科技部補助專題研究計畫成果報告
期末報告

釐清白色念珠菌CDC4在形態生成及逆境反應之對話所扮演的角色 (GM3)(第3年)

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中 華 民 國 105 年 03 月 31 日

中 文 摘 要 ： 白色念珠菌編碼屬Skp1-cullin-F-box（SCF）E3泛素連接酶中具受質專一性的F-box 蛋白質的CDC4 (CaCDC4)基因能遏止酵母菌與菌絲形態轉換，也發現Dbf4依賴之Cdc7激酶所屬之DBF4及CDC7，在已知高度保留的DNA複製起始角色外，具備負調節者菌絲形成的功能。我們以親合力純化法鑑定出新穎之CaCdc4 的結合蛋白Gph1及Thr1。兩者與肝糖分解為葡萄糖及蘇氨酸生合成有關，分別受HOG及TOR路徑之調節使其存活在逆境及營養缺乏環境。我們因而提出假說:CaCdc4在白色念珠菌中具協調形態生成及逆境反應和營養感應的重要角色而以三年期的研究計劃驗證此假說。本期為計劃第三年，並延半年。我們先進一步驗證CaCdc4是含WD40 重複及Fbox功能域的蛋白質具抑制菌絲生成及絮凝的角色，又確認CaCDC4是生物膜形成的負調節者。我們發現白色念珠菌Thr1在與CaCDC4過量表現下方能被檢測到，顯示Thr1並未經CaCdc4進行泛素依賴蛋白酶體降解，而CaCdc4是正向調節Thr1表現。CaCDC4表現抑制產生菌絲型態能以持續表現Thr1所遏止，顯示兩者在形態生成角色上具關聯性。THR1剔除之白色念珠菌無法在菌絲誘導條件的血清誘導成菌絲，印證了THR1具菌絲誘導正調節角色。CaCDC4剔除之白色念珠菌與THR1剔除之白色念珠菌對氧化壓力具敏感性，證實兩者間在抗逆境角色上具關聯性。另外，我們發現白色念珠菌THR1受TOR營養感應路徑透過GCN4而誘導表現，定性報導基因結果發現Gcn4蛋白質不但能直接轉錄活化THR1表現也能轉錄活化應為HOG逆境反應路徑下游的GPH1表現，暗示白色念珠菌TOR營養感應路徑與逆境反應路徑有交互作用。而缺乏GCN4的細胞能拯救細胞在缺乏THR1而於影響threonine生合成路徑特定營養條件下的致死現象，暗示GCN4調節廣泛threonine生合成路徑基因甚至及於其他由threonine衍生之生合成路徑。白色念珠菌Gph1在表現被抑制方能被檢測到下，但Gph1並未能偵測到泛素化現象，暗示Gph1並經CaCdc4進行非泛素依賴之蛋白質降解。CaCDC4表現抑制產生菌絲型態也能以持續表現Gph1所遏止，亦暗示兩者在形態生成角色上具關聯性。GPH1 剔除之白色念珠菌對逆境包括抗真菌藥物不具影響，暗示存在GPH1替代功能基因或路徑，將進一步分析其它外表型特性如絮凝力、表面疏水性、黏附力、生物膜形成能力，外表型轉換力及細胞壁或膜相關特性，如碳源利用及脂質代謝等以便確認白色念珠菌GPH1的角色。此外，我們針對研究營養感應路徑使用營養缺陷品系可能造成結果評估的困擾及操作野生型白色念珠菌的實用性發展了顯性選擇記號抗生素基因做為篩選品系之依據。建立剔除品系及以四環黴素抑制表現系統構築必要基因品系，試著解答TOR營養感應路徑三個基因功能的關係。計畫的初步結論是白色念珠菌形態生成與逆境反應及營養感應能透過CaCDC4整合，而CaCDC7/ CaDBF4整合白色念珠菌形態生成與基因毒性及環境因子的可能性則有待進一步探討。

中 文 關 鍵 詞 ： 白色念珠菌；CDC4；CDC7；DBF4；GPH1；THR1；HOG逆境反應；TOR營養感應；蘇胺酸生合成路徑；顯性選擇；四環黴素抑制表現系統

英 文 摘 要 ： Candida albicans CDC4 (CaCDC4), encoding the F-box protein for substrate-specificity of the Skp1-cullin-F-box (SCF) E3 ubiquitin ligase complex, suppresses yeast-to-filament

transition in C. albicans. Additionally, CaCDC7 and CaDBF4 genes coding the Dbf-dependent kinase that is highly concerned in the initiation of DNA replication are found to be the negative regulators of filamentation. By affinity purification, we identified two novel CaCdc4-associated proteins encoded by GPH1 and THR1 that are requires for glycogen-to-glucose catabolism and threonine synthesis and are involved in HOG and the TOR pathways for the survival of cells during stress and nutrient limitation, respectively. Hence, we hypothesize that CaCdc4 is important in coordination of morphogenesis and stress response as well as nutrient sensing in C. albicans. To validate this hypothesis, we propose a three-year project. The proposal has been extended for one-and-half-a-year. We first verified that CaCdc4 containsWD40 repeats and F-box functional domain, which are required for suppression of filamentation and cellular flocculation. We then confirmed that CaCDC4 is the negative regulator of filamentation.We found that Thr1protein was detectable only under CaCDC4 over-expressed condition, suggesting that Thr1 is not a CaCdc4-dependent degradation target mediated by ubiquitin-proteasom and CaCDC4 positively controls the expression of THR1. Also, we observed that the expression of THR1 is activated concurrently with GCN4 during nutrient depletion. Upon TOR pathway activation, Gcn4 transactivates the expression of THR1 and GPH1, suggesting that the TOR and HOG pathways are interconnected. Cells lacking GCN4 could rescue the lethality of cells absence of THR1 in condition accumulating homoserine in the threonine biosynthesis pathway, suggesting that GCN4 regulates the expression of many genes on thronine biosynthesis pathway upstream of THR1 or those on other biosynthesis pathway with thronine being the precursor. Moreover,
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. Our conclusion is that the morphogenesis and stress/nutrient response are mediated by CaCDC4 and CaCDC7 and CaDBF4 may integrate morphogenesis ans genotoxic and environmental cues but these are required further study.

英文關鍵詞：Candida albicans; CDC4; CDC7；DBF4；GPH1; THR1; morphogenesis; stress response; nutrient sensing; HOG pathway; TOR pathway; dominant selection; Tet-off expression system

附件一

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

釐清白色念珠菌 *CDC4* 在形態生成及逆境反應之對話所扮演的角色

Deciphering the role of *CaCDC4* on crosstalk between morphogenesis and stress response in *Candida albicans* (GM3) (3/3)

計畫類別：■個別型計畫　□整合型計畫
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計畫主持人：謝家慶
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計畫參與人員：賴威仲、許婉華、方怡雅、林沂霈、李宜樺、謝伯思、林珮璇、吳蔚航、翁尚楣、許小琪

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■出席國際學術會議心得報告 x2
□國際合作研究計畫國外研究報告

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中　華　民　國 105 年 03 月 31 日

摘要

關鍵字: 白色念珠菌；*CDC4*；*CDC7*；*DBF4*；*GPH1*；*THR1*；HOG 逆境反應；TOR 營養感應；蘇胺酸生合成路徑；顯性選擇；四環黴素抑制表現系統

白色念珠菌編碼屬 Skp1-cullin-F-box (SCF) E3 泛素連接酶中具受質專一性的 F-box 蛋白質的 *CDC4* (*CaCDC4*)基因能遏止酵母菌與菌絲形態轉換，也發現 Dbf4 依賴之 Cdc7 激酶所屬之 *DBF4* 及 *CDC7*，在已知高度保留的 DNA 複製起始角色外，具備負調節者菌絲形成的功能。我們以親合力純化法鑑定出新穎之 *Ca*Cdc4 的結合蛋白 Gph1 及 Thr1。兩者與肝糖分解為葡萄糖及蘇氨酸生合成有關，分別受 HOG 及 TOR 路徑之調節使其存活在逆境及營養缺乏環境。我們因而提出假說:*Ca*Cdc4 在白色念珠菌中具協調形態生成及逆境反應和營養感應的重要角色而以三年期的研究計劃驗證此假說。本期為計劃第三年，並延半年。我們先進一步驗證 *Ca*Cdc4 是含 WD40 重複及 Fbox 功能域的蛋白質具抑制菌絲生成及絮凝的角色，又確認 *CaCDC4* 是生物膜形成的負調節者。我們發現白色念珠菌 Thr1 在與 *CaCDC4* 過量表現下方能被檢測到，顯示 Thr1 並未經 *Ca*Cdc4 進行泛素依賴蛋白酶體降解，而 *Ca*Cdc4 是正向調節 Thr1 表現。*CaCDC4* 表現抑制產生菌絲型態能以持續表現 Thr1 所遏止，顯示兩者在形態生成角色上具關聯性。*THR1* 剔除之白色念珠菌無法在菌絲誘導條件的血清誘導成菌絲，印證了 *THR1* 具菌絲誘導正調節角色。*CaCDC4* 剔除之白色念珠菌與 *THR1* 剔除之白色念珠菌對氧化壓力具敏感性，證實兩者間在抗逆境角色上具關聯性。另外，我們發現白色念珠菌 *THR1* 受 TOR 營養感應路徑透過 *GCN4* 而誘導表現，定性報導基因結果發現 Gcn4 蛋白質不但能直接轉錄活化 *THR1* 表現也能轉錄活化應為 HOG 逆境反應路徑下游的 *GPH1* 表現，暗示白色念珠菌 TOR 營養感應路徑與逆境反應路徑有交互作用。而缺乏 *GCN4* 的細胞能拯救細胞在缺乏 *THR1* 而於影響 threonine 生合成路徑特定營養條件下的致死現象，暗示 *GCN4* 調節廣泛 threonine 生合成路徑基因甚至及於其他由 threonine 衍生之生合成路徑。白色念珠菌 Gph1 在表現被抑制方能被檢測到下，但 Gph1 並未能偵測到泛素化現象，暗示 Gph1 並經 *Ca*Cdc4 進行非泛素依賴之蛋白質降解。*CaCDC4* 表現抑制產生菌絲型態也能以持續表現 Gph1 所遏止，亦暗示兩者在形態生成角色上具關聯性。*GPH1* 剔除之白色念珠菌對逆境包括抗真菌藥物不具影響，暗示存在 *GPH1* 替代功能基因或路徑，將進一步分析其它外表型特性如絮凝力、表面疏水性、黏附力、生物膜形成能力，外表型轉換力及細胞壁或膜相關特性，如碳源利用及脂質代謝等以便確認白色念珠菌 *GPH1* 的角色。此外，我們針對研究營養感應路徑使用營養缺陷品系可能造成結果評估的困擾及操作野生型白色念珠菌的實用性發展了顯性選擇記號抗生素基因做為篩選品系之依據。建立剔除品系及以四環黴素抑制表現系統構築必要基因品系，試著解答 TOR 營養感應路徑三個基因功能的關係。計畫的初步結論是白色念珠菌形態生成與逆境反應及營養感應能透過 *CaCDC4* 整合，而 *CaCDC7/ CaDBF4* 整合白色念珠菌形態生成與基因毒性及環境因子的可能性則有待進一步探討。

Abstract

Keywords: *Candida albicans*; *CDC4*; *CDC7*；*DBF4*；*GPH1*; *THR1*; morphogenesis; stress response; nutrient sensing; HOG pathway; TOR pathway; dominant selection; Tet-off expression system

Candida albicans CDC4 (*CaCDC4*), encoding the F-box protein for substrate-specificity of the Skp1-cullin-F-box (SCF) E3 ubiquitin ligase complex, suppresses yeast-to-filament transition in *C. albicans*. Additionally, *CaCDC7* and *CaDBF4* genes coding the Dbf-dependent kinase that is highly concerned in the initiation of DNA replication are found to be the negative regulators of filamentation. By affinity purification, we identified two novel *Ca*Cdc4-associated proteins encoded by *GPH1* and *THR1* that are requires for glycogen-to-glucose catabolism and threonine synthesis and are involved in HOG and the TOR pathways for the survival of cells during stress and nutrient limitation, respectively. Hence, we hypothesize that *Ca*Cdc4 is important in coordination of morphogenesis and stress response as well as nutrient sensing in *C. albicans*. To validate this hypothesis, we propose a three-year project. The proposal has been extended for one-and-half-a-year. We first verified that *Ca*Cdc4 containsWD40 repeats and F-box functional domain, which are required for suppression of filamentation and cellular flocculation. We then confirmed that *CaCDC4* is the negative regulator of filamentation.We found that Thr1protein was detectable only under *CaCDC4* over-expressed condition, suggesting that Thr1 is not a *Ca*Cdc4-dependent degradation target mediated by ubiquitin-proteasom and *CaCDC4* positively controls the expression of *THR1*. Also, we observed that the expression of *THR1* is activated concurrently with *GCN4* during nutrient depletion. Upon TOR pathway activation, Gcn4 transactivates the expression of *THR1* and *GPH1*, suggesting that the TOR and HOG pathways are interconnected. Cells lacking *GCN4* could rescue the lethality of cells absence of *THR1* in condition accumulating homoserine in the threonine biosynthesis pathway, suggesting that *GCN4* regulates the expression of many genes on thronine biosynthesis pathway upstream of *THR1* or those on other biosynthesis pathway with thronine being the precursor. Moreover,

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. Our conclusion is that the morphogenesis and stress/nutrient response are mediated by *CaCDC4* and *CaCDC7* and *CaDBF4* may integrate morphogenesis ans genotoxic and environmental cues but these are required further study.

目錄

I. Introduction 前言

The opportunistic human fungal pathogen *Candida albicans*, a natural diploid with atypical sexual cycle, causes disease in healthy and immunocompromised hosts. Using budding yeast *Saccaromyces cerevisiae* as a comparative model for *C. albicans*, great effort has been made to elucidate the molecular mechanism underlying morphogenesis in *C. albicans* as it is known to be coupled with virulence and pathogenesis. Research progress has revealed surprising complexity in that at least three positive and five negative pathways control morphological transition in *C. albicans* [1-3]. To add even more obstacles, many Cdks and cyclins with their regulators have also been shown to control morphological plasticity in *C. albicans* [4]. As such, a key issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incomplete understood. Intriguingly it has recently been revealed by us and others that some key cell cycle genes conserved throughout evolution play no essential role on cell cycle but morphogenesis in *C. albicans* [5-10].

Hence, our long term overall research goal is to use budding yeast *S. cerevisiae* as a comparative model in exploring link between cell cycle progression and morphogenesis in *C. albicans*. How are the diverse morphological states coordinated with cell cycle progression and the extent of divergence in regulations between *C. albicans* and *S. cerevisiae* are to be addressed.

II. Objective and specific scientific aims 研究目的

In the current proposal, with our preliminary data and by reviewing literature we postulate that *Ca*Cdc4 is a vital component used to coordinate morphogenesis and stress/nutrient response and set a goal to decipher the role of *CaCDC4* on crosstalk between morphogenesis and stress response in *C. albicans*. According to the proposal we aim to (a) verify the presence of crosstalk between HOG/TOR pathways, (b) assess the lethality-suppression of hyperactivation of HOG/TOR pathways, and (c) clarify if the stress response (SR)/nutrient limitation (NL)/filamentous growth (FG) due to loss of *HOG1*/*TOR1* is suppressed by *Ca*Cdc4 overexpression (o/p). During the process of the proposal execution, the role of domains of *Ca*Cdc4 in negative regulating filamentation and flocculation was sought to determine. The ability of *CaCDC4* to suppress biofilm formation was sought to assess.

Additionally, we have established an efficient deletion strategy and a system that can conditionally switch off essential genes, which are incorporated with dominant selection markers, for the study of wild-type strain devoid of the use of the auxotrophic strains in which interpretation of the results involved in nutrient conditions can be problematic.

Moreover, we have investigated the *Ca*Dbf4-dependnet kinase encoded by *C. albicans CDC7* and *DBF4* (*CaCDC7* and *CaDBF4*) whose homologs across evolutionary spectrum is known to be essential in the initiation of DNA replication. Intriguingly, the expression of *CaCDC7* and *CaDBF4* appeared to be affected by the hyphal induction agents [11]. We therefore investigate the role of *CaCDC7* and *CaDBF4* in regulating morphogenesis.

III. Literature Review 文獻探討

*Ca*Cdc4 contains specific domains of WD40–repeat and F-box, whose homologous ones are required for interacting with the Skp1, one of the component of the Skp1-Cdc53/Cul1-F-box (SCF) complex, and the substrate [12], respectively. It is essential to assess indeed *CaCDC4* is a canonical F-box protein of SCF ubiquitin ligase [13], termed the SCF^{CaCDC4}. This will clarify the possible

biochemical regulation of SCF^{CaCDC4} with it targets, such as Thr1 and Gph1. We observed that *C. albicans* cells defected in *CaCDC4* enhance both filamentation and flocculation. This prompted us to examine the *Ca*Cdc4 domains associated with filamentation and flocculation [14-16]. In addition to filamentation [17-19], flocculation, is also tightly associated with biofilm formation [20-22]. It became important for us to evaluate if *CaCDC4* has a role in biofilm formation in *C. albicans*.

We identified Gph1 and Thr1 as *Ca*Cdc4-associated proteins [23]. *GPH1* and *THR1* encode glycogen phosphorylase required for conversion of glycogen to glucose and homoserine kinase for threonine synthesis [24-26], critical to the survival of cells during nutrient limitation [25-29], which involves HOG [30, 31] and TOR[32, 33] pathways, respectively. Since *CaCDC4* suppresses filamentation, we reason that morphological alteration of *C. albicans* is a result of responding environmental cues in which the availability of required molecules in cells is reprogrammed such that the cellular structures can be reorganized. Therefore, the common targets shared by morphological transition, stress response, and nutrient limitation are rational.

Whereas inactivation of TOR activates Gcn4 translation via Gcn2[34], Gcn4 transcriptionally modulates expression of genes involved in amino acids biosynthesis in the budding yeast, which links TOR pathway directly to *THR1* gene. Moreover, *C. albicans thr1* homozygous null mutant shows a variety of stress sensitivity[26], suggesting that *THR1* is involved in HOG pathway in *C. albicans*. Inactivation of *C. albicans* Gcn4 blocks amino acid starvation-induced morphogenesis, providing a direct link of *C. albicans GCN4* between morphogenetic and metabolic responses [35]. In contrast, rapamycin inactivated TOR pathway leads to glycogen accumulation [36], in which Gph1 is inactivated by Pho85/cyclin Pcl6 [37] or is transcriptional inactivated by retention of Msn2/Msn4 transcriptional activator of *GPH1* in the cytoplasm [38]. Msn2/Msn4 is one of target of HOG pathway in budding yeast [30], suggesting that *GPH1* might be regulated by HOG pathway. Moreover, *CDC4* mutation prevents the degradation of Msn2/Msn4 that induces PNC1, which in turn activates Sir2 histone deacetylase [39], protecting against Hog1-induced programmed cell death. These results indicate a possible role of *CaCDC4* in coordinating the function of *GPH1* through Msn2/Msn4. Notably, it has recently been shown that reduced TOR signaling sustains hyphal growth in *C. albicans* by lowing Hog1 basal activity [40], demonstrating crosstalk between HOG and TOR pathways. We therefore postulate that *Ca*Cdc4 is a key component used to coordinate morphogenesis and stress/nutrient response and set a goal to decipher the role of *CaCDC4* on crosstalk between morphogenesis and stress response in *Candida albicans*. We have assessed the role of *GPH1* and *THR1* in stress response (SR)/nutrient limitation (NL) by examine known genes on HOG and TOR pathways in association with *THR1* and *GPH1* along with examining the possible regulation mechanism of *Ca*Cdc4 to either Thr1 or Gph1.

