation that strain WCL 308 (*gcn4Δ/gcn4Δ*) was sensitive to 3-AT causing starvation and WCL315 (*pcl5Δ/pcl5Δ*) was resistant to 3-AT (Fig. 4a) is consistent with the negative regulation between *CaGCN4* and *CaPCL5* (Gildor et al. 2005). Interestingly, strain WCL324 (*gcn4Δ/gcn4Δ pcl5Δ/pcl5Δ*) was sensitive to 3-AT and responded similarly to strain WCL308 (*gcn4Δ/gcn4Δ*) (Fig. 4b), which suggests that the epistatic state of *CaGCN4* is a master regulator in nutrient starvation. To assess the effect of dominant selection markers, the strains WCL322 (*gcn4Δ/gcn4Δ PCL5/pcl5ΔH*) and WCL323 (*gcn4Δ/gcn4Δ pcl5ΔH/pcl5ΔS*) bearing the markers were compared with WCL324 (*gcn4Δ/gcn4Δ pcl5Δ/pcl5Δ*) bearing no markers. As shown in Fig. 4a, these strains, WCL323 and WCL324, displayed similar colony morphology, which suggests that the presence of dominant selection markers does not interfere with the functional interaction between *CaGCN4* and *CaPCL5* in *C. albicans*. Moreover, *C. albicans* cells lacking *CaGCN4*, irrespective of the presence or the absence of *CaPCL5,* exhibited impairment of filamentation on Lee's medium, notably in pH 4.5 at 30 °C (Fig. 4a, c), which suggests that *Ca*Gcn4 serves as an epistatic regulator of filamentation in response to nutrient signalling.

In *S. cerevisiae*, Pho85p plays a pivotal role in the metabolism of carbon, nitrogen and phosphate via forming complexes with the cyclins Pcl6p/Pcl7p, Pcl5p and Pho80p (Huang et al. 2007). To understand how the function of *CaPHO85* is influenced by *CaGCN4* in response to nutrient starvation, the strains WCL303 (P_{TET}-*PHO85/pho85Δ*), WCL308 (*gcn4Δ/gcn4Δ*) and WCL318 (P_{TET}-*PHO85/pho85Δ gcn4Δ/gcn4Δ*), along with WCL316 (P_{TET}-*PHO85/pho85Δ GCN4/gcn4ΔH*) and WCL317 (P_{TET}-*PHO85/pho85Δ gcn4ΔH/gcn4ΔS*) that were the intermediate strains in strain construction grown in YPD medium with Dox overnight were serially diluted and spotted onto agar plates with or without 3-AT and Dox for 3 days. Due to the slow growth resulting from repression of *CaPHO85* by the Tet promoter, the strains bearing Tet-repressible *CaPHO85*, WCL303, WCL316, WCL317 and WCL318 grew as smaller colonies on SC agar plates with Dox than on those without Dox (Fig. 4d). Interestingly, colonies of strain WCL303 (P_{TET}-*PHO85/pho85Δ*) grew larger than those of the wild-type strain on agar with 3-AT at 30 °C, notably without Dox (Fig. 4d, upper panel, e), but on agar with 3-AT and Dox at 37 °C, the WCL303 colonies became weaker than wild-type colonies (Fig. 4d, lower panel, e). This finding suggested that *CaPHO85* plays a primary role in adaption to high temperature and in resisting nutrient starvation. Because Cdc28p and Pho85p share many substrates involving cell cycle in *S. cerevisiae* (Carroll and O'Shea 2002; Huang et al. 2007; Jimenez et al. 2013) and *Ca*Pho85p is able to complement *Scpho85* mutant (Miyakawa 2000), *Ca*Pho85p also possibly acts as a regulator in cell cycle under the nutrient starvation and thus increases the colony size of WCL303 under the unrepressed condition. Moreover, it is noteworthy that the deletion of

CaGCN4 made the cells of all strains sensitive to nutrient starvation (Fig. 4d, e), which suggests that *CaGCN4* is epistatic to *CaPHO85* in nutrient signalling.