It is a challenge to explain the results without misinterpretation when the auxotrophic strains deficient in genes responsible for the biosynthetic pathways are used. To effectively study essential genes in the wild-type *C. albicans*, the compatible effects of the antibiotics hygromycin B [41] and nourseothricin [42, 43], the recyclable *CaSAT1*-flipper [42] and the tetracycline-repressible (Tet-off) system [44] have the great advantage. To allow deleting two alleles at once, creation of a cassette with antibiotic genes, such as a *C. albicans HygB* resistance gene (*CaHygB*), flanked with the *FLP* recombinase target sites that can be operated alongside the established *CaSAT1*-flipper is valuable. Also, to enable conditionally switching off essential genes, a *CaHygB* based Tet-off cassette that

consistes of the *CaTDH3* promoter, which is used for the constitutive expression of the tetracycline-regulated transactivator and a tetracycline response operator can be commonly applicable since it does not rely on a specific strain, which separates expression of the tetracycline-regulated transactivator from a tetracycline response operator where the expression of target gene is under control [44].

In budding yeast, *CDC4* function before *CDC7* in the mitotic cell division cycle. Because *C. albicans CDC4* (*CaCDC4)* negative regulate filamentation, we were interested in determining if *C. albicans CDC7* (*CaCDC7*) has a role in the yeast-to-hypha transition. Given that the expression of *CaCDC7* appeared to be affected by the hyphal induction agents of serum and α-MEM that are dependent on Efg-1 [11], we also postulated that *CaCDC7* and *CaDBF4* may involve in environmental cues and suppress the yeast-to-hypha transition. Research for the assessment of the function of *CaCDC7* and *CaDBF4* in morphogenesis has been designed and conducted.

IV. Experimental Design 研究方法

1. **Strain construction. Auxotrophic strain-independent.** The homozygous null mutants are constructed by a modified *SAT1* flipper method[42] with two rounds of integration/excision using the *C. albicans*-adpated *FLP* gene driven by BSA-inducible SAP2 promoter (C-Y., Lan, personal commun.) or maltose-inducible promoter[42]. *C. albicans* homozygous null mutant is made by the *SAT1* flipper method, the 5'- and 3'-flanking regions of gene of interest is PCR-amplified and cloned into pSFS1A plasmid sequentially. The *CaSAT1*-cassette contains a dominant nourseothricin resistance (Nou^r) marker (*CaSAT1*) for selection. *C. albicans*-adapted *FLP* gene that allows subsequent excision of the cassette, flanked by the 5'- and 3'-flanking region of the gene, is released by *Kpn*I and *Sac*II. The *CaSAT1*-cassette was introduced into cells for Nou^r. The *CaSAT1*-cassette is popped out form the first allele when grown on plate with maltose or BSA for nourseothricin sensitivity (Nou^s). The deletion of the 2nd allele is carried out subsequently. The constructed strains can be verified by PCR or Southern blotting. Inability of the strains to express the gene can be examined by RT-PCR.
2. **Alternative strain construction. Auxotrophic dependent.** The auxotrophic strain BWP17 (*ura3/ura3 his1/his1 arg3/arg3*) [45] was used to construct the heterozygous null mutant strain of the *CaCDC4*, *CaCDC7* or *CaDBF4* by using mini-Ura-blaster [46]. Cells of the heterozygous null mutant strain were plated with 5-FOA to induce recombination between two copies of dpl200 flanking the mini-Ura-blaster for a loss of *CaURA3* to generate the Ura^- strain. The plasmid pFA-HIS1-MET3p (*HIS1* selection marker) with a partial target gene coding sequence for N-terminal gene product was linearized at specific site and used to transform the Ura^- heterozygous null mutant to generate $Ura^- His^+$. These strains are capable of repressing target gene expression in the presence of methionine and cysteine.
3. **The doxycycline-inducible system.** To allow the expression of cassettes encoding assorted *Ca*Cdc4 domains in *C. albicans*, a Tet-on plasmid, pTET25M [47], which is derived from pTET25 [48] for inducing gene expression with doxycycline (Dox), has been developed. To regulate *CaCDC4* expression by the Tet-on system, the coding sequence of *CaCDC4* or the segment of *CaCDC4* was PCR-amplified and constructed into the plasmid pTET25M. The plasmids bearing those *CaCDC4* segments or *CaCDC4* flanked with common *C. albicans ADH1* (*CaADH1*) sites were digested with *Sac*II and *Kpn*I, each of which was transformed into *C. albicans* for integration at the *CaADH1* locus. The strains are capable of Dox-induced expression of the target genes or gene segments.

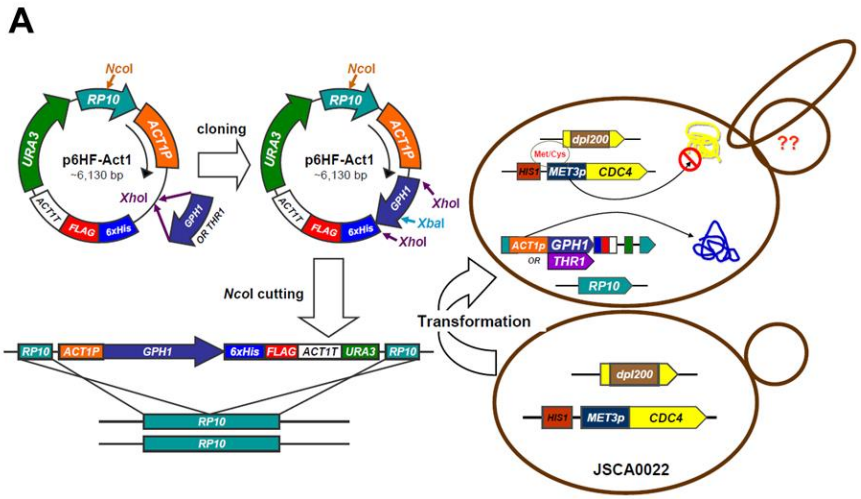
4. **To construct strain carrying reporter plasmid and reporter assay.** The upstream sequence of *THR1* and *GPH1* were PCR-generated and cloned into reporter plasmid pCR-*LacZ* at the *Sal*I and *Pst*I sites. Each of the recombinant plasmids was linearized with *Bam*HI at the *ADE2* on the plasmid and introduced into *ADE2* locus of strain CAI8 (*ura3*::imm434/*ura3*::imm434 *iro1::imm434/ iro1::imm434 ade2::hisG/ade2::hisG*) for ADE^{+}. The cells with the reporter were plated onto plate with SC containing X-gal in nutrient limited or stress conditions.
5. **To introduce gene under the control of tetracycline-induced system into *C. albicans*.** The coding sequence of gene is cloned into the Tet-on plasmid established previously by us [47], the cassette can be released from the plasmid by specific restriction enzymes and introduced into *C. albicans* cells targeting specifically at the *ADH1* locus to generate a stable integrant capable of doxcycycline-induced the expression of the gene, which is likely to the level much higher than the endogenous level. The interference of overexpression of the gene to particular phenotypes can be used to decipher the functional interaction of the genes with others.
6. **To determine the nutrient/stress sensitivity of *C. albicans*.** To assess survival in semi-solid medium, the strains to be tested are grown in medium at 30°C to mid-exponential phase. The spotting assay was used. Cells are diluted in 10^6 cells /ml, and 10-fold dilutions thereof, are spotted in 5 μl onto various agar plates containing the stress agents to be tested at various concentrations. The plates are incubated at 30°C for up to 3 days **[**49].
7. **To evaluate the morphological alteration of *C. albicans*.** The morphological alterations of cells in liquid culture are visualized with microscope of Nikon 50i or ZEISS AXioskop2. DIC micrographs using ZEISS LSM 510 META are taken to reveal characteristic features of hyphae or pseudohyphae. The formation of colony on semi-solid media of agar plates is examined by presence of crenulated appearance, ability to invade the agar substratum, and existence of projections extending from the colonies.
8. **To create mutations of gene of interest.** To achieve hyperactivation of HOG pathway, *C. albicans* $Pbs2^{DD}$ (S355D, T359D), equivalent to *S. cerevisiae* (S515D, T519D)[50], is needed. The wild-type version of gene had been made in the Tet-on expression system based on what we had created previously [47] followed by site-directed mutagenesis with oligonucleotide primers for specific nucleotide change by QuikChange Site-Directed Mutagenesis Kit (Strategene).
9. **To establish the new systems that allow manipulate genes in the wild-type strain**. Plasmids with cassettes combined dominant selection, recyclability, Tet-off control when the essential is encountered were created. To validate the new systems, all strains were constructed based on the wild-type strain SC5314 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.

V. Results and Discussions 結果與討論

1. **The dependency of *Ca*Cdc4 domains in morphogenesis and flocculation in *C. albicans*.** A *C. albicans* strain, with one *CaCDC4* allele deleted and the other under the repressible *C. albicans MET3* promoter (*CaMET3*p) control, was made before introducing cassettes capable of doxycycline (Dox)-induced expression of various *C. albicans* Cdc4 (*Ca*Cdc4) domains. Cells from

each strain could express a specific CaCdc4 domain under Dox-induced, but *CaMET3-CaCDC4* repressed conditions. Cells expressing domains without either the F-box or WD40-repeat exhibited filamentation and flocculation similarly to those lacking *CaCDC4* expression, indicating the functional essentiality of the F-box and WD40-repeat. Notably, cells expressing the N-terminal 85-amino acid truncated *Ca*Cdc4 partially reverse the filament-to-yeast and weaken the ability to flocculate compared to those expressing the full-length *Ca*Cdc4, suggesting that N-terminal 85-amino acid of *Ca*Cdc4 regulates both morphogenesis and flocculation. We conclude that the F-box and the WD40-repeat of *Ca*Cdc4 are essential in inhibiting yeast-to-filament transition and flocculation and the N-terminal region (1-85) of *Ca*Cdc4 also has a positive role for its function, loss of which impairs both the ability to flocculate and to reverse filamentous growth in *C. albicans*. This part of work has been published **[51]** and is attached in the end of this report (**Appendix I**).

2. ***CaCDC4* negatively regulate biofilm formation in *C. albicans***. Because we observed that cells deleted with *CaCDC4* increase their ability to flocculate, we were interested to determine if *CaCDC4* plays a role in biofilm formation in *C. albicans*. To elucidate the role of *CaCDC4* in biofilm formation, *Cacdc4* null mutant strains were constructed by using the min-Ura-blaster method [46]. To create a *CaCDC4* rescued strain, a plasmid p6HF-*ACT1*p-*CaCDC4* [52] capable of constitutively expressing *CaCDC4* was introduced into the *Cacdc4* homozygous null mutant. To determine the biofilm formation ability, an *in vitro* XTT reduction assay was used [53, 54]. Compared to the parental auxotrophic strain BWP17, the *Cacdc4* homozygous null mutant was able to enhance biofilm formation significantly. We concluded that *CaCDC4* has a role in suppressing biofilm formation in *C. albicans*. This part of work has also been published **[55]** and is attached in the end of this report (**Appendix II**).
3. **The expression of *THR1* or *GPH1* suppresses filamentous growth due to repressed expression of *CaCDC4* in *C. albicans*.** To understand *functional association between CaCDC4* and *THR1* and *GPH1*. *C. albicans* cells capable of repressing ***CaCDC4*** expression in the presence of methionine and cysteine (Met/Cys) and constitutively expressing either *THR1* or *GPH1* were constructed (**Fig. 1A**). To assess the effect of expression of *THR1* or *GPH1* on the filamentous growth of cells with *CaCDC4* expression being repressed, the cells were plated on plate of rich media or were grown in the minimum media with or without 2.5 mM Met/Cys. (**Fig.1**). The constitutive expression of either *THR1* or *GPH1* suppresses the filamentous mode of growth when the expression of *CaCDC4* is repressed, suggesting that *THR1* and *GPH1* are functionally related to *CaCDC4* regarding the control morphogenesis and that *THR1* and *GPH1* positively modulate hyphal formation.



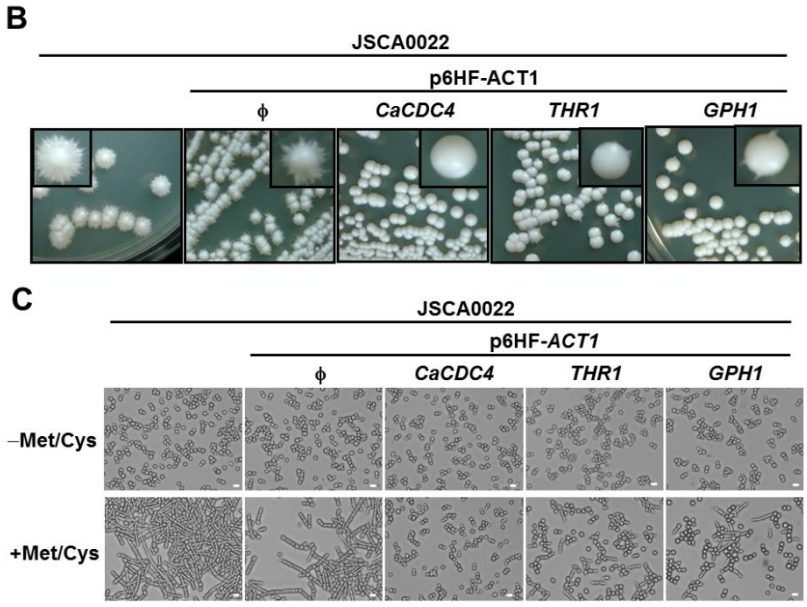


Figure 1. The constitutive expression of either *THR1* or *GPH1* suppresses the filamentous mode of growth when the expression of *CaCDC4* is repressed. **(A)** Diagram to illustrate strains. The cells were (**B**) plated on YPD plate or were (**C**) grown in the SC with or without 2.5 mM Met/Cys. "Φ" represent empty plasmid p6HF-*ACT1*.

4. **The *Ca*Cdc4 regulates Thr1 and Gph1 divergently in *C. albicans*.** To assess possible regulation of *Ca*Cdc4 and Thr1 and Gph1, cells of the same strains as shown in **Fig 1A** were grown in the minumum media with or without Met/Cys and the proteins were extracted and subjected to Western blotting analysis (**Fig. 2**). Repressed expression of *CaCDC4* led to increase of Gph1 level, but reduction of Thr1 level. The results suggest that the *Ca*Cdc4 appears to negatively regulate the level of Gph1 protein but positively regulate the level of Thr1 protein.

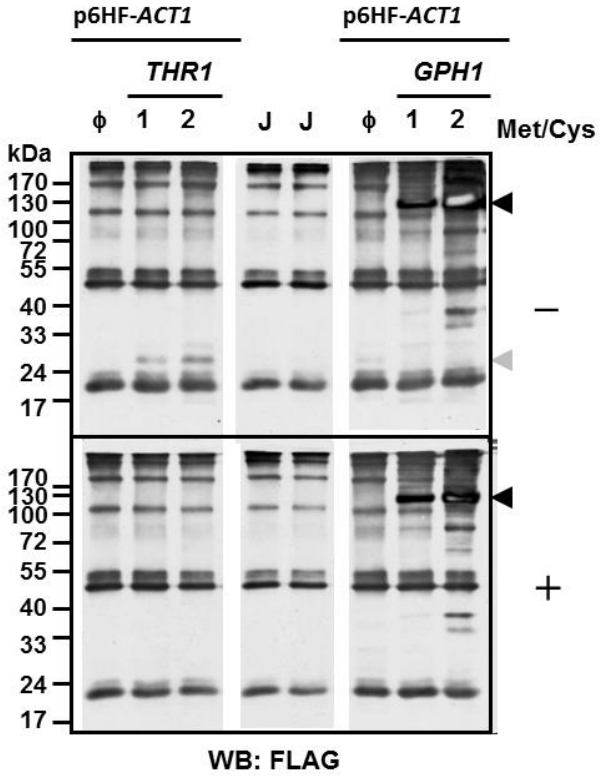


Figure 2. The mode of regulation between *Ca*Cdc4 and Thr1 or Gph1 is different. Cells of strains as in Fig. 1 were grown in SC with or without 2.5 mM Met/Cys before subjecting to Western blotting analysis. Anti-FLAG antibody was used as the Thr1 and Gph1 are tagged with FLAG. Triangles in black indicate the migrated position of Gph1 protein. Triangle in grey indicates the migrated position of Thr1 protein.

5. ***C. albicans* cells lost *CaCDC4* or *THR1* enhance the biofilm formation.** Because *CaCDC4* negatively regulate biofilm formation, we wonder if *THR1* plays a similar role. Cells of homozygous null *Cacdc4* and *Thr1* were subjected to biofilm formation XTT assay (**Fig. 3**). As shown in **Fig 3**, cells lost either *CaCDC4* or *THR1* exhibit ability in enhanced bioflm formation with those lacking *CaCDC4* show a greater extent of enhancement. These results suggest both *CaCDC4* and *THR1* negatively regulate bioflm formation but *CaCDC4* plays a major role.

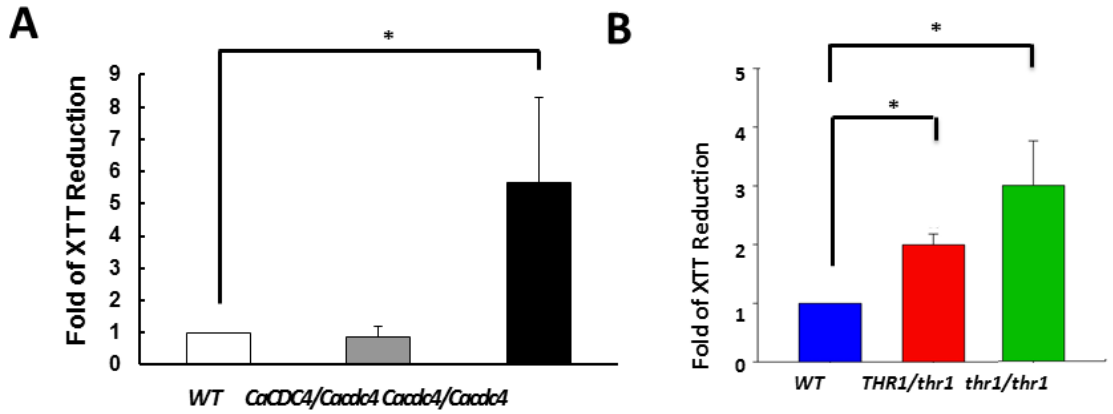


Figure 3. *CaCDC4* and *THR1* suppress bioflm formation. (**A**) Cells of the wild-type SC5314, heterozygous *CaCDC4* null mutant (*CaCDC4/Cacdc4)*, and homozygous *CaCDC4* null mutant (*Cacdc4/Cacdc4*) and (**B**) the wild-type SC5314, heterozygous *THR1* null mutant (*THR1/thr1*), and homozygous *THR1* null mutant (*thr1/thr1*) were subjected to *In vitro* XTT reduction assay for biofilm formation.

6. ***C. albicans* cells lost *CaCDC4* or *THR1* are sensitive to oxidative stress.** Because we have previously observed that cells lost *THR1* were sensitive to oxidative stress, we were interested in learning if *CaCDC4* also has a similar role. Cells of homozygous null *Cacdc4* and *Thr1* were subjected to the spotting assay on plate containing H_2O_2 (**Fig. 4**). As shown in **Fig 4**, cells lost either *CaCDC4* or *THR1* shows sensitivity to H_2O_2 with those lacking *CaCDC4* exhibit a greater extent of sensitivity. These results suggest that Both *CaCDC4* and *THR1* are required for growth under oxidative stress although *CaCDC4* is the key player.

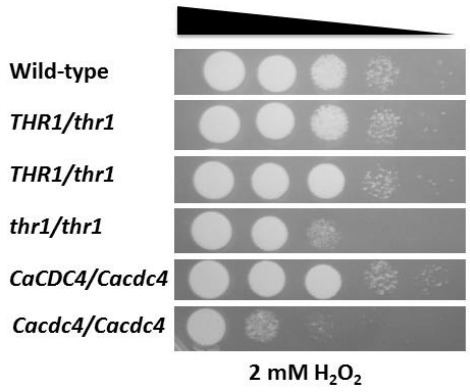


Figure 4. Cells lacking either *CaCDC4* or *THR1* are sensitive to H_2O_2 with those lacking *CaCDC4* being more sensitive. Cells are diluted in 10^6 cells /ml, and 10-fold dilutions thereof, are spotted in 5 µl onto agar plates containing 2 mM H_2O_2. The plates are incubated at 30°C for up to 3 days

7. ***C. albicans* cells lost *GPH1* impair the ability to form germ tube in the hyphal induction condition.** Cells lacking *GPH1* appeared resistance to many stress agents such as oxidative stress, osmotic stress, heat shock, antifungal drugs. We have therefore sought to test other possible affected phenotype in cells lacking *GPH1*. As cells constitutive expression of *GPH1* suppresses the filamentation of those cells repressed the expression of *CaCDC4*, though cells lacking *GPH1* has no morphological alteration, we wonder *GPH1* may still affect the morphology under specific condition. To test this possibility, cells of homozygous null *gph1* mutant were subjected to germ tube induction condition and the ability cells in germ tube formation were determined. As shown in **Fig. 5**, it appears that cells lacking one allele of *GPH1* (*CaGPH1* +/－; or the one allele complement one gph1－/*re1*) can impair the ability to form germ tube similar as those of homozygous null mutant (*CaGPH1* －/－) under the hyphal induction condition. The results suggest that *GPH1* has a role in the suppression of hyphal formation.

	SC5314	gcn4	CaGPH1-/-	CaGPH1-/+	gph1-/re1
mean	0.12338	0.10369	0.1108852	0.1082453	0.110891
SD.	0.03044	0.02355	0.0205005	0.0202408	0.024708
p-value	1	0.000285	0.01083187	0.003321709	0.0293175
		***	*	**	*
			1	0.491732665	0.9988976

p<0.05	*
p<0.01	**
p<0.001	***

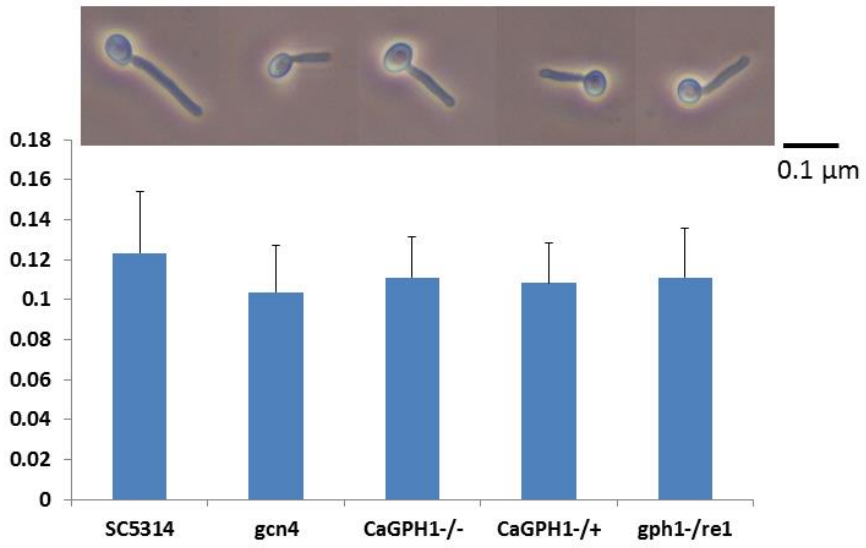


Figure 5. Cells lacking either *GPH1* are decicient in germ tube formation. sensitive to H_2O_2 with those lacking *CaCDC4* being more sensitive. To induce germ tube formation, 1×10^6 *C. albicans* yeasts/ml are transferred into the cell culture medium RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) and seeded into a 24-well plate. After 1 h incubation at 37°C, pictures were taken by phase contrast microscopy and germ tube length was determined with the Photoshop 6 software. The gcn4 null mutant, known to impair germ tube formation, was used as a control.

8. ***THR1* and *GCN4* are concurrently induced expression upon nutrient depletion in *C. albicans*.** To learn if TOR pathway-activation induced expression of *THR1* via *GCN4*, expression of those two genes in cells under nutrient limited conditions was examined. Wild-type SC5314 cells were grown to mid-log phase, followed by treatment with either rapamycin or 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of the product of the *HIS3* gene, known to activate the TOR pathway [35], and the cells were collected at various times and subjected to RT-PCR (**Fig. 6**). As shown in **Fig. 6B**, 3-AT activates the expression of both *GCN4* and *THR1*, indicating that *GCN4* might be the direct transcription factor that activates the expression of *THR1*. In contrast, cells treated with rapamycin activates the expression of *GCN4* but not *THR1* **Fig. 6A**, perhaps rapamycin activates the *GCN4* expression transcriptionally but inhibits Gcn2 known to regulate *GCN4* translationally, leading to insignificant transcriptional activation of *THR1*.

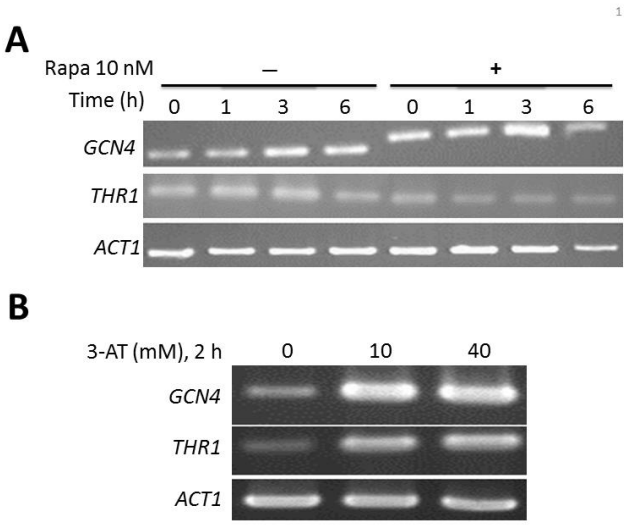


Figure 6. Nutrient limitation induces the expression of *GCN4* and *THR1*. **(A)** RT-PCR analyses of *CaGCN4* and *CaTHR1* mRNA levels were performed following 0, 1, 3, and 6 h of growth in YPD with or without 10 nM rapamycin (Rapa), using *ACT1* mRNA as a loading control. (B) RT-PCR analyses of *CaGCN4* and *CaTHR1* mRNA levels were performed following 2 h of growth in SC in the presence of absence of 10 and 40 mM 3-Amino-1,2,4-triazole (3-AT), using *ACT1* mRNA as a loading control.

9. **Gcn4 transactivates the expression of both *THR1* and *GPH1* in *C. albicans*.** To verify the Gcn4 directly activating the expression of *THR1* transcriptionally, cells from strains carrying reporter pCR-*LacZ* with promoter region of about 1 kb in each of two alleles of *THR1* (there are differences) and *GPH1* were used to assay for their ability to activate the reporter induced in the presence of 3-AT. As shown in **Fig. 7A**, several isolates of each of the two alleles of *THR1* were able to activate the reporter that allows colonies to become blue, suggesting that the Gcn4 is a transcription factor for *THR1*. In parallel, the reporter assay showed that Gcn4 is also a transcription factor for *GPH1* (**Fig.7B**). We note that GCRE element (TGACTC) was found only in one allele of *THR1*, suggesting that Gcn4 is able to bind non-canonical GCRE elements.

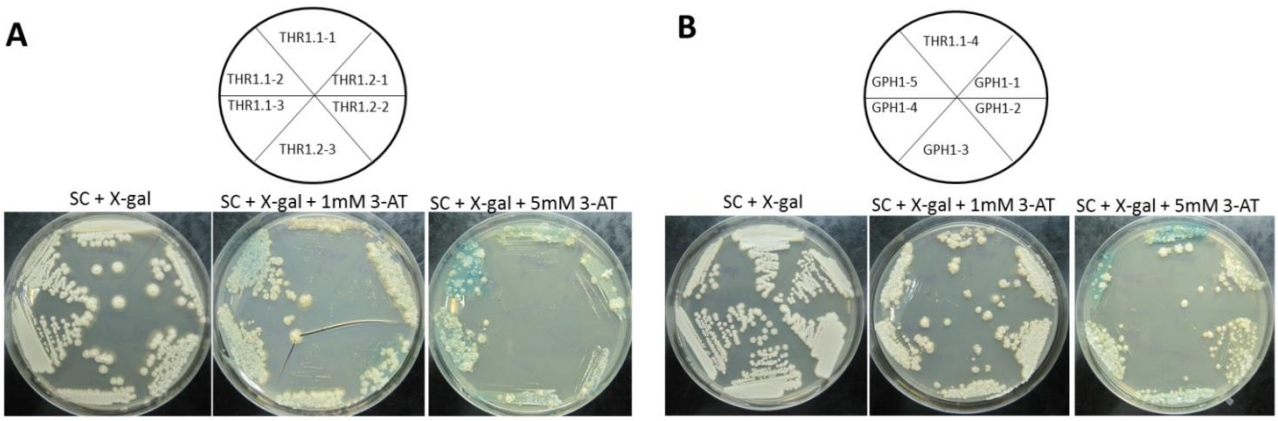


Figure 7. Gcn4 transactivates the expression of both *THR1* and *GPH1*. The upstream sequence of *THR1* and *GPH1* genes were cloned into pCR-*LacZ* and introduced into strain CAI8. The cells with the reporter were plated onto plate with SC containing X-gal in nutrient limited or stress conditions. **A.** Assay for *THR1* promoter. THR1.1 and THR1.2 represent two different alleles. Three isolates shown as THR1.1-1~3 and THR1.2-1~3 were used to perform the assay. **B.** Assay for *GPH1* promoter. Five isolates and one shown as GPH1-1~5 and one THR1.1

10. ***C. albicans*** **cells lacking** ***THR1*** **impair growth on nutrient limitation or deprived of and interfering with amino acids supply but can be rescued by the absence of** ***GCN4***. As stress sensitivity of the *thr1* homozygous mutant has been reported previously [25, 26], we were interested to know if *thr1* homozygous mutant is also sensitive to nutrient limitation or diverse conditions regarding amino acids supply. As shown in **Fig. 8A**, cells lacking *THR1* greatly impaired their growth ability in either rich medium (YPD) or minimum defined medium with amino acids (SC). The growth of cells further weakened in the presence of rapamycin and 3-AT, with 3-AT exhibited far-greater effect. However, such growth inhibition could be partly released to some extent when simultaneously deleted with *GCN4*, suggesting that growth defect due to absence of *THR1*, most likely via accumulation of homoserine,in the threonine biosynthesis pathway (**Fig. 8B**), can be rescued by deletion of *GCN4*, which might be a result of blocking expression of genes upstream of *THR1* in the threonine biosynthesis pathway or directing to other biosynthesis pathway at or down-stream *THR1*. Indeed, while addition of aspartate led to complete inhibition of growth in cells lacking *THR1* or both *THR1* and *GCN4* in minimum medium with amino acids (SD), addition of thronine rescued the growth defect, even in the presence of aspartate (**Fig. 8C**), confirming that relief of homoserine accumulation free cells from toxicity effect.

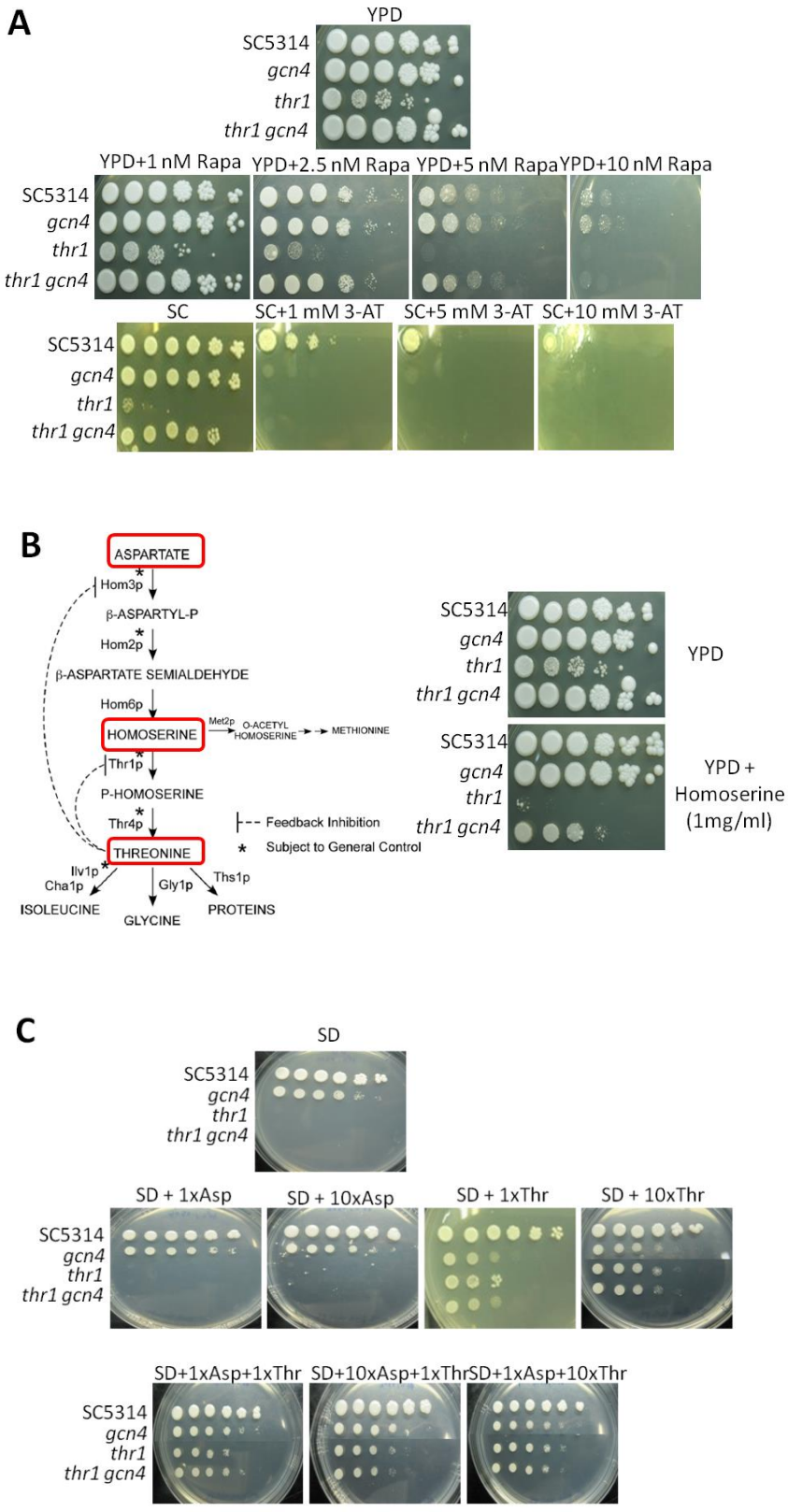


Figure 8. Growth of *C. albicans* strains assayed by series dilution of cells on nutrient limitation conditions. **(A)** Wild-type, null mutants of *gcn4* , *thr1*, *gcn4 thr1* were grown on YPD with or without 1 nM, 2.5 nM, 5nM, and 10nM Rapa plate or SC with or without 1mM, 5mM, 10mM 3-AT plate in 30 ℃ for 2 days; **(B)** on YPD with or without homoserine, the known threonine biosynthesis pathway is shown alongside. **(C)** in 30 ℃ for 2 days on SD plate without or with 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. SD, synthetic complete medium; 3-AT, 3-Amino-1,2,4-triazole; Rapa, rapamycin; SD, synthetic defined medium lacking amino acids; 1x: 80 ng/ml; 10x: 800 ng/ml.

11. **Doxycycline-inducing Pbs2^{DD} (S355D, T359D) or N-terminal truncated Ssk2 was unable to suppress filamentous growth in cells repressed *CaCDC4* expression and activate the expression of *GPH1* due to not being able to hyperactivate HOG pathway.** Although the overexpression of Pbs2^{DD} was verified both in transcriptional and translational levels, the HOG pathway was not hyperactivated confirmed by level of Hog1 phosphorylation level remaining the same under doxycycline (Dox)-induced Pbs2^{DD} expression (data not shown). We have also worked on construction of strain deleted with *C. albicans SSK2* but being capable of Dox-induced expression of N-terminal truncated SSK2 (SSK2ΔN), which has been shown to hyperactivate HOG pathway [56] that might facilitate verification the activation of *GPH1*. We were unable to observe the hyperactivation of the HOG pathway as the level of Hog1 phosphorylation remained the same compared with that in the wild-type strain.
12. ***Candida albicans DBF4* gene inducibly duplicated by the mini-Ura-blaster is involved in hypha-suppression.** The *C. albicans DBF4* gene, a *Saccharomyces cerevisiae* homolog that encodes a regulatory subunit of Cdc7 kinase that is known to initiate DNA replication. We made a *C. albicans* strain, with one *CaDBF4* allele deleted by the mini-Ura-blaster and the other controlled by a repressible promoter. We also found a third *CaDBF4* copy that was later verified to be inducibly duplicated by targeted recombination with the min-Ura-blaster. Surprisingly, the strain deleted with the third *CaDBF4* copy exhibited hyphal growth under repressed conditions. We conclude that the *CaDBF4* gene is prone to being duplicated by the mini-Ura-blaster and that it suppresses hyphal growth in *C. albicans*. This part of work has also been published [57] and is attached in the end of this report (**Appendix III**)..
13. ***Candida albicans* Dbf4-dependent Cdc7 kinase plays a novel role in the inhibition of yeast-to-hypha transition.** The Dbf4-dependent protein kinase encoded gene *CDC7* is conserved in initiating DNA replication. We made a *Candida albicans* strain, with one *C. albicans CDC7* allele deleted and the other's expression repressible, whose cells grew as hypha under the repressed condition, albeit *Cacdc7* homozygous null was unobtainable. The *in vitro* kinase assays confirmed that *Ca*Cdc7 (K232) and *Ca*Cdc7 (T437) are critical for the catalytic activity and the phophoacceptor of activation, respectively. *C. albicans* cells formed hypha when expressed either the catalytically inactive *Ca*Cdc7 (K232R) or the phophoacceptor deficient *Ca*Cdc7 (T437A). While *Ca*Cdc7 interacted with *Ca*Dbf4, neither cells of the strain repressing *CaCDC7* were rescued by constitutively expressing *C. albicans DBF4* nor vice versa. We conclude that *CaDBF4*-dependent *CaCDC7* being an essential gene suppresses yeast-to-hypha transition. **This part of work is currently under submission**.
14. **A new rapid and efficient system with dominant selection developed to inactivate and conditionally express genesnin *Candida 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. This part of work has also been published [58] and is attached in the end of this report (**Appendix IV**).

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Appendix

Appendix I
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Appendix II
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Appendix III
Mutation Research 779: 78–85 (2015)

Appendix IV
Curr Genet 62:213–235 (2016)

Conference report 1

Conference report 2

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Dissection of the *Candida albicans* Cdc4 protein reveals the involvement of domains in morphogenesis and cell flocculation

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Abstract

Background: *CDC4*, which encodes an F-box protein that is a member of the Skp1-Cdc53/Cul1-F-box (SCF) ubiquitin E3 ligase, was initially identified in the budding yeast *Saccharomyces cerevisiae* as an essential gene for progression through G1-S transition of the cell cycle. Although *Candida albicans CDC4* (*CaCDC4*) can release the mitotic defect caused by the loss of *CDC4* in *S. cerevisiae*, *CaCDC4* is nonessential and suppresses filamentation.