In addition to the cross-talk between *CaPHO85* and *CaGCN4*, the cyclin *Ca*Pcl5p encoded by *CaPCL5* is a key member with *Ca*Pho85p for phosphorylation of the substrate *Ca*Gcn4p (Simon et al. 2013). To characterize the effect of *Ca*Pho85p-*Ca*Pcl5p on nutrient starvation by 3-AT, the strains WCL303 (P_{TET}-*PHO85/pho85Δ*), WCL315 (*pcl5Δ/pcl5Δ*) and WCL321 (P_{TET}-*PHO85/pho85Δ pcl5Δ/pcl5Δ*), accompanied with WCL319 (P_{TET}-*PHO85/pho85Δ PCL5/pcl5ΔH*) and WCL320 (P_{TET}-*PHO85/pho85Δ pcl5ΔH/pcl5ΔS*) that were the intermediate strains in strain construction grown overnight in YPD medium with Dox were spotted onto SC agar plates with 3-AT and Dox for 3 days. Dependency on the resistance to nutrient starvation by 3-AT of the strains could be observed. Strain WCL315 (*pcl5Δ/pcl5Δ*) was more resistant to 3-AT than was the wild type, even at high concentrations of 3-AT (Fig. 4f, g). However, the colonies of strain WCL315 were less wrinkled than those of strains WCL303 (P_{TET}-*PHO85/pho85Δ*) and the wild-type at 30 °C (Fig. 4f, upper panel, g). Compared with the wild-type, strain WCL321 (P_{TET}-*PHO85/pho85Δ pcl5Δ/pcl5Δ*) showed only slight growth defect with Dox at 30 °C, which suggests that *CaPHO85* might have a minor role on growth related to cell cycle. Along with the increased concentration of 3-AT at 30 °C, strain WCL321 (P_{TET}-*PHO85/pho85Δ pcl5Δ/pcl5Δ*) was more resistant to 3-AT than strain WCL303 (P_{TET}-*PHO85/pho85Δ*) and developed wrinkled colonies under *CaPHO85* repression by Dox, but strain WCL315 (*pcl5Δ/pcl5Δ*) developed less wrinkled colonies (Fig. 4f, g). Unexpectedly, the colonies of strain WCL321 were unable to exhibit more resistance to nutrient starvation (Fig. 4g). It is possible that *CaPHO85* serves as a regulator for adaption to temperature stress but that its response to 3-AT could be diverse depending on the associated cyclin partner such that the deletion of *CaPCL5* in the conditional Tet-off *CaPHO85* strain prevented it from displaying increased resistance to 3-AT. Taken together, *CaPHO85* appears to play an important role in adaption to high temperature, and its cyclin *Ca*Pcl5p encoded by *CaPCL5* acts as a partner of *Ca*Pho85p to direct certain mechanisms in the response to nutrient starvation. In comparison with *CaPCL5* and *CaPHO85*, *CaGCN4* appears to act as an epistatic regulator of growth and filamentation regarding nutrient signalling.

Discussion

The major aim of this study was to establish an efficient approach for constructing null mutants and conditional Tet-off strains from any prototrophic strain in *C. albicans*, including laboratory strains and clinical isolates. Three plasmids, pHB1S, pSFS2AS and pWTF1, containing a *CaHygB* cassette, a *CaSAT1*-flipper cassette and a *CaHygB*-based Tet-off cassette, were established and their outcomes examined via deletion of *CaGCN4* and *CaPCL5* and creation of a conditional Tet-off *CaPHO85* mutant in the genetic background of the wild-type strain SC5314. In serial dilution spotting assays, these mutants displayed differences in colony morphology and in growth on agar. These consequences revealed roles of *CaGCN4*, *CaPCL5* and *CaPHO85* and their relationships in nutrient sensing by *C. albicans*. Particularly, we confirm that *CaPHO85* has an important role in adaption to high temperature and *CaPCL5* that encodes a cyclin partner of *Ca*Pho85p assists it to direct certain mechanisms in the response to nutrient starvation. Additionally, *CaGCN4* functions as an epistatic regulator of growth and filamentation regarding nutrient signalling in comparison with *CaPCL5* and *CaPHO85*. Thus, we confirmed that the new system works efficiently for switching off gene expression for functional analysis. Our system that is devoid the use of auxotrophic strains is particularly suitable for study of genes associated with nutrient response. The genomic structure of the constructed strains based on auxotrophic strains are not close to that of the wild-type strain since mutations of genes involved in the metabolic pathways are present in the auxotrophic strains that might interfere with analyses or elucidations in nutrient-related studies.

Recently, a new *Clox* system and a CRISPR system for gene manipulation were published (Shahana et al. 2014; Vyas et al. 2015). The *Clox* system is made up of four selectable markers, *CaURA3*, *CaHIS1*, *CaARG4* and *CaNAT1*, flanked by *loxP* sequences, and a Cre recombinase driven by the *CaMET3* promoter. It allows multiple gene disruption of both alleles by four selectable markers and recycles the markers through the intron-containing Cre for recombination. The benefits of this system are that it provides multiple gene deletion at once and recycles used markers for another target. However, this toolkit relies on auxotrophic strains such as RM1000. The CRISPR system has been widely used in many eukaryotic cells for genomic editing (Doudna and Charpentier 2014). This system has been designed for *C. albicans* with a plasmid carrying a codon-optimized Cas9 nuclease gene, which targets the *CaENO1* locus, and a plasmid bearing a synthetic guide RNA (sgRNA) to direct Cas9 to cleave the target region, which targets *CaRP10*. The advantage of CRISPR is that it permits not only target gene knockout but also the mutagenesis of essential genes in the laboratory and clinical strains. In comparison with their strategies, our dominant selection system is able to delete both alleles of a target gene with the *CaHygB* and *CaSAT1* markers in wild-type strains and to recycle the markers simultaneously to enhance the benefit of the *CaSAT1*-flipper, similar to the *Clox* system but

with dominant selection. Moreover, a *CaHygB*-based Tet-off system for conditional gene regulation was incorporated into the *CaSAT1*-flipper for the analysis of essential genes. Two major components of Tet-off system, the Tet transactivator and the Tet-responsive operator, are combined into a DNA cassette to address the limitations of strain CaSS1 in the GRACE method (Roemer et al. 2003) and strain THE1 used for the Tet-off system (Nakayama et al. 2000), which use auxotrophic markers. By integrating the Tet-off system at one allele of a locus with the other allele being deleted, the genomic structure of the Tet-regulated mutant compared with that of the wild-type strain is kept unaltered, except for the locus of the target gene. In contrast, the CRISPR system leaves some modules required for the function of CRISPR in the genome. Therefore, we have designed two efficient strategies for switching off gene expression but not for the mutagenesis of essential genes.

We have successfully applied PCR-based gene targeting with dominant selection to analyse the *Ca*Pho85p-*Ca*Pcl5p-*Ca*Gcn4p circuitry in response of nutrient starvation. Our system can be used in clinical isolate other than auxotrophic strains. This should be particularly beneficial to apply our system in the study of the antifungal drug resistance when nutrient signalling pathways, specifically the lipid biosynthesis pathway (Prasad and Singh 2013) are involved. Indeed, a recent report that *C. albicans* lacking *CaERG3* exhibited azole resistance but was abolished when compromised by abrogation of nutrient signalling (Robbins et al. 2010) confirms the need of our system. Moreover, our system can be combined with the CRISPR system to create specific point mutations on genes in which mechanistic details of functional interactions among genes can be analysed. In conclusion, we set up a system for gene deletion and conditional Tet-off regulation with dominant selection, which facilitates strain construction in wild-type genetic backgrounds and allows subsequent convenient performance of epistatic analysis.

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