Results: To further elucidate the function of *CaCDC4*, a *C. albicans* strain, with one *CaCDC4* allele deleted and the other under the repressible *C. albicans MET3* promoter (*CaMET3*p) control, was made before introducing cassettes capable of doxycycline (Dox)-induced expression of various *C. albicans* Cdc4 (*Ca*Cdc4) domains. Cells from each strain could express a specific *Ca*Cdc4 domain under Dox-induced, but *CaMET3-CaCDC4* repressed conditions. Cells expressing domains without either the F-box or WD40-repeat exhibited filamentation and flocculation similarly to those lacking *CaCDC4* expression, indicating the functional essentiality of the F-box and WD40-repeat. Notably, cells expressing the N-terminal 85-amino acid truncated *Ca*Cdc4 partially reverse the filament-to-yeast and weaken the ability to flocculate compared to those expressing the full-length *Ca*Cdc4, suggesting that N-terminal 85-amino acid of *Ca*Cdc4 regulates both morphogenesis and flocculation.

Conclusions: The F-box and the WD40-repeat of *Ca*Cdc4 are essential in inhibiting yeast-to-filament transition and flocculation. The N-terminal region (1–85) of *Ca*Cdc4 also has a positive role for its function, lost of which impairs both the ability to flocculate and to reverse filamentous growth in *C. albicans*.

Keywords: *Candida albicans*, *CDC4* domains, Morphogenesis, Flocculation

Background

Candida albicans is a natural diploid without a complete sexual cycle and exists as yeast, pseudohyphal, and hyphal cells [1]. It is capable of a morphological switch induced by environmental stimuli [2], essentially via cAMP-mediated and MAPK signaling pathways [3]. Importantly, its ability to alter morphology among cell types is associated with virulence to humans [4]. Many cell cycle regulators including cyclins are also known to control morphogenesis in *C. albicans* [5].

Recently, an F-box protein encoded *C. albicans CDC4* (*CaCDC4*) has been shown to play a role in filamentous development [6,7]. Cdc4, originally identified in the budding yeast *Saccharomyces cerevisiae*, encodes ubiquitin E3 ligases, which belongs to a member of the Skp1-Cdc53/Cul1-F-box (SCF) complex. This complex is known to play a role in ubiquitin-proteasome dependent degradation of regulatory proteins in eukaryotes [8]. A specific SCF complex is designated by its associated F-box protein. This protein is variable with two interacting domains of F-box for Skp1 and WD40-repeat (or LRR) for specific substrates [9], such that Cdc4 can be named SCF^{Cdc4}. To progress through the G1-S transition in *S. cerevisiae*, SCF^{Cdc4} is required to degrade Sic1 [10] and Far1 [11], which are the cyclin-dependent kinase inhibitors. Therefore, *S. cerevisiae CDC4* (*ScCDC4*) is essential in *S. cerevisiae*.

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Although *Ca*Cdc4 is a structural homolog of *S. cerevisiae* Cdc4 (*Sc*Cdc4) and is capable of rescuing the mitotic defect caused by the loss of *ScCDC4* in *S. cerevisiae* [7], the functions of *Ca*Cdc4 and ScCdc4 are dissimilar as the null *Cacdc4* mutant is viable and the depletion of *Ca*Cdc4 causes the accumulation of Sol1 (Sic1 like) for hyphal development rather than initiation of cell cycle arrest [6]. This verifies that *CaCDC4* is nonessential and suppresses filamentation and suggests that controlling the degradation on Sol1 in *C. albicans* by *Ca*Cdc4 is important for inhibition of filamentation. Therefore, while *C. albicans* Sol1 is likely a substrate of SCF^{CaCdc4}, which can be demonstrated by the reduction of Sol1 when *Ca*Cdc4 is overexpressed [6], there has not been any direct evidence to support this hypothesis. Additionally, the filamentous properties for mutants of *Cacdc4* null and *Cacdc4 sol1* double null were comparable. This refutes the idea that Sol1 is the sole target of *Ca*Cdc4. Indeed, with an affinity-purification approach, we have isolated at least two novel *Ca*Cdc4-associated proteins [12] that are potential substrates of *Ca*Cdc4.

To further elucidate the role of *CaCDC4* and its mediation through a characteristic F-box protein of SCF ubiquitin E3 ligase in *C. albicans*, we have sought to dissect the *Ca*Cdc4 domains associated with filamentation. In this study, we made a *C. albicans* strain with one deleted *CaCDC4* allele and repressed the other by *CaMET3* promoter (*CaMET3*p) using methionine and cysteine (Met/Cys). We used this strain to introduce plasmids capable of inducing expression of various *Ca*Cdc4 domains with doxycycline (Dox). We observed the roles of F-box and WD40-repeat for *Ca*Cdc4 function and the possible role of the N-terminal 85-amino acid for morphogenesis. We also showed that *C. albicans* cells that lacked *Ca*Cdc4 triggered flocculation. Moreover, we found that N-terminal 85-amino acid of *Ca*Cdc4 is required for inhibition of both filamentation and flocculation.

Methods

Strains and growth conditions

E. coli strain DH5α was used for the routine manipulation of the plasmids. They were grown at 37°C in LB broth medium [13] or on plates containing 1.5% agar (Difco, BD Biosciences), with 50 μg/ml ampicillin or 30 μg/ml kanamycin. All *C. albicans* strains (Table 1) were derived from auxotrophic strain BWP17 (*arg4/arg4 his1/his1 ura3/ura3*) [14]. They were grown at 30°C in either yeast extract-peptone-dextrose (YEPD) or supplemented minimal synthetic defined (SD) medium with 2% glucose with or without 2% agar [15]. While Ura^+ prototrophs were selected on SD agar plates without uridine, His^+ prototrophs were selected on SD plates without histidine. Selection for the loss of the *C. albicans URA3* (*CaURA3*) marker was performed on plates with 50 μg/ml uridine and 1 mg/ml 5-fluoroorotic acid (5-FOA, MD Bio). To repress the *CaCDC4* expression that was controlled by *CaMET3*p, strains were grown on SD medium or on plates with 2.5 mM Met/Cys, which has been shown to optimally switch off the expression of the *CaMET3*p-driven downstream gene [16]. To induce gene expression under the Tet-on system, 40 μg/ml Dox (Sigma) was added to YEPD or SD media.

Plasmid DNA manipulation

Plasmid DNA was extracted routinely from *E. coli* cultures using Gene-Spin™ MiniPrep purification Kit-V^2 (PRO TECH, Taipei, Taiwan) and the instructions provided by the manufacturer. *E. coli* was transformed with plasmid DNA by using $CaCl_2$. The DNA cassettes were introduced into *C. albicans* by the lithium acetate method as described previously [17].

Construction of *C. albicans* strains

Initially, a strain with repressed *CaCDC4* expression was made. A mini-Ura-blaster cassette, flanked with 60-bp sequences homologous to *CaCDC4,* was PCR-amplified using a template of plasmid pDDB57 and long primers of CaCDC4-URA3-F and CaCDC4-URA3-R (Table 1). BWP17 was transformed by integration of the cassette into the *CaCDC4* locus to generate Ura^+ strain JSCA0018. The plasmid pFA-HIS1-MET3p-CaCDC4, with a partial *CaCDC4* coding sequence for N-terminal *Ca*Cdc4 (1–563), was linearized with *Bsp*EI and used to transform JSCA0018 to generate His^+ JSCA0021 (Figure 1A; Table 1). Cells of JSCA0021 were plated with 5-FOA to induce recombination between two copies of *dpl200* flanking the mini-Ura-blaster for a loss of *CaURA3* to generate JSCA0022.

To allow the expression of cassettes encoding assorted *Ca*Cdc4 domains in *C. albicans*, a Tet-on plasmid, pTET25M [18], which is derived from pTET25 [19] for inducing gene expression with Dox, has been developed. To regulate *CaCDC4* expression by the Tet-on system, the coding sequence of *CaCDC4* was PCR-amplified using plasmid CaCDC4-SBTA bearing *CaCDC4* (Lai WC, unpublished results), primers CaCDC4-SalI and CaCDC4-BglII (Table 2), and *Pfu* polymerase (5 U/μl, MD bio), digested with *Sal*I and *Bgl*II for cloning into pTET25M, from which pTET25M-CaCDC4 was generated. Moreover, *CaCDC4*-6HF, which encodes 6×histidine and FLAG (6HF) tags at the C-terminal of *Ca*Cdc4, was PCR-amplified with primers CaCDC4-6HF SalI and CaCDC4-6HF BglII (Table 2), followed by digestion with *Sal*I and *Bgl*II and cloning into pTET25M to obtain pTET25M-CaCDC4-6HF.

To define the function of the distinct *Ca*Cdc4 domains (Figure 2A), different *CaCDC4* portions were used to replace the full length *CaCDC4* coding sequence on

Table 1 *Candida albicans* strains used in this study

Systemic name of the strain	Parental strain	Name relevant to genotype	Genotype
BWP17		*CaCDC4* +/+	*ura3::imm434/ura3:imm434 his1::hisG / his1::hisG arg4::hisG/arg4::hisG*
JSCA0018	BWP17	*CaCDC4* +/*U3*−	*CaCDC4/cdc4::CaURA3-dpl200*
JSCA0021	JSCA0018	*CaCDC4 M3/U3*−	*Cacdc4::URA3-dpl200/P_{MET3}-CaCDC4:HIS1*
JSCA0022	JSCA0021	*CaCDC4 M3*/−	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1*
JSCA0023	JSCA0022	*CaCDC4 M3*/− | Tet-*CaCDC4*	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 | CaADH1/adh1::P_{TET}-CaCDC4:CaURA3*
JSCA0024	JSCA0022	*CaCDC4 M3*/− | Tet-*CaCDC4*-6HF	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 | CaADH1/Caadh1::P_{TET}-CaCDC4-6HF:CaURA3*
JSCA0025	JSCA0022	*CaCDC4 M3*/− | Tet-ΔN-6HF	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 | CaADH1/Caadh1::P_{TET}-CaCDC4(85–768)-6HF:CaURA3*
JSCA0026	JSCA0022	*CaCDC4 M3*/− | Tet-F-box-6HF	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 | CaADH1/Caadh1::P_{TET}-CaCDC4(241–392)-6HF:CaURA3*
JSCA0027	JSCA0022	*CaCDC4 M3*/− | Tet-WD40-6HF	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 | CaADH1/adh1::P_{TET}-CaCDC4(393–768)-6HF:CaURA3*
JSCA0030	JSCA0022	*CaCDC4 M3*/− | Tet-ΔNF-6HF	*Cacdc4::dpl200/P_{MET3}-CaCDC4:HIS1 |CaADH1/Caadh1::P_{TET}-CaCDC4(85–392)-6HF:CaURA3*

pTET25M-CaCDC4-6HF. By using the primer sets listed in Table 2, the following constructs were made: pTET25M-ΔNCaCDC4-6HF (with primers CaCDC4 ΔN AatII and CaCDC4 ΔN XhoI), which encodes the N-terminal truncated *Ca*Cdc4; pTET25M-F-6HF (with primers CaCDC4 F-box AatII and CaCDC4 F-box XhoI), which encodes the F-box domain with flanking regions; pTET25M-WD40-6HF (with primers CaCDC4 WD40 AatII and CaCDC4 ΔN XhoI), which encodes eight copies of WD40-repeat; and pTET25M-ΔNF-6HF (with primers CaCDC4 ΔN AatII and CaCDC4 F-box XhoI), which encodes truncated N-terminal *Ca*Cdc4 and the F-box domain. All inserts of the constructs were released with *Aat*II and *Xho*I to replace the full-length *CaCDC4* on pTET25M-CaCDC4-6HF. Consequently, plasmids bearing those *CaCDC4* segments flanked with common *C. albicans ADH1* (*CaADH1*) sites were digested with *Sac*II and *Kpn*I, each of which was transformed into *C. albicans* for integration at the *CaADH1* locus. All strains were verified by colony PCR with specific primers before subjecting to Southern blotting analysis.

Southern blotting analysis

Genomic DNA from the *C. albicans* strains was isolated by the MasterPure™ Yeast DNA Purification Kit (Epicentre®, an Illumina company) according to the manufacture's instruction. Southern blotting was performed with the aid of the Rapid Downward Transfer System (TurboBlotter™, Whatman) using 10 μg of the restriction enzyme-digested genomic DNA. The DNA on the blot was hybridized with a probe amplified by the PCR DIG probe synthesis kit (Roche) with the primers CaCDC4_Probe_F and CaCDC4_Probe_R for *CaCDC4* locus or CaADH1 Probe_F and CaADH1 probe_R for *ADH1* locus (Table 2) using DIG Easy Hyb (Roche). To reveal the structure of gene locus, the DIG Luminescent Detection Kit (Roche) was used after hybridization, and the luminescent images of blot were captured with the imaging analysis system (ImageQuant LAS4000 mini, GE Healthcare Life Sciences).

Protein extraction and Western blot analysis

Cultured cells were collected, and the total protein from each sample was extracted as described previously [20]. The proteins were resolved by 10% SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, USA). Proteins on the membranes were probed with polyclonal antibody to FLAG (Sigma) in 1:2000 dilution and detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (PIERCE). These were recorded with the Luminescent Image Analyzer (FUJIFILM LAS-1000) and analyzed by ImageGauge 3.46 and L Process v 1.96 (FUJIFILM).

Flocculation assay by low-speed centrifugation

The cells of strains were streaked on YPD agar plate for 3 days and colonies were picked and inoculated into SD medium with required supplements for 48 hrs. Next, the cultures were diluted into fresh SD medium to 0.1 of an initial OD_{600} with required supplements. To simultaneously repress the expression of *CaMET3*p-driven *CaCDC4* and to induce the expression of various *CaCDC4* segments encoding series of *Ca*Cdc4 domains, 2.5 mM Met/Cys and 40 μg/ml Dox were also added into the SD medium. After 48 hrs, the cultures were spun down for

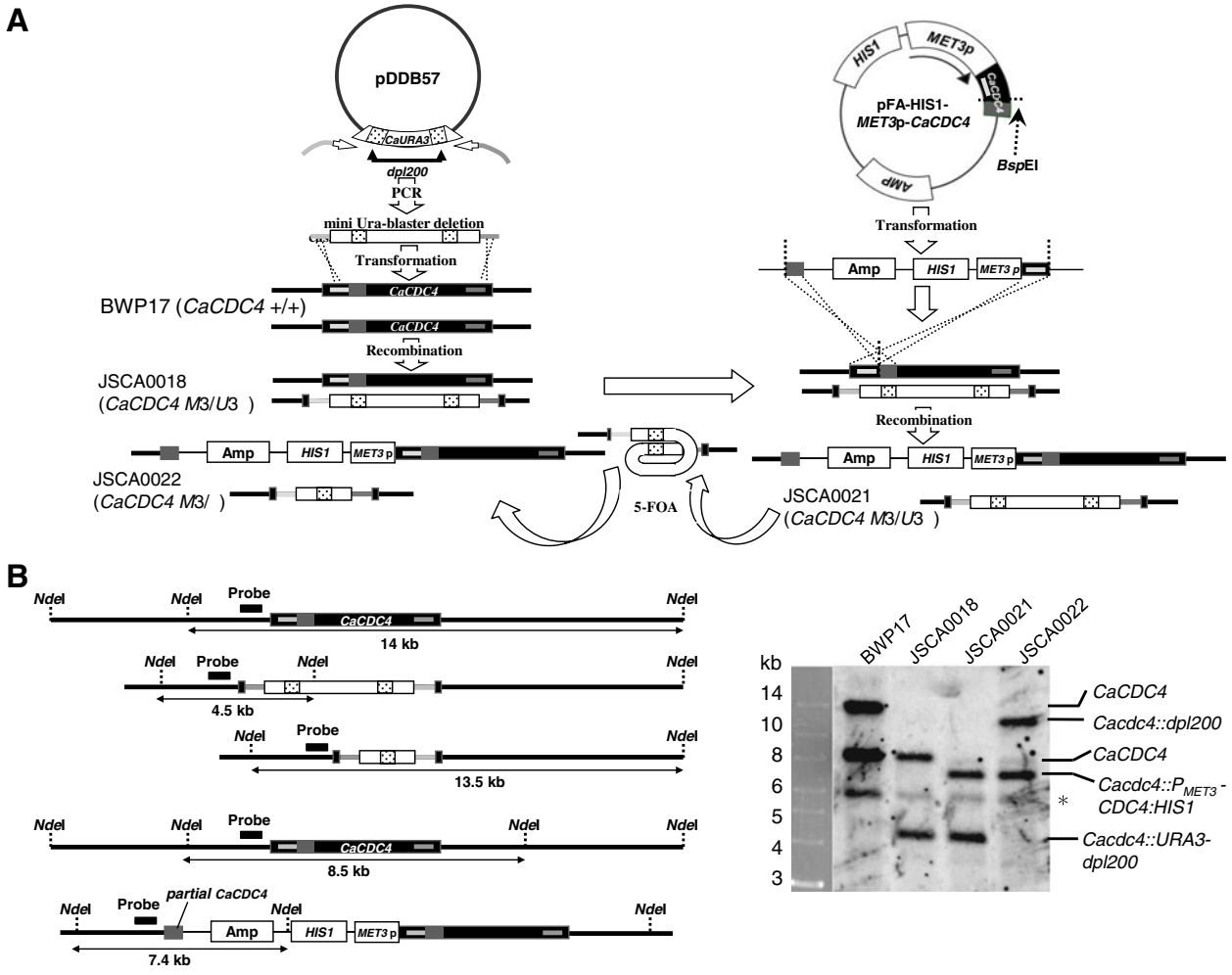

Figure 1 Construction of a *C. albicans* strain for repressibly expressing *CaCDC4*. (A) Strain construction (detailed in the Methods). The first *CaCDC4* allele on BWP17 was deleted by mini-Ura-blaster to obtain JSCA0018. Plasmid pFA-HIS1-MET3p-CaCDC4 containing partial *CaCDC4* coding sequence was linearized at a unique site for introducing into strain JSCA0018 to generate JSCA0021. 5-FOA was used to counter-select *CaURA3* removal to obtain JSCA0022 for re-introducing the Tet-on plasmid with a *CaURA3* marker. **(B)** Verification of constructed strains by Southern blotting analysis. Organization of the *CaCDC4* locus with respect to *Nde*I sites is shown. The relative positions of the probe used and the predicted *Nde*I-digested pattern of the *CaCDC4* locus are indicated. Two *Nde*I-fragments of 14 kb and 8.5 kb, specific to *CaCDC4*, could be detected in genomic DNA from BWP17 digested with *Nde*I; two *Nde*I-fragments of 8.5 kb and 4.5 kb, specific to *CaCDC4* and *Cacdc4::URA3-dpl200*, respectively, could be detected in JCSA0018; two *Nde*I-fragments of 4.5 kb and 7.4 kb specific to *Cacdc4::URA3-dpl200* and *Cacdc4::*P_{MET3} *-CDC4:HIS1*, respectively, could be detected in JSCA0021; and two *Nde*I-fragments of 13.5 kb and 7.4 kb specific to *Cacdc4::dpl200* and *Cacdc4::*P_{MET3} *-CDC4:HIS1*, respectively, could be detected in JSCA0022. A non-specific *Nde*I-fragment is indicated as "*" can be detected in all strains tested.

1 minute at 500 rpm, and the suspensions of the cultures were sampled to determine their optical density at OD_{600}. Three independent assays were conducted and each sample was assayed in duplication. A paired Student t test with $p < 0.05$ was considered significance.

Ca^{2+}-initiated flocculation assay

The *FLO*-encoded flocculins are known to be essential for flocculation in *S. cerevisiae* [21]. Functional homologues of *FLO* genes have been found in *C. albicans*. In particular, the important *S. cerevisiae* gene *FLO11* responsible for flocculation has *C. albicans* functional counterpart *ALS1* [22]. Since *FLO11*-associated flocculation is dependent on the presence of Ca^{2+}, we adopted an alternative flocculation assay in which the rate of flocculation is initiated by Ca^{2+} and the optical density was assessed within a short time-frame [23]. Briefly, to initiate flocculation, an aliquot of 800 μl deflocculated cell suspension was transferred into a 1-ml cuvette, followed by addition of 200 μl of 100 mM $CaCl_2$. The cuvette was mixed robustly by pipetting and the absorbance (OD_{600}) was assessed instantly at 30-s intervals for 5 minutes using a spectrophotometer (DU800, Beckman Coulter, Inc.). All assays were conducted in triplicate.

Table 2 Oligonucleotides used in this study

Name	Sequence[a]
CaCDC4 XhoI F	GAA*CTCGAG*ATGGATAAGAAATCAAAG
CaCDC4 XhoI R	GAA*CTCGAG*CTGTAAAAGTGGTTGACT
CaCDC4 SalI	TAGC*GTCGAC*ATGGATAAGAAATCAAAGC
CaCDC4 BglII	TCG*AGATCT*TCACTGTAAAAGTGGTTGAC
CaURA3-dpl200 BamHI	AAT*GGATCC*CCAGATATTGAAGGTAAAAGG
CaURA3-dpl200 XhoI	ATT*CTCGAG*CTAGAAGGACCACCTTTGAT
TET25M KpnI	CAA*GGTACC*GAACCATCGTGAGTGTAA
TET25M BamHI	GAA*GGATCC*CGACATTTTATGATGGAA
CaCDC4-6HF SalI	GCGT*GTCGAC*GTCATGGATAAGAAATCAAAGCTA
CaCDC4-6HF[b] BglII	TCG*AGATCT*ttatttatcatcatcatctttataatcACCACC gtggtggtggtggtggtg*CTCGAG CGGCCGC*TGTAA AAGTGGTTGACTGAAATC
CaCDC4 ΔN AatII	AATA*GACGTC*CTTATGCCCTCATGTGACGAC
CaCDC4 ΔN XhoI	ATC*CTCGAG*CTGTAAAAGTGGTTGACTGA
CaCDC4 F-box AatII	AAGC*GACGTC*ATGAGCAATGAACCTACT
CaCDC4 F-box XhoI	GCCA*CTCGAG*CCACCTATTGACAATTAT
CaCDC4 WD40 AatII	GCTA*GACGTC*ATGGATCCAAAGTTCAAAC
CaCDC4-URA3-F	ATGGATAAGAAATCAAAGCTATTCAAATATCCTTT GAGCGAGGAGACGGCTAAATTTGAGGTTTTCCCA GTCACGACGTT
CaCDC4-URA3-R	TCACTGTAAAAGTGGTTGACTGAAATCTAGAATCT CAATAAACGTTTCACCTTCATCTTCTGTGGAATTGT GAGCGGATA
CaADH1_probe_F	GGAGTATTGGCATTGTTGGG
CaADH1_probe_R	AAGCTTGCTTGCATGACGAG
CaCDC4_probe_F	GGTTTCCAACACTTTCCCAG
CaCDC4_probe_R	CACTACTAGTTGGTTGCTGT

[a]Restriction enzyme sites are in italics.
[b]Sequences complementary to those encoding 6×His and FLAG are in lower case letters. The italics has been used for restriction enzymes as in note "a". The underline is new replaced with lower case letters.

Results

Constructing a *C. albicans* strain capable of conditionally repressing the expression of *CaCDC4*

To establish *C. albicans* strains capable of expressing *CaCDC4* and its domains solely controlled under a *Tet* promoter directly in *C. albicans*, BWP17, with both alleles of *CaCDC4* deleted, was constructed to accommodate Tet-on plasmid cassettes capable of expressing assorted *Ca*Cdc4 domains induced by Dox. The first allele of *CaCDC4* was deleted in BWP17 by mini-Ura-blaster [24] to generate the JSCA0018 strain (Figure 1A; Table 1). This strain was used to delete the second *CaCDC4* allele to obtain a *Cacdc4* null mutant. However, *Cacdc4* null mutant cells growing as filamentous form with toughened cell walls obstructed transformation.

To overcome this problem, the strain JSCA0021 (Figure 1A; Table 1) was created that had one *CaCDC4* allele deleted and the other under *CaMET3* control that was Met/Cys repressible. To allow the introduction of Tet-on cassettes with the same *CaURA3* selectable marker as the mini-Ura-blaster on JSCA0021, 5-FOA was used as a counter-selection agent to remove *CaURA3* from JSCA0021, from which JSCA0022 was obtained (Figure 1A; Table 1). The strains were PCR-confirmed with specific primers before subjecting to Southern blotting analysis. The *CaCDC4* locus from BWP17 strain could detect two *Nde*I-digested fragments with size of 14 kb and 8.5 kb, respectively (Figure 1B). The size shifting of *Nde*I-fragment flanking *CaCDC4* from 14 kb to 4.5 kb demonstrated that one *CaCDC4* allele was integrated with the mini-Ura-blaster cassette as in strain JSCA0018 (Figure 1B). The size shifting of *Nde*I-fragment flanking *CaCDC4* from 8.5 kb to 7.4 kb demonstrated that the other *CaCDC4* allele integrated with the *MET3*-diven *CaCDC4* plasmid as in strain JSCA0021 (Figure 1B). Strain JSCA0021 could be further popped out the mini-Ura-blaster cassette to obtain strain JSCA0022 in which the size shifting of *Nde*I-fragment flanking *CaCDC4* from 4.5 kb to 13.5 kb (Figure 1B). These results indicate that all strains constructed have expected organizations in their genome.

Phenotypic verification of *C. albicans* strains capable of conditionally repressing the expression of *CaCDC4*

It has been shown that Ura^{-} auxotrophic mutants are avirulent [25] and other virulence-associated features can be influenced by the level of *CaURA3* gene expression [26]. To assess presence of *CaURA3* having effect on yeast-to-filament transition, the yeast-to-filament transitions between strain JSCA0021 and JSCA0022 were compared, cells of those strains were assessed under *CaMET3*p repressed or de-repressed conditions. Cells of both strains on SD plates without Met/Cys grew as circular colonies with smooth surfaces (Figure 2). By contrast, cells on plates with Met/Cys formed irregular colonies with filaments (Figure 2). Under the microscope, these strains exhibited equivalent filamentous forms, suggesting a comparable ability to deplete *CaCDC4* for expression and inability of *CaURA3* interfering with yeast-to-filament transition *in C. albicans*. Subsequently, JSCA0022 was used as a parental strain to introduce the Tet-on cassettes (with *CaURA3* marker) that encoded assorted *Ca*Cdc4 domains.

Establishment of Tet-on cassettes capable of expressing assorted *CaCDC4* domains in *C. albicans* reveals that both the F-box and WD40-repeat are required for *Ca*Cdc4 function

The filamentous development of JSCA0022 under *CaMET3*p-*CaCDC4* repressed conditions, with Met/Cys and the Tet-on system, allows us to study the function of the *Ca*Cdc4 domains. A set of Tet-on cassettes (obtained from pTET25M-CaCDC4-6HF, pTET25M-ΔN-6HF, pTET25M-F-box-6HF, pTET25M-WD40-6HF, and pTET25M-ΔNF-6HF) that encoded each of the

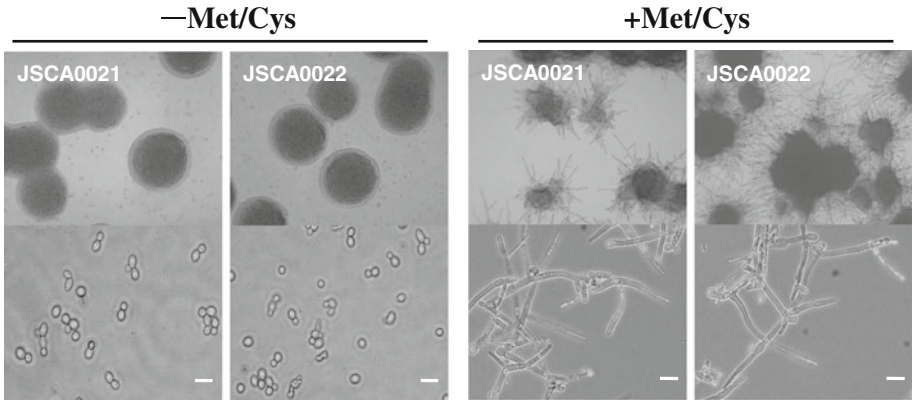

Figure 2 Morphological analysis of the constructed *CaCDC4* repressible strains. Cells of strains JSCA0021 and JSCA0022 were grown on SD medium or plates with (+) or without (–) Met/Cys. Colonies were photographed with MEIJI stereoscopic microscope EMZ5 at 40× magnification (top panel). Cells in liquid culture were visualized and recorded with a Nikon 50i microscope at 400× magnification (bottom panel). Bars represent 10 μm.

assorted domains of *Ca*Cdc4 (Figure 3A) were used to transform JSCA0022 (which contained a *CaMET3*p-repressible *CaCDC4*) to Ura^+ by integration at the *CaADH1* locus (Figure 3B). The correctness of the strains was confirmed by yeast colony PCR with specific primers before Southern blotting analysis. The *CaADH1* locus from strain JSCA0022 could detect a *Spe*I-digested fragment with size of 3.3 kb (Figure 3C). The *CaADH1* locus from strains JSCA0023 and JSCA0024 detected an increased *Spe*I-digested fragment of 9.4 kb due to the integration of Tet-on cassettes of either pTET25M-CaCDC4 or pTET25M-CaCDC4-6HF (Figure 3C). The *CaADH1* locus from other strains also showed expected alteration in size according to the size of different *CaCDC4* domains (Figure 3C). These results confirmed the correctness of the strains.

The JSCA0022 strain, which expressed the non-tagged and repressible *Ca*Cdc4, was used as a negative control. The sample obtained from JSCA0022 contained two prominent proteins of approximately 55 kDa and 72 kDa (Figure 4A) which were presumably a result of cross-reactivity to the anti-FLAG antibody. Those two proteins were used as an internal control. The F-box and WD40-repeat proteins from strains JSCA0026 and JSCA0027 migrated to their expected positions of approximately 19 kDa and 43 kDa (Figure 4A), respectively. However, the full-length *Ca*Cdc4 and the N-terminus truncated *Ca*Cdc4 (ΔN) from strains JSCA0024 and JSCA0025 exhibited signals at positions corresponding to 100 kDa and over 100 kDa (Figure 4A), respectively, as opposed to 86 kDa and 77 kDa, respectively. Three distinctive signals (Figure 4A) were observed for strain JSCA0030 expressing ΔNF of *Ca*Cdc4, but none of them matched the expected size of 34 kDa; however, the signal at the lowest position could be meaningful. These patterns of expression were similar to strains expressing each of the domains, with either BWP17 or JSCA0021 as a parental strain (Lai WC, unpublished results). Therefore, even though some of the strains expressed domains with unexpected size, they were unique from the negative control of JSCA0022. We concluded that the Tet-on system functions in JSCA0022 and that *Ca*Cdc4 might be undergoing undefined modifications.

To determine the function of the assorted *Ca*Cdc4 domains, JSCA0022-based strains capable of repressing *CaCDC4* and inducing expression of assorted *Ca*Cdc4 domains were grown in SD medium with or without Met/Cys and in the presence or absence of Dox. Cells from strains in SD medium without Met/Cys grew as yeast in the presence or absence of Dox (Figure 4B). By contrast, cells from strains in medium with Met/Cys grew with filaments (Figure 4B). As expected, cells of JSCA0023 and JSCA0024 growing on medium with Met/Cys and Dox and that expressed the full-length *Ca*Cdc4 with or without tag grew as yeast. Disregarding the full-length *Ca*Cdc4, cells from all strains, except JSCA0025 expressing assorted domains, still grew as filaments (Figure 4B). Under Met/Cys and Dox conditions, cells from JSCA0025 expressing the N-terminal 85-amino acid truncated *Ca*Cdc4 seemed to have an ability to suppress filamentation but not complete back to the yeast form (Figure 4B). This is in consistent with our previous observation in which, comparing with cells capable of expressing the full-length *Ca*Cdc4 under the *CaMET3*p repressible control, those cells expressing the N-terminal 85-amino acid truncated *Ca*Cdc4 lagged behind in reaching exponential stage (Additional file 1: Figure S1) and converted to filamentous form earlier (Additional file 2: Figure S2) in the repressed condition.

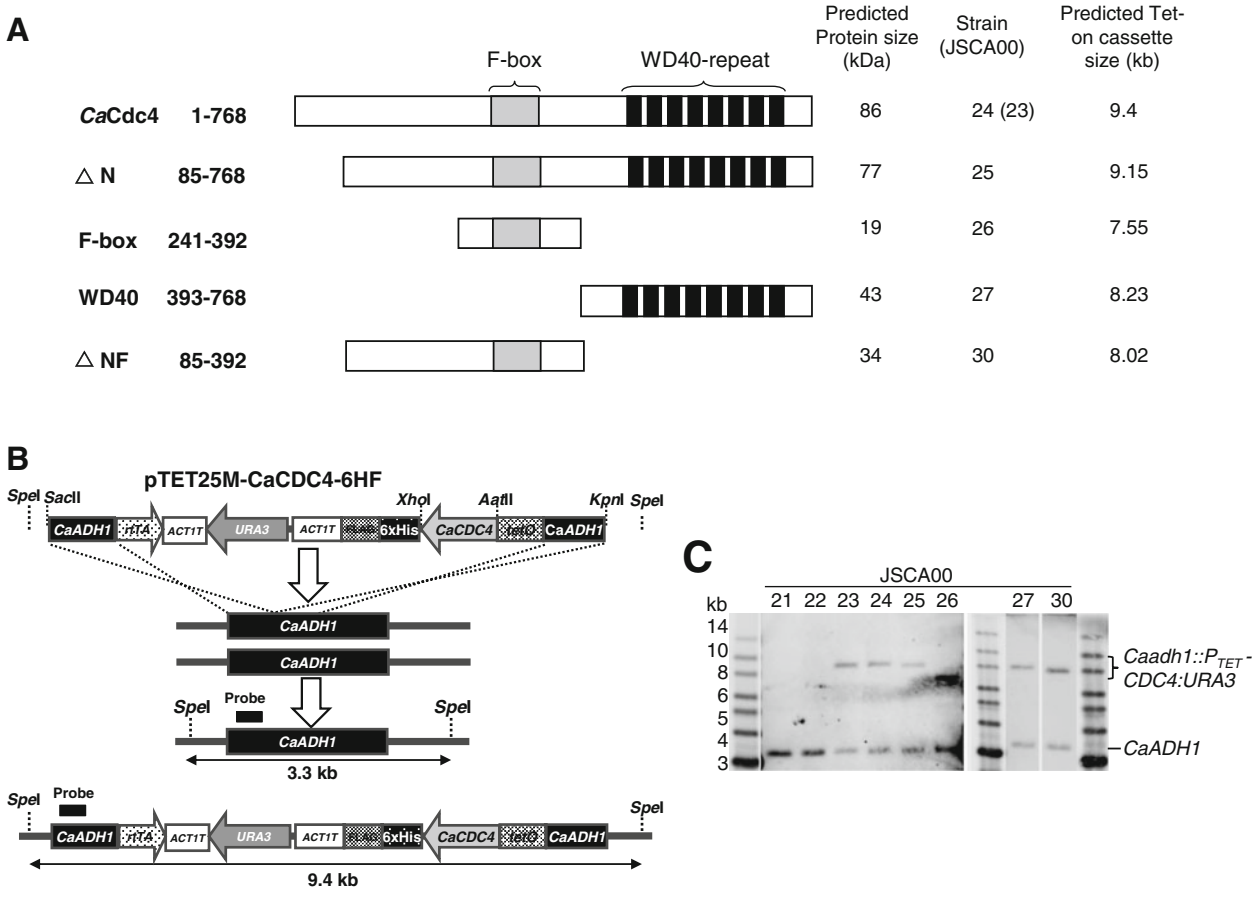

Figure 3 Construction of *C. albicans* strains for Dox-inducing the expression of assorted *CaCDC4* domains. (A) Schematic representation of *Ca*Cdc4 domains expressed from the Tet-on system. The strains with which they are derived and predicted Tet-on cassette size are shown **(B)** Generation of Tet-on cassettes for expressing assorted CaCdc4 domains. Different portions of *CaCDC4* were PCR-generated with primer sets (Table 2) containing common *Aat*II and *Xho*I sites for replacing full-length *CaCDC4* on pTET25M-CaCDC4-6HF as described in the Methods. By digestion with *Sac*II and *Kpn*I, each cassette was used to transform *C. albicans* strain JSCA0022 for integration into the *CaADH1* locus. (**C**) Verification of Tet-on cassettes being integrated into *CaADH1* locus by Southern blotting analysis. Organization of the *CaADH1* locus with respect to *Spe*I sites is shown in Figure 3B. The relative positions of the probe used and the predicted *Spe*I-digested pattern of the *CaADHI* locus are indicated in Figure 3B. One *Spe*I-fragment of 3.3 kb specific to *CaADH1* locus could be detected in genomic DNA from strain JSCA0022 and its derivatives digested with *Spe*I. The correctness of integration of the cassette into the *CaADH1* locus of various strains was confirmed by alteration of the *Spe*I-fragment from size of 3.3 kb to 9.4 kb (Figure 3B) or various sizes as indicated in Figure 3A.

C. albicans CDC4 negatively regulating cell flocculation

Significant differences in the ability among strains to form suspensions (to resist flocculation) were observed. The extent of flocculation among strains was observed after resuspending the cells in cuvettes, where they remained for 30 seconds. When cells were grown under the Met/Cys and Dox conditions, only those from JSCA0023 and JSCA0024 were somewhat easier to maintain as a suspension. To exclude the possibility that this was a result of increases in cell density, cells from all strains were initially grown to saturation, and the cultures were subsequently diluted to the same initial optical density and grown exponentially to similar optical density. The extent of flocculation among strains was observed after spinning the cells for 1 minute at 500 rpm. The suspended cells were sampled for determination of their optical density. Cells resisted in flocculation would remain in suspension upon centrifugation. Under the *CaMET3*p de-repressed condition and in the presence or absence of Dox, all strains exhibited a similar degree of suspension. However, under the *CaMET3*p repressed condition, JSCA0026, JSCA0027, and JSCA0030 displayed flocculation similar to JSCA0022 regardless of the presence or absence of Dox (Figure 5A). While more cells of strains JSCA0023, JSCA0024 maintained as suspension, those of JSCA0025 with some filamentous cells, showed comparable extent of flocculation to JSCA0022 under *CaMET3*p repressed but Tet-on induced conditions (Figure 5).

To solidify our observations, an alternative flocculation assay where flocculation is initiated by addition of

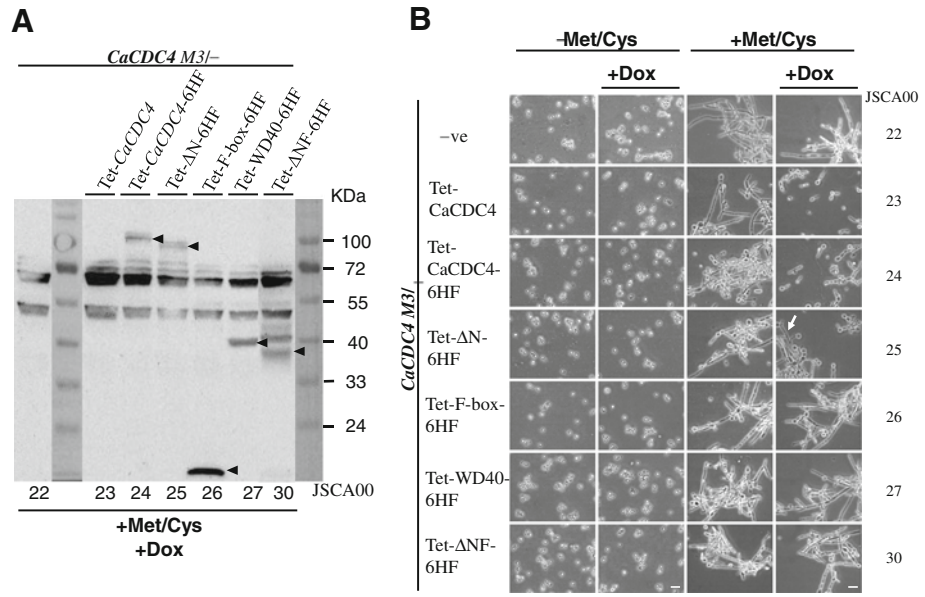

Figure 4 Morphological analysis of *C. albicans* strains capable of Dox-inducing the expression of assorted *CaCDC4* domains. Cells were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration. Cells were grown exponentially in SD in the absence of 2.5 mM Met/Cys, with or without 50 μg/ml Dox (–Met/Cys + Dox or –Met/Cys), or in the presence of 2.5 mM Met/Cys, with or without 50 μg/ml Dox (+Met/Cys + Dox or + Met/Cys). **(A)** The Dox-inducibly expressing assorted *Ca*Cdc4 protein domains under *CaMET3-CaCDC4* repressed conditions was verified by Western blotting with polyclonal antibody to FLAG. The non-specific signals between 72 and 55 kDa, and between 55 and 40 kDa are served as a loading controls. **(B)** The images were visualized and recorded with a Nikon 50i microscope at 400× magnification. The arrow in white indicates filamentous cells. Bars represent 10 μm. The designations of strains are the same as in Table 1.

Ca^{2+} to the culture medium being depleted with Ca^{2+} beforehand was used [23]. Only cells of JSCA0023 and JSCA0024 remained resistance in flocculation during the time-frame of 5-minute assay compared with those of the rest of strains (Figure 6), which were consistent with the results shown in Figure 5. However, both strains JSCA0025 and JSCA0027 exhibited greater ability to resist flocculation than that of JCSA 0026 and JSCA0030 when considering the differences in OD_{600} from the initial to the end points.

Discussion

In this study, we aimed to dissect the function of *Ca*Cdc4 domains by introducing a Tet-on system with cassettes that encoded for a variety of *Ca*Cdc4 domains in a *C. albicans* mutant of *Cacdc4* null. However, the *Cacdc4* null mutant with a filamentous form could not be easily used to introduce the Tet-on cassettes; therefore, we constructed the JSCA0022 strain, where *CaURA3* was released from the strain JSCA0021, and *CaCDC4* expression was repressible. Under repressed conditions, the JSCA0022 strain showed similar filamentous morphology (Figure 2) to those from previous reports of cells with *CaCDC4* repressed strain [6,7] and of *cacdc4* null mutant [6] (Tseng TL, Hsu WH, and Shieh JC, unpublished results). We confirmed that the JSCA0022 strain under repressed conditions was equivalent to a strain that had completely lost *CaCDC4* function. Hence, by introduction of the Tet-on cassettes into JCSA0022 strain, each of the strains was capable of expressing individual *Ca*Cdc4 domains in the presence of Met/Cys and Dox for functional comparisons.

To verify the ability of the Tet-on cassettes in *C. albicans*, each of the cassettes encoding various *Ca*Cdc4 domains was transformed into BWP17 and JSCA0021 before introducing them into JSCA0022 at the *CaADH1* locus. Individual *Ca*Cdc4 domains from relevant strains were all detectable, suggesting that the Tet-on system functions in *C. albicans*. However, while cells expressing the F-box and the WD40 repeat could be detected as their expected sizes, those expressing the full-length *Ca*Cdc4, the N-terminus truncated *Ca*Cdc4 (ΔN), and the ΔNF of *Ca*Cdc4 could be detected at positions higher than anticipated (Figure 4A). In particular, the sample from strain JSCA0030 expressing the ΔNF could be detected three signals (Figure 4A), all of which were greater than the predicted sizes. These results suggest that the N-terminal *Ca*Cdc4 from residue 85 to 241 (Figure 3A) might be undergoing post-translational modification during the Tet-on-induced expression,

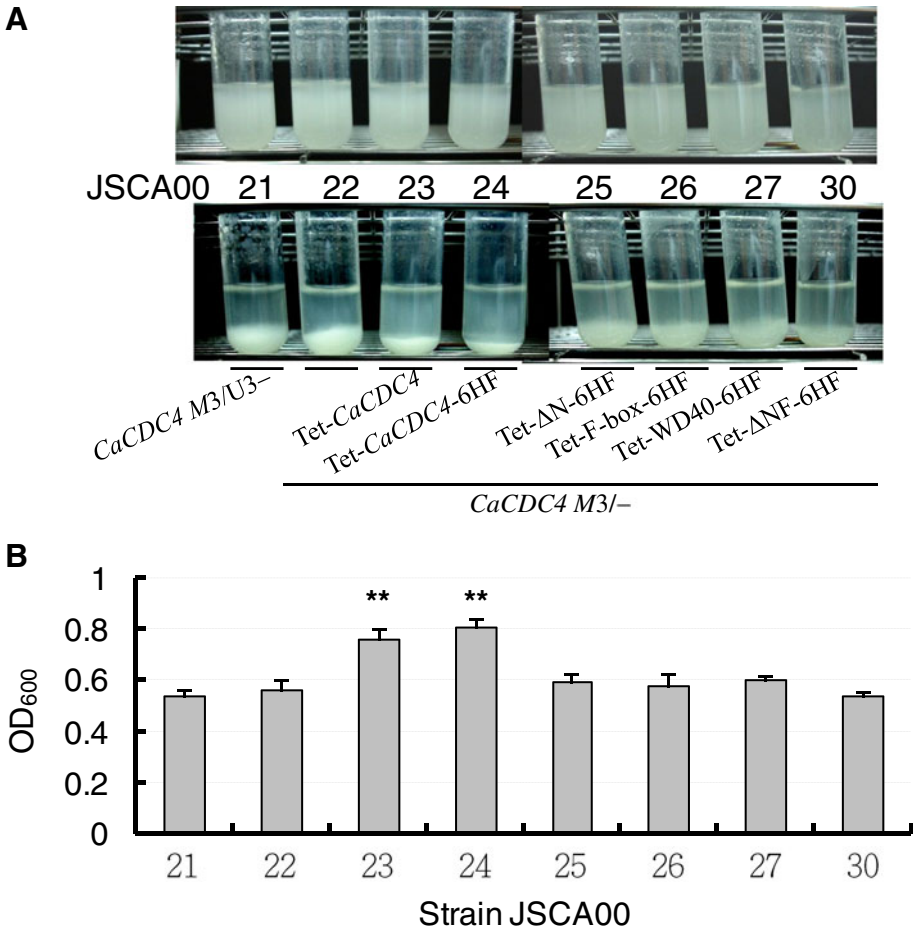

Figure 5 Analysis of cell flocculation by low-speed centrifugation. Cultures of the indicated strains were grown in SD medium with histidine, arginine, uridine for 2 days before diluting into the SD medium to an initial $OD_{600} = 0.1$ with addition of 2.5 mM Met/Cys to repress the expression of *CaMET3*p-driven *CaCDC4* and 40 μg/ml Dox to induce the expression of *Ca*Cdc4 domains tested for 48 hrs to $OD_{600} \approx 1.6$. Cultures were photographed before and after centrifugation. **(A)**. A representative of the cultures. Upper panel: two-day culture. Bottom panel: cultures being spun down with 500 rpm for 1 minute. **(B)**. Quantitative results. Data are represented as means with standard deviation from three independent experiments, each sample was in duplication. The data from JSCA0022 were compared with those of other strains. **: $P < 0.01$. The designations of strains are the same as in Table 1.

although its functional significance is unknown. Interestingly, the region between residue 85 and 241 of *Ca*Cdc4 contains abundant serine and threonine residues, the majority of which are homologous to *S. cerevisiae* Cdc4 [7]. This implies possible phosphorylations or other modifications on these residues that is specific to *C. albicans*. However, the genuine nature of these residues remains to be determined, and their functional significance of this N-terminal *Ca*Cdc4 requires further study.

With regards to integration of *CaADH1* locus by the Tet-on cassette, it is known that *C. albicans adh1* homozygous null mutant gains the ability to form biofilm both *in vitro* and *in vivo* [27], suggesting a possible role of *CaADH1* in flocculation. However, the heterozygous *CaADH1* null mutant with which the homozygous *adh1* null mutant is reintegrated a functional copy of *CaADH1* to the *CaADH1* locus appears to be similar in biofilm formation as its isogenic wild-type strain. In addition, disruption of *CaADH1* has no consequence of morphology alteration in *C. albicans* [27] (Lai WC, unpublished results). Therefore, the possible effect of Tet-on cassette on flocculation and filamentation by integration, hence disruption of a copy of *CaADH1* locus can be excluded.

Under the Met/Cys and Dox conditions, cells expressing F-box, WD40 repeat, and the ΔNF of *Ca*Cdc4 exhibited filamentous forms similar to those of JSCA0022, whose *CaCDC4* was repressed, compared to those expressing the full-length *Ca*Cdc4 without or with tag (JSCA0023 and JSCA0024), which exhibited yeast forms

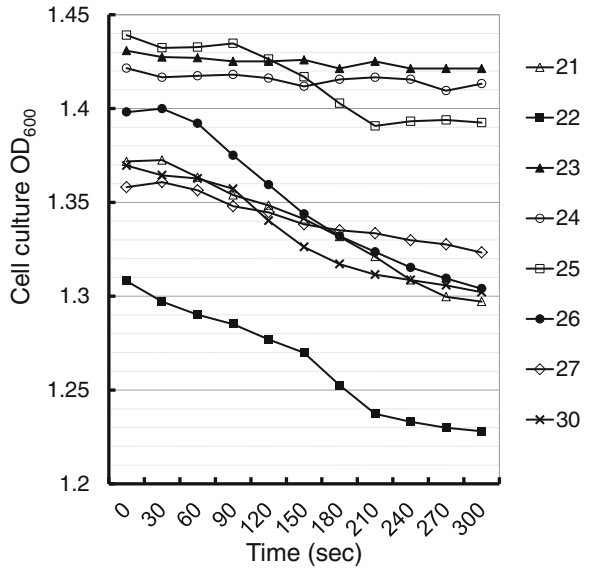

Figure 6 Analysis of Ca^{2+} initiated cell flocculation. The strains were grown as described in Figure 5 to saturation at $OD_{600} \approx 1.4$. The cultures were harvested and washed twice with deflocculation buffer, followed by initiation of flocculation as described in the Methods. The assays were conducted in triplicate. The name of each strain shows only the last two numbers.

(Figure 4B). These results suggest that both the WD40 repeat and F-box are essential to suppress the yeast-to-filament transition. Cells from strain JSCA0025 expressing the ΔN of *Ca*Cdc4, which were grown in the presence of Met/Cys and Dox, were only partially able to reverse filamentous cells to yeast cells, suggesting that the N-terminal 85-amino acid of *Ca*Cdc4 plays a role in the yeast-to-filament transition in *C. albicans*. The role of the N-terminal 85-amino acid of *Ca*Cdc4 for growth was observed previously, in which cells expressing N-terminal 85-amino acid truncated *Ca*Cdc4 lagged slightly in proliferation during the exponential stage (Additional file 1: Figure S1), and repression of the expression of the N-terminal 85-amino acid truncated *Ca*Cdc4 resulted in prominently lagging behind in growth, which was presumably due to the morphological alteration of cells to filaments in advance (Additional file 2: Figure S2) that delays proliferation as compared to those of yeast cells. Since the N-terminal 85-amino acid of *Ca*Cdc4 is unique compared to that of the *S. cerevisiae* Cdc4 [7], our finding reveals a role of N-terminal 85-amino acid of *Ca*Cdc4 on morphogenesis, which is unknown previously.

Importantly, cells of all JSCA0022-based strains exhibited flocculation in medium with Met/Cys, but the strains JSCA0023 (CaCDC4) and JSCA0024 (CaCDC4-6HF) exhibited less flocculation by adding Dox simultaneously (Figure 5). Unlike cells of JSCA0023 and JSCA0024, those of JSCA0025 expressing N-terminal 85-amino acid truncated *Ca*Cdc4 were unable to totally overturn filamentous-to-yeast cells, suggesting that N-terminal 85-amino acid is required for full activity of *CaCDC4* function in *C. albicans* to inhibit filamentation. However, if flocculation is tightly associated with filamentation, we expect to see the extent of flocculation in JCSA0025 (ΔN 6HF) being greater than that of JSCA0022 but less than that of JSCA0023 and JSCA0024 in the presence of Met/Cys and Dox. This was not revealed by the low speed-centrifugation method but by the Ca^{2+}-initiation assay. Importantly, both JSCA0025 and JSCA0027 expressing *Ca*Cdc4 lacking N-terminal 85-amino acid (Figure 3A) exhibits similar extent of flocculation. Moreover, JSCA0025 that expressing *Ca*Cdc4 lacking N-terminal 85-amino acid could only partially suppress filamentation yet JSCA0027 that expressing *Ca*Cdc4 lacking N-terminal 85-amino acid and F-box with flanking regions completely lose the ability to inhibit filamentation (Figure 3A and Figure 4B). These results imply that N-terminal 85-amino acid of *Ca*Cdc4 has a role in inhibition of cell flocculation in *C. albicans* and that the F-box and its flanking region in addition to the N-terminal 85-amino acid of *Ca*Cdc4 might be associated with proper control of both morphogenesis and flocculation.

Conclusions

Therefore, we conclude that F-box and WD40-repeat are important in suppressing yeast-to-filament transition and flocculation and that the N-terminal region (1–85) has a positive role in *CaCDC4* function, lost of which impairs reverse of filament-to-yeast and reduces the ability to flocculate in *C. albicans*. Moreover, the function of *Ca*Cdc4 for suppressing flocculation that is related to cell-cell adhesion [21] implies a role of *CaCDC4* in biofilm formation [28] that is under investigation.

Additional files

Additional file 1: Figure S1. N-terminal 85-amino acid of *Ca*Cdc4 is required for normal growth of *C. albicans*. Strains: BWP17, heterozygous null mutant *CaCDC4* +/−, M3*CaCDC4* +/− carrying *CaMET3*-full-length *CaCDC4*, and M3NT*CaCDC4* +/− carrying *CaMET3*-partial *CaCDC4* (capable of expressing N-terminal 85-amino acid of truncated *Ca*Cdc4). Cells of the strains were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration. Cells were grown for 12 hrs in SD either with or without 2.5 mM Met/Cys (−Met/Cys or + Met/Cys) and at each 2-hr interval the cells were sampled to determine the optical density of 595 nm (O.D. 595) in which the growth curves could be plotted.

Additional file 2: Figure S2. N-terminal 85-amino acid of *Ca*Cdc4 is required for suppression of yeast-to-filament transition in *C. albicans*. Cells of the strains were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration. Cells were grown for 8 hrs in SD either with or without 2.5 mM Met/Cys (−Met/Cys or + Met/Cys). The images were visualized and recorded with a Nikon 50i microscope at 400× magnification. Bars represent 10 μm. The designations of strains are the same as in Additional file 1: Figure S1.

Abbreviations

SCF: Skp1-Cdc53/Cul1-F-box; *CaCDC4*: *Candida albicans CDC4*; *CaMET3*p: *C. albicans MET3* promoter; Dox: Doxycycline; *Ca*Cdc4: *C. albicans* Cdc4; *ScCDC4*: *S. cerevisiae CDC4*; *Sc*Cdc4: *S. cerevisiae* Cdc4; Met/Cys: Methionine and cysteine; YEPD: Yeast extract-peptone-dextrose; SD: Synthetic defined; *CaURA3*: *C. albicans URA3*; *CaADH1*: *C. albicans ADH1*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CC, WCL, JCS, TLL conceived and designed the experiments. CC, WCL, and TLT performed the experiments. CC, WCL, JCS, and TLT analyzed the data. WCL, TLL, and TLT contributed reagents and materials. JCS wrote the paper. All authors read and approved the final manuscript.

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ARTICLE

A role of *Candida albicans* CDC4 in the negative regulation of biofilm formation

Tzu-Ling Tseng, Wei-Chung Lai, Tai-Lin Lee, Wan Hua Hsu, Yu Wen Sun, Wan Chen Li, Chun-Wen Cheng, and Jia-Ching Shieh

Abstract: The *CDC4* gene is nonessential in *Candida albicans* and plays a role in suppressing filamentous growth, in contrast to its homologues, which are involved in the G1–S transition of the cell cycle. While characterizing the function of *C. albicans CDC4* (*CaCDC4*), we found that the loss of *CaCDC4* resulted in a reduction in cell flocculation, indicating a possible role for *CaCDC4* in biofilm formation. To elucidate the role of *CaCDC4* in biofilm formation, *Cacdc4* null mutant strains were constructed by using the mini-Ura-blaster method. To create a *CaCDC4* rescued strain, the plasmid p6HF-*ACT1*p-*CaCDC4* capable of constitutively expressing *CaCDC4* was introduced into the *Cacdc4* homozygous null mutant. To determine the biofilm formation ability, an in vitro XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium-5-carboxanilide) reduction assay was used. Compared with the parental auxotrophic strain BWP17, the *Cacdc4* homozygous null mutant was able to enhance biofilm formation significantly. This enhancement of biofilm formation in the *Cacdc4* homozygous null mutant could be reversed by constitutively expressing *CaCDC4*. We conclude that *CaCDC4* has a role in suppressing biofilm formation in *C. albicans*.

Key words: Candida albicans CDC4, XTT reduction assay, biofilm formation.

Résumé : Le gène *CDC4* est non essentiel chez *Candida albicans* et joue un rôle dans la suppression de la croissance filamenteuse, contrairement à ses homologues impliqués dans la transition G1–S du cycle cellulaire. Lors de la caractérisation de la fonction du *CDC4* de *C. albicans* (*CaCDC4*), nous avons constaté que la perte de *CaCDC4* entraînait une diminution de la floculation cellulaire, laissant entrevoir un rôle possible de *CaCDC4* dans la formation de biofilm. Afin de préciser le rôle de *CaCDC4* dans la formation de biofilm, on a créé des souches mutantes à délétion *Cacdc4* au moyen de la méthode mini-Ura-blaster. Pour obtenir une souche ayant récupéré le *CaCDC4*, on a introduit dans le mutant délété homozygote *Cacdc4* la plasmide p6HF-*ACT1*p-*CaCDC4* conférant l'expression constitutive de *CaCDC4*. On a eu recours à une analyse de réduction du XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium-5-carboxanilide) afin de mesurer la capacité de formation de biofilm. Le mutant délété homozygote *Cacdc4* a démontré une formation de biofilm significativement supérieure comparativement à la souche parentale BWP17. Cette intensification de la formation de biofilm chez le mutant délété homozygote *Cacdc4* a su être annulée par l'expression constitutive de *CaCDC4*. Nous déduisons que *CaCDC4* joue un rôle dans la suppression de la formation de biofilm chez *C. albicans*. [Traduit par la Rédaction]

Mots-clés : Candida albicans CDC4, analyse de réduction du XTT, formation de biofilm.

Introduction

The opportunistic human fungal pathogen *Candida albicans* is a natural diploid that lacks a complete sexual cycle. Because of its unique biological features and its ability to cause human diseases, ranging from superficial mucosal infections to life-threatening systemic disorders, substantial research has focused on the molecular mechanisms that control *C. albicans*. The morphological transition has been of particular interest because it is thought to be connected to virulence and pathogenesis, although morphogenesis and virulence can be separated (Noble et al. 2010). At least 3 positive and 5 negative pathways in *C. albicans* interact with environmental cues to control morphogenesis (Biswas et al. 2007; Whiteway and Bachewich 2007). Many cyclin-dependent kinases and their regulators also dictate the morphological transition in *C. albicans* (Berman 2006). These genes and environmental factors are intimately intertwined to modulate morphogenesis, making the complete elucidation of morphogenesis difficult.

In addition to flocculation and adhesion (Verstrepen and Klis 2006), the ability of yeast, hyphae, and pseudohyphae cells to undergo morphological alterations is essential for the formation of biofilms, which are surface-attached microbial communities (Cos et al. 2010). Biofilms are enclosed in a matrix of exopolymeric substances, a distinction that confers survival advantages over planktonic forms (Flemming and Wingender 2010). *Candida* biofilms are highly prevalent on catheter surfaces (Douglas 2003) and are resistant to antifungal drugs (Sanglard and Odds 2002), which can lead to superficial (Jayatilake and Samaranayake 2010) and systemic candidiasis (van de Veerdonk et al. 2010). A variety of

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

Strain	Parental strain	Genotype
BWP17		*ura3*::*imm434*/*ura3*::*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*
CaCDC4 +/–::*CaURA3*	BWP17	*CaCDC4*/*Cacdc4*::*URA3-dpl200*
CaCDC4 +/–	*CaCDC4* +/–::*CaURA3*	*CaCDC4*/*Cacdc4*::*dpl200*
CaCDC4 –/–::*CaURA3*	*CaCDC4* +/–	*Cacdc4*::*URA3-dpl200*/*Cacdc4*::*dpl200*
CaCDC4 –/–	*CaCDC4* –/–::*CaURA3*	*Cacdc4*::*dpl200*/*Cacdc4*::*dpl200*
CaCDC4 –/–::p6HF	*CaCDC4* –/–	*RPS1*/*RPS1*::p6*HF*
CaCDC4 –/–::*CaCDC4*	*CaCDC4* –/–	*RPS1*/*RPS1*::p6*HF-CaCDC4*
SC5314		Wild type
CaCDC4 +/S	SC5314	*CaCDC4*/*Cacdc4*::*SAT1-FRT*
CaCDC4 +/F	*CaCDC4* +/S	*CaCDC4*/*Cacdc4*::*FRT*
CaCDC4 S/F	*CaCDC4* +/F	*Cacdc4*::*SAT1-FRT*/*Cacdc4*::*FRT*
CaCDC4 F/F	*CaCDC4* S/F	*Cacdc4*::*FRT*/*Cacdc4*::*FRT*

genes, including those encoding transcription factors, cell-wall-related proteins, protein kinases, and drug efflux pumps, are required for biofilm formation in *C. albicans* because of the consecutive stages of adherence, initiation, maturation, and dispersal that occur during biofilm development (Finkel and Mitchell 2011). The mechanisms by which these genes and their encoded proteins are intertwined to modulate biofilm development remain unclear.

We have used comparative genomics of the budding yeast *Saccharomyces cerevisiae* and *C. albicans* to elucidate the function of *C. albicans* genes, particularly those controlling the G1-to-S phase transition of the cell division cycle, which is the determinant of differentiation (Merlini et al. 2013), and those coordinating the diverse morphological states of *S. cerevisiae* (Howell and Lew 2012). By analogy, cell cycle genes involved in the G1-to-S phase transition could be involved in morphogenesis in *C. albicans*. We and others have found that *C. albicans* gene *CDC4* (*CaCDC4*), which encodes the F-box protein that determines the substrate specificity of the Skp1–cullin–F-box (SCF) ubiquitin E3 ligase complex, negatively modulates the yeast-to-filament transition in *C. albicans* (Atir-Lande et al. 2005; Shieh et al. 2005), as opposed to the G1-to-S phase transition of the cell cycle, as in the case of *S. cerevisiae* and other eukaryotes (Reed 2003). This functional discrepancy between *CaCDC4* and its homologues cannot be fully explained by the identification of the *C. albicans* homologue of *S. cerevisiae SIC1*, termed *SOL1*, whose protein product is a target of *Ca*Cdc4 (Atir-Lande et al. 2005). This is because the hyperfilamentation phenotype of the *Cacdc4* –/– and *sol1* –/– double mutant is similar to that of the *Cacdc4* –/– single mutant (Atir-Lande et al. 2005). In addition, affinity purification of *Ca*Cdc4 identified 2 associated proteins encoded by *GPH1* and *THR1* (Tseng et al. 2010), the glycogen phosphorylase required for the conversion of glycogen to glucose and the homoserine kinase for threonine biosynthesis, respectively (Murad et al. 2001; Kingsbury and McCusker 2010*a*, 2010*b*). Gcn4-dependent, hence TOR-pathway-related, expression of *THR1* (Tournu et al. 2005) and HOG-pathway-dependent expression of *GPH1* (Enjalbert et al. 2006) have been revealed by transcriptional profiling in *C. albicans*. This revelation together with the recent finding that reduced TOR signaling decreases Hog1 basal activity in *C. albicans* (Su et al. 2013) and that *C. albicans* mutants lacking *GCN4* are sensitive to hydrogen peroxide stress (Sundaram and Grant 2014) suggest a possible role of *GPH1* and *THR1* for cellular survival under nutrient limitation and stress. However, the precise mechanism by which *CaCDC4*, *GPH1*, and *THR1* are involved in morphogenesis and stress remains to be elucidated. In addition, to confirm that *CaCDC4* functions as a characteristic F-box protein of the SCF ubiquitin E3 ligase in *C. albicans*, we dissected the *CaCDC4* domains associated with filamentation and found that both the F-box and WD40-repeat of *CaCDC4* are essential domains that are involved in morphogenesis and cell flocculation and that the N-terminal 85-amino acid region is important in their regulation (Chin et al. 2013), implying a role for *CaCDC4* in biofilm formation.

In this report, we characterized *CaCDC4* with respect to biofilm development. By using *Cacdc4* homozygous null mutants constructed either with or without constitutively expressing *CaCDC4*, we were able to analyze the ability of the cells to form biofilms with a colorimetric 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium-5-carboxanilide (XTT) reduction assay. We found that cells lacking *CaCDC4* could advance biofilm formation, indicating that *C. albicans CDC4* plays a novel role in negative regulation of biofilm formation.

Materials and methods

Strains and growth conditions

The *C. albicans* strains used in this study were the wild-type strain SC5314 (Gillum et al. 1984), the auxotrophic strain BWP17 (*ura3*/*ura3 his1*/*his1 arg4*/*arg4*) (Wilson et al. 1999), and the 2 *Cacdc4* homozygous null mutants of BWP17 described in the next section. The strains were routinely inoculated into fresh YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose) from overnight cultures and grown at 30 °C on a shaker at 200 r/min. Uridine prototrophy (Ura^+) was selected for on synthetic complete plates that lacked uridine and uracil (6.7 g yeast nitrogen base without amino acid, 1.92 g yeast synthetic drop-out media supplement without uracil, 2% agar, and 100 mL of 20% glucose per litre). Uridine auxotrophy (Ura^-) was assessed on synthetic defined (SD) plates containing 80 μg/mL arginine, 80 μg/mL histidine, 50 μg/mL uridine, and 1 mg/mL 5-fluoroorotic acid (5-FOA). Nourseothricin-resistant (Nou^+) *C. albicans* cells were selected for on YEPD plates containing 200 μg/mL nourseothricin. To select for *SAT1* pop-out cells (Nou^-), cells of the Nou^+ strain were grown for 5 days in YCB–BSA medium containing 23.4 g/L yeast carbon base and 4 g/L bovine serum albumin.

Strain construction

The nucleotide sequence for *C. albicans* was obtained from the *Candida* Genome Database (http://www.candidagenome.org/). *CDC4* was deleted in the *C. albicans* auxotrophic strain BWP17 with the mini-Ura-blaster cassette *dpl200-CaURA3-dpl200*, derived from pDDB57 (Wilson et al. 2000). Briefly, the Ura-blaster cassette flanked by 60 bp of upstream and downstream homology with the *CaCDC4* open reading frame (CR_01680C_A, 2307 nucleotides in length) was amplified by PCR with the primers CaCDC4_URA3_F and CaCDC4_URA3_R (see Table 2). The Ura-blaster cassette flanked by the short homology regions of *CaCDC4* was then transformed into BWP17 cells using the LiAc/PEG/ssDNA method (Gietz and Woods 2006) to produce the strain containing the heterozygous deletion of *CaCDC4*. The Ura^+ transformants were then treated with 1 mg/mL 5-FOA to generate *CaCDC4* +/– (Table 1) that made the *CaURA3*

Table 2. Primers used in this study.

Name of the primer	Sequence[a]
CaCDC4_URA3_F	5′-atggataagaaatcaaagctattcaaatatcctttgagcgaggagacggctaaatttgag**GTTTTCCCAGTCACGACGTT**-3′
CaCDC4_URA3_R	5′-tcactgtaaaagtggttgactgaaatctagaatctcaataaacgtttcaccttcatcttc**TGTGGAATTGTGAGCGGATA**-3′
CaCDC4-N-XhoI-F	5′-GAA*CTCGAG*atggataagaaatcaaag-3′
CaCDC4-N-XhoI-R	5′-GAA*CTCGAG*ctgtaaaagtggttgact-3′
CaCDC4-UR-KpnI-F	5′-CGG*GGTACC*gtatagtcggcaacaaaggt-3′
CaCDC4-UR-XhoI-R	5′-CCG*CTCGAG*atgagctgtgatgagacttg-3′
CaCDC4-DR-SacII-F	5′-TCC*CCGCGG*cgttaaggatttgggatgcta-3′
CaCDC4-DR-SacI-R	5′-CTAG*GAGCTC*agaactaatttagtactcactg-3′
CaCDC4_Probe_F[b]	5′-GGTTTCCAACACTTTCCCAG-3′
CaCDC4_Probe_R[b]	5′-CACTACTAGTTGGTTGCTGT-3′
CaCDC4-detect-F	5′-caagtctcatcaacagctcatttg-3′
CaCDC4-detect-R	5′-gattgagtagtaggttcattgctc-3′

[a]The lowercase regions correspond to the coding regions of the *CaCDC4* gene. The regions with italic uppercase letters are restriction enzyme sites. The regions with bold-typed uppercase letters correspond to the coding regions of the *Candida albicans URA3* gene.
[b]These are upstream of the *CaCDC4* coding region between –1573 and –1065 bp.

marker available for constructing the homozygous null mutant of *CaCDC4*. The second *CaCDC4* allele was deleted as described above. The homozygous null mutants of *CaCDC4* (either *CaCDC4* –/–::*CaURA3* or *CaCDC4* –/–) with or without *CaURA3* (Table 1) the mini-Ura-blaster cassette were confirmed by yeast colony PCR and Southern blotting analysis. The *CaCDC4* rescued strain was made using the *CaCDC4* –/– as a parental strain. The protein coding region of *CaCDC4* was cloned from plasmid pCR2.1-TOPO-*CaCDC4* (T.L. Tseng, unpublished results) with the primer pair CaCDC4-N-XhoI-F and CaCDC4-N-XhoI-R (Table 2) into plasmid p6HF-*ACT1*p (Kaneko et al. 2004) to generate p6HF-*ACT1*p-*CaCDC4*, capable of constitutively expressing *Ca*Cdc4 tagged C-terminally with epitope of 6× His-FLAG in *C. albicans* cells. This along with the empty plasmid p6HF-*ACT1*p was linearized with *Nco*I and introduced into cells of strain *CaCDC4* –/– and selected for Ura$^+$ transformants to generate *CaCDC4* –/–::*CaCDC4* and *CaCDC4* –/–::p6HF (Table 1), respectively.

Moreover, *CDC4* was deleted in the *C. albicans* wild-type strain SC5314 with the *SAT1*-flipper method (Reuss et al. 2004). Briefly, both the upstream and downstream regions of *CaCDC4* were amplified with primer pairs CaCDC4-UR-KpnI-F/CaCDC4-UR-XhoI-R and CaCDC4-DR-SacII-F/CaCDC4-DR-SacI-R, respectively (Table 2), and with template DNA of the genomic DNA extracted from SC5314. These were sequentially cloned into plasmid pSF1A at sites of *Kpn*I/*Xho*I, and *Sac*II/*Sac*I to generate plasmid pSF1A-*CaCDC4*-k/o. A cassette released from pSF1A-*CaCDC4*-k/o by using *Kpn*I/*Sac*II was introduced into SC5314 by electroporation (Walther and Wendland 2003) and selected for Nou$^+$ to generate the *Cacdc4* single-deletion mutant *CaCDC4* +/S. The *SAT1*-popped out strain *CaCDC4* +/F was obtained by induction in YCB–BSA. To generate *Cacdc4* homozygous null mutant *CaCDC4* S/F, the cassette pSF1A-*CaCDC4*-k/o was introduced into *CaCDC4* +/F and selected for Nou$^+$. The *SAT1*-popped out strain *CaCDC4* F/F was obtained by induction in YCB–BSA.

Isolation of genomic DNA and Southern blotting

Genomic DNA from the *C. albicans* strains was isolated by the MasterPure™ Yeast DNA Purification kit (Epicentre). Southern blotting was performed according to standard protocols with the aid of the Rapid Downward Transfer System (TURBOBLOTTER™) using 10 μg of the restriction enzyme-digested genomic DNA. The DNA on the blot was hybridized with a probe amplified by the PCR DIG Probe Synthesis kit (Roche) with the primers CaCDC4_Probe_F and CaCDC4_Probe_R (Table 2) using DIG Easy Hyb (Roche). To reveal the gene deletion, the DIG Luminescent Detection kit (Roche) was used after hybridization, and the blot was exposed to X-ray film for up to 24 h as appropriate.

RT–PCR analysis

Cells were grown to mid-log phase and subjected to extraction of total RNA by using the MasterPure™ Yeast RNA Purification kit (Epicentre) according to the manufacturer's instruction. Five micrograms of total RNA was used to convert into cDNA by using SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instruction. The cDNA was then subjected to PCR with the *CaCDC4*-specific primers CaCDC4-detect-F and CaCDC4-detect-R (Table 2).

Western blot analysis

The total protein was extracted from cultured cells as previously described (Shieh et al. 2007) and was resolved by 10% SDS–PAGE. Proteins were transferred to PVDF membranes (PerkinElmer, Boston, Massachusetts, USA) and probed with anti-FLAG polyclonal antibody (Sigma) in 1:2000 dilution and visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Proteins detected were recorded with the Luminescent Image Analyzer (Fujifilm LAS-1000) and analyzed by ImageGauge 3.46 and L Process version 1.96 (Fujifilm).

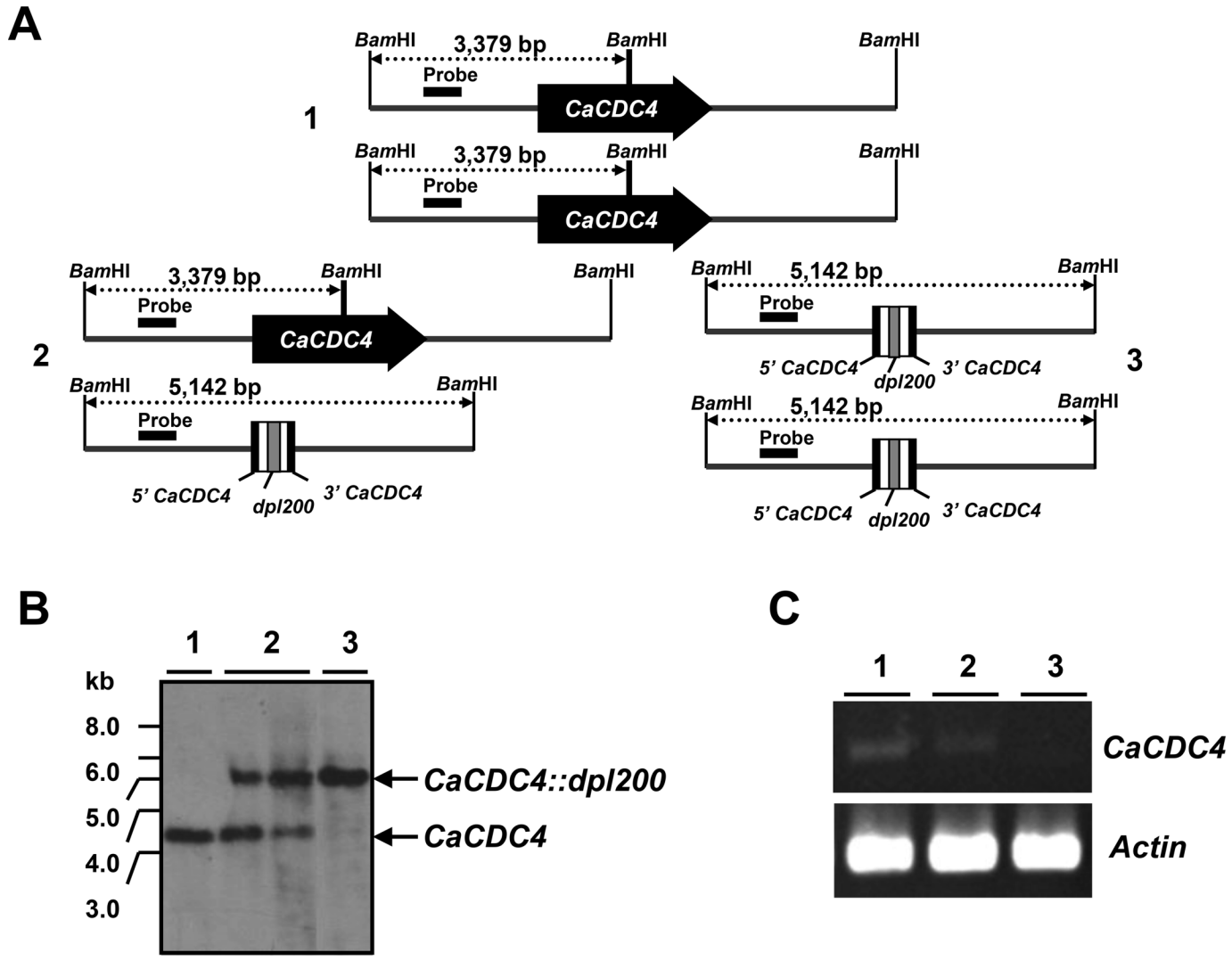
Growth rate determination

To accurately assess the growth rate of strains, the alamarBlue® (Invitrogen) test was adopted to exclude the discrepancy caused by using optical density as a determinant, since strains compared were in different morphological states. The doubling times of the *C. albicans* strains were determined from their growth in YEPD medium at 30 °C on a shaker at 200 r/min. At 2 h intervals, an aliquot was removed and frozen at –80 °C with glycerol. The frozen cells were recovered on ice, pelleted by centrifugation at 12 000*g*, washed twice with 1× PBS, and resuspended in an aliquot of 1× PBS equal in volume to the original aliquot. From each cell suspension, 200 μL was transferred into a well of a sterilized, nonpyrogenic polystyrene, flat-bottom 96-well microtiter plate (Costar), followed by the addition of 20 μL of alamarBlue® cell viability reagent (1/10 volume of cell suspension) (Invitrogen) to each well; 200 μL of 1× PBS was used as a negative control. The plate was incubated in the dark at 37 °C for 1–4 h. The subsequent solution color change was measured spectrophotometrically (Molecular Devices/Spec384) at 570 nm.

Biofilm formation assay

Biofilm formation was assessed for the different strains on sterilized, nonpyrogenic polystyrene, flat-bottom 96-well microtiter plates (Costar). Individual colonies from fresh plates were separately inoculated into 5 mL of YEPD medium and incubated at 30 °C overnight. The overnight cultures were refreshed by subculturing in 5 mL of fresh YEPD medium with an OD_{600} adjusted to 1 and incubated at 30 °C for 2 h. To standardize the cell number among the different strains with or without filamentous cells, after 2 h of incubation 1–2 mL of cell suspension containing 20% glycerol was frozen at –80 °C, and the concentration of the frozen cells was determined from the number of colony-forming units. The frozen cells with known concentrations were recovered

Fig. 1. Construction of the *Cacdc4* homozygous null mutant using the mini-Ura-blaster method. (A) Organization of the *CaCDC4* locus with respect to *Bam*HI sites is shown. The first allele of the *CaCDC4* open reading frame in BWP17 was replaced by the Ura-blaster cassette followed by popping out the *CaURA3* with 5-FOA (5-fluoroorotic acid) in which only 1 of 2 copies of *dpl200* remained to generate strain *CaCDC4* +/–. The second allele of *CaCDC4* was replaced by the Ura-blaster cassette followed by excision of *CaURA3* with 5-FOA to generate strain *CaCDC4* –/–. The relative positions of the probe used and the predicted *Bam*HI-digested pattern of the *CaCDC4* locus in different strains are indicated. (B) Southern blotting analysis. Using the specific probe shown in panel A, a *Bam*HI fragment of 3379 bp, specific to *CaCDC4*, could be detected in genomic DNA from BWP17 digested with *Bam*HI; 2 *Bam*HI fragments of 3379 bp and 5142 bp, specific to *CaCDC4* and *Cacdc4*::*dpl200*, respectively, could be detected in *CaCDC4* +/–; and a *Bam*HI fragment of 5142 bp specific to *Cacdc4*::*dpl200* could be detected in *CaCDC4*–/–. (C) RT–PCR analysis. Cells of strains BWP17, *CaCDC4* +/–, and *CaCDC4* –/– were grown to exponential phase and subjected to RNA extraction, reverse-transcription as described in the Materials and methods. The Arabic numerals 1, 2, and 3 denote strains BWP17, *CaCDC4* +/–, and *CaCDC4* –/–, respectively.



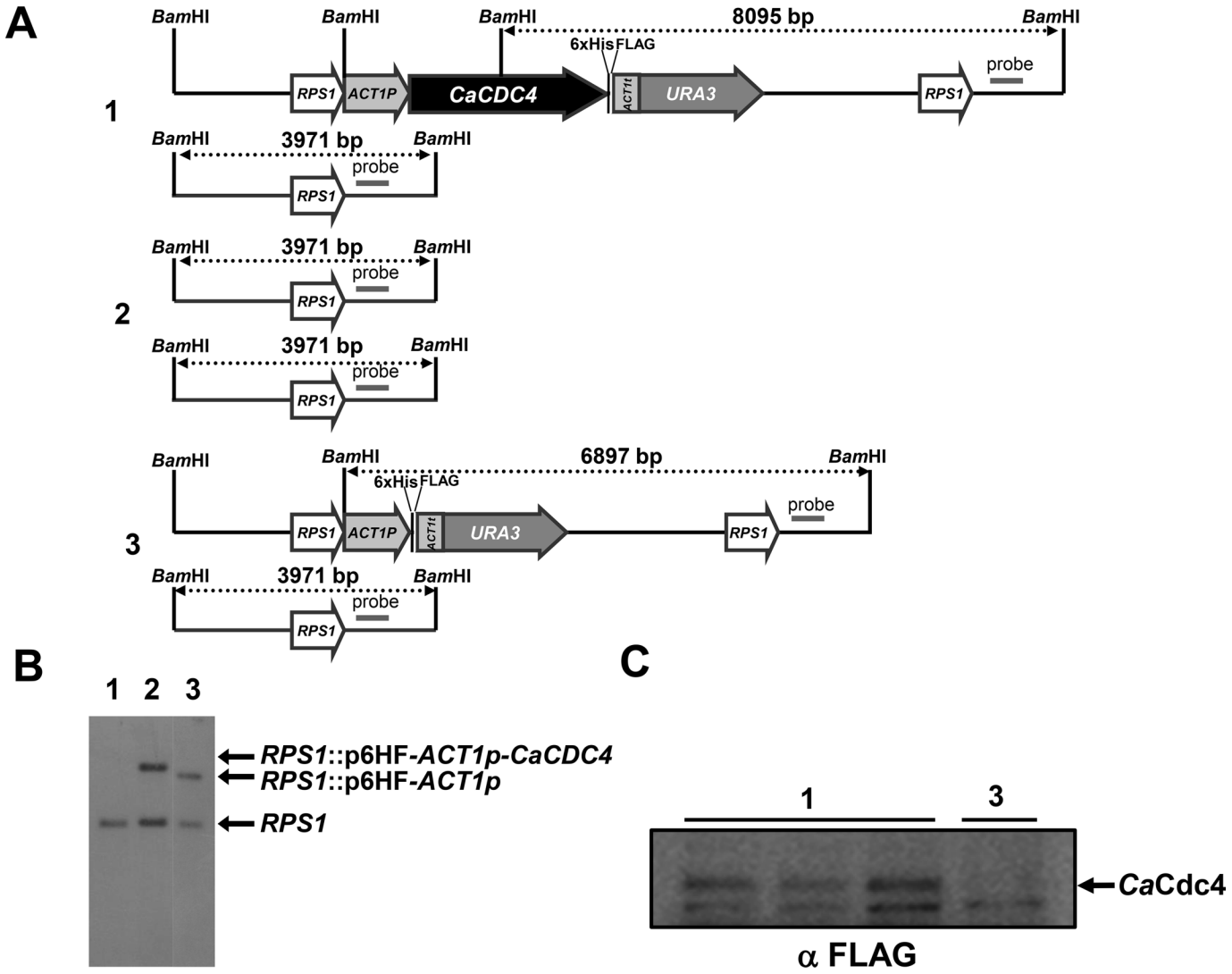
on ice and pelleted by centrifugation at 12 000*g*. Each cell pellet was washed twice with 1× PBS, and the concentration was adjusted to 1 × 10^6 cells/mL for a standardized cell suspension. To assess the abilities of the different strains to metabolize XTT (Sigma), 200 μL of a standardized cell suspension from each strain was subjected directly to the XTT reduction assay, as described in the next section.

To evaluate the abilities of the different strains to form biofilms, the cells need to induce biofilm formation before performing XTT reduction assay. Briefly, 200 μL of a standardized cell suspension from each strain was transferred into the wells of a microtiter plate and incubated at 37 °C with shaking at 100 r/min for 1 h to allow the cells to adhere to the surface of the wells. Following adherence, the supernatants were aspirated, and each well was washed twice with 200 μL of 1× PBS to remove loosely adherent cells. A 200 μL aliquot of YEPD medium was transferred into each of the wells, and the plate was incubated at 37 °C with shaking at 100 r/min for 48 h to allow filamentous growth (Kadosh and Johnson 2005) that triggers the biofilms to develop (Finkel and Mitchell 2011). Finally, the biofilm cells were quantified by the XTT reduction assay, as described below.

XTT reduction assay

Prior to the XTT reduction assay, stock solutions of 1 mg/mL XTT (Sigma) in 1× PBS and 5 mmol/L menadione (Sigma) in acetone were prepared and stored at –20 °C and –80 °C, respectively. Before each assay, the 1 mg/mL XTT stock solution was prewarmed in a 37 °C water bath until completely solubilized, and the XTT–menadione solution was immediately prepared at a ratio of 499:1 by volume from the stocks and filtered through a 0.2 μm mem-

Fig. 2. Construction of the *CaCDC4* rescued strains. (A) Organization of the *RPS1* locus with respect to *Bam*HI sites with Southern blotting analysis. The *Nco*I linearized plasmid of either the p6HF-*ACT1*p-*CaCDC4* or empty vector p6HF-*ACT1*p was introduced into the strain *CaCDC4* –/– at the *RPS1* locus to generate *CaCDC4* –/–::*CaCDC4* and *CaCDC4* –/–::p6HF (Table 1), respectively. (B) Southern blotting analysis. The corresponding size alterations in 1 allele of the *RPS1* locus are shown. (C) Western blot analysis. Three isolates of strain *CaCDC4* –/–::*CaCDC4* and 1 isolate of strain *CaCDC4* –/–::p6HF were used. Cells were grown to exponential phase and subjected to protein extraction for western blot analysis. Anti-FLAG antibody that recognizes the FLAG-tagged *Ca*Cdc4, indicated by arrow, was used. The anti-FLAG antibody appeared to cross-react with a protein smaller in the size than *Ca*Cdc4. The Arabic numerals 1, 2, and 3 denote strains *CaCDC4* –/–::*CaCDC4*, *CaCDC4* –/–, and *CaCDC4* –/–::p6HF, respectively.



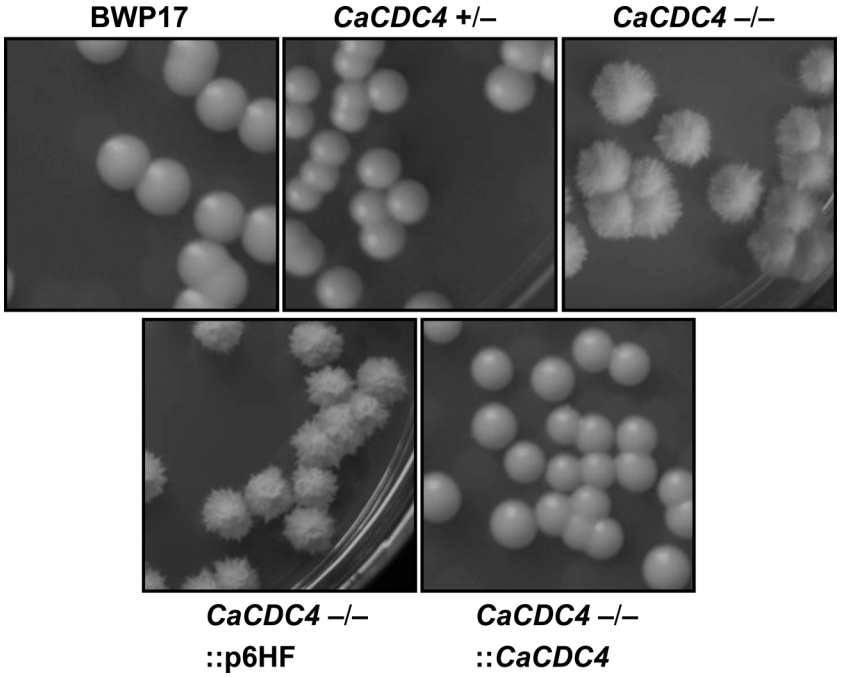
brane (Acrodisc). The biofilm cells were washed twice with 200 μL of 1× PBS, and 200 μL of XTT–menadione solution was then added to each well, including the empty wells used as the negative controls. The plate was incubated in the dark at 37 °C for 1 h. After the incubation, 100 μL aliquots were transferred to new wells, and the change in the color of the solution was measured in a spectrophotometer (Molecular Devices/Spec384) at 490 nm. To measure the XTT metabolism rates of the different strains, 200 μL of a standardized cell suspension of each strain was pelleted by centrifugation at 12 000*g* and washed 2 times with 1× PBS, and the absorbance value was then measured by the same method described above.

Results and discussion

Construction of *Cacdc4* null mutants and *CaCDC4* rescued strains

The previous construction of a *CaCDC4*-repressible strain demonstrated that *CaCDC4* is a nonessential gene (Shieh et al. 2005). We therefore constructed *C. albicans* strains containing deletions of the 2 alleles of *CaCDC4* to determine the role of *CaCDC4* in biofilm formation. The first allele of *CaCDC4* was deleted in the auxotrophic *C. albicans* BWP17 with the mini-Ura-blaster (Wilson et al. 2000) to generate the *Cacdc4* single-deletion mutant strain *CaCDC4* +/–::*CaURA3* (Table 1). To permit deletion of the second allele of *CaCDC4*, 5-FOA was used as a counter-selection agent to remove *CaURA3* from the mini-Ura-blaster cassette of the single-deletion mutant *CaCDC4* +/– (Table 1). The second *CaCDC4* allele was then deleted to obtain the *Cacdc4* homozygous null mutant *CaCDC4* –/–::*CaURA3* (Table 1). The *CaURA3* in the mini-Ura-blaster cassette of the *CaCDC4* –/–::*CaURA3* was further removed by treatment with 5-FOA to obtain the *Cacdc4* homozygous null mutant *CaCDC4* –/–. Prior to Southern blotting analysis, several isolates of each of the constructed strains were initially verified by yeast colony PCR with primers that could distinguish the genomic structure of the deleted *CaCDC4* (data not shown). Southern blotting

Fig. 3. Colony morphologies of the *Candida albicans* strains. Cells of different strains, including BWP17, *CaCDC4* +/–, *CaCDC4* –/–, *CaCDC4* –/–::*CaCDC4*, and *CaCDC4* –/–::p6HF (Table 1), were initially plated on YEPD semisolid agar plates to form individual colonies. A single colony of each strain was then streaked out on a fresh YEPD plate to form colonies. Images of representative colonies from each strain were photographed.



analysis was performed on BWP17 and the *Cacdc4* heterozygous and homozygous null mutants *CaCDC4* +/– and *CaCDC4* –/–, respectively (Fig. 1A). While the probe hybridized to a 3379 bp fragment of the *Bam*HI-digested genomic DNA from BWP17, the probe hybridized to a 5142 bp fragment of the genomic DNA from either the *CaCDC4* +/– or the *CaCDC4* –/– due to presence of *dpl200* within the *CaCDC4* locus by sequential insertion of the mini-Ura-blaster cassette and treatment with 5-FOA (Fig. 1B). To further check if the null mutant cells indeed lost the ability to express *CaCDC4*, RT–PCR, where *CaCDC4* mRNA was reverse transcribed, followed by PCR was used to determine the relative amount of *CaCDC4*. As expected, the expression of the *CaCDC4* transcript was completely lost in the *Cacdc4* homozygous null mutant *CaCDC4* –/– (Fig. 1C). These results confirmed that the strains were constructed correctly. To rescue loss of *CaCDC4* in the *Cacdc4* homozygous null mutant *CaCDC4* –/–, a plasmid cassette p6HF-*ACT1*p-*CaCDC4* (Table 1) was introduced into cells of *CaCDC4* –/– at the *RPS1* locus to generate *CaCDC4* –/–::*CaCDC4* in which the C-terminal FLAG-tagged *CaCDC4* was constitutively expressed. The empty plasmid p6HF-*ACT1*p (Table 1) was also introduced to generate *CaCDC4* –/–::p6HF, which was used as a control. Southern blotting analysis was performed on the strains. Whereas the probe hybridized to a 3971 bp fragment at the *RPS1* locus of the *Bam*HI-digested genomic DNA from BWP17, it hybridized, as expected, to a 8095 bp and a 6897 bp fragment at the *RPS1* loci of *CaCDC4* –/–::*CaCDC4* and *CaCDC4* –/–::p6HF, respectively (Figs. 2A and 2B). The ability of *CaCDC4* –/–::*CaCDC4* but not *CaCDC4* –/–::p6HF to express *Ca*Cdc4 protein was confirmed by Western blotting analysis (Fig. 2C).

Cacdc4 homozygous null mutants exhibit filamentous growth

The constructed strains exhibited the expected morphological alterations relative to the wild-type strains. The *Cacdc4* single-deletion mutant strain *CaCDC4* +/– formed colonies with a circular shape and smooth surface on YEPD that were indistinguishable from those of the auxotrophic strain BWP17 (Fig. 3). In contrast, the *Cacdc4* homozygous null mutant *CaCDC4* –/– formed colonies that exhibited an irregular shape with filaments (Fig. 3), similar to the previously reported *CaCDC4* repressed (Shieh et al. 2005) and deleted strains. The *CaCDC4* rescued strain *CaCDC4* –/–::*CaCDC4* formed colonies that were essentially the same as those of the auxotrophic strain BWP17 (Fig. 3) but the strain *CaCDC4* –/–::p6HF, where *CaCDC4* –/– carried an empty plasmid p6HF-*ACT1*p, formed colonies of irregular shape with filaments. These results indicate that the constructed strains exhibited expected morphological changes and that *CaCDC4* is indeed required for cells to suppress filamentous growth.

The ability to proliferate was comparable among the constructed *Cacdc4* mutants and the parental strain

The repression of *CaCDC4* expression (Chin et al. 2013) or the lack of *CaCDC4* (T.L. Tseng and J.C. Shieh, unpublished results) in *C. albicans* enhances cell flocculation, a cell–cell interaction event associated with quorum sensing (Nobile and Mitchell 2006) that governs both the aggregation and dispersal of biofilm cells. To understand the role of *CaCDC4* in biofilm formation, the *Cacdc4* homozygous null mutant strains were compared with their parental auxotrophic strain, BWP17. A soluble tetrazolium/formazan of the XTT reduction assay (Kuhn et al. 2003) was used to quantify biofilm formation among the different *C. albicans* strains, as it has been shown that the formazan signal relates agreeably with cell number (Hawser 1996) and the signal increases when a biofilm develops (Lal et al. 2010). Importantly, the XTT assay appears to provide the most reproducible, accurate, and efficient method, as compared with crystal violet staining, DNA quantification, qPCR, protein quantification, dry cell weight measurement, and viable colony counting (Taff et al. 2012).

Before using the XTT reduction assay with biofilm cells, we first assessed whether the growth rates and the reduction of XTT by the mitochondrial dehydrogenases of metabolically active cells were different among the strains (Kuhn et al. 2003). To determine the growth rates of the different strains, the alamarBlue cell viability assay was used to quantitatively measure the doubling time for cellular proliferation, as it has been used to quantify in vitro viability of various cells (Rampersad 2012) including *C. albicans* (Le Lay et al. 2008). By this method, the doubling time of BWP17

Fig. 4. Determination of the biofilm formation abilities of the *Candida albicans* strains based on auxotrophic strain BWP17. Cells from each strain were used to form biofilms, and the number of biofilm cells was assessed with the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium-5-carboxanilide) reduction assay. The values were normalized by the growth rate and the XTT reduction capability of each of the strains. The biofilm formation ability of each strain was expressed as the fold difference in XTT reduction relative to that of BWP17. ***, indicates a significant difference ($P < 0.001$) between the test strain and BWP17. Representative data from 1 of the 3 independent experiments are shown.

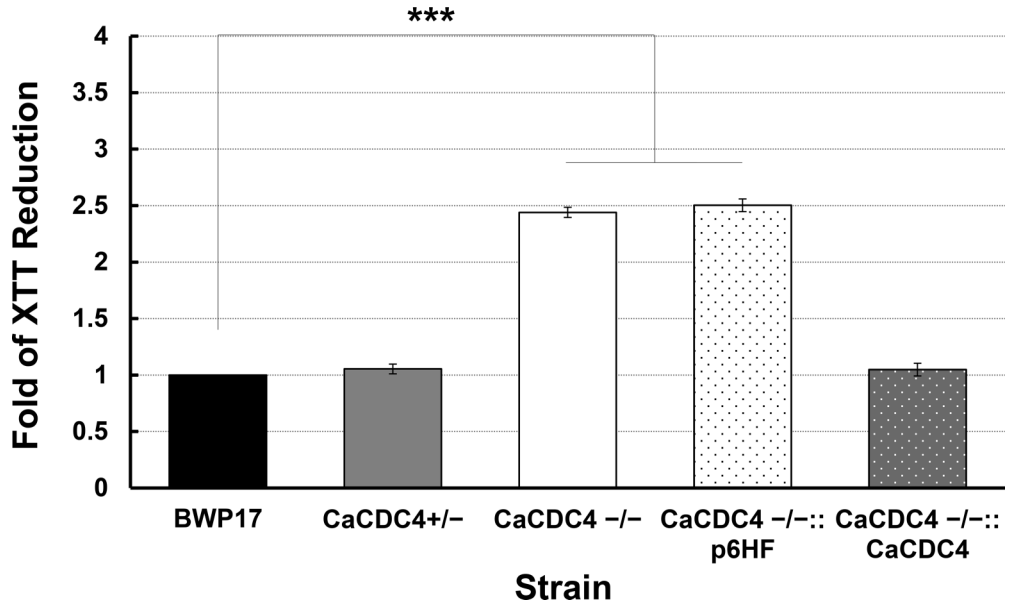


Table 3. Growth rate.

Strain	Doubling time[a] (h)
BWP17	1.812±0.183
CaCDC4 +/–	1.732±0.011
CaCDC4 –/–	1.729±0.054
CaCDC4 –/–::p6HF	1.719±0.080
CaCDC4 –/–::*CaCDC4*	1.746±0.023

[a]Mean of 3 independent experiments with standard deviation.

Table 4. Capability of XTT metabolism.

Strain	OD_{490}[a]
BWP17	0.335±0.011
CaCDC4 +/–	0.337±0.015
CaCDC4 –/–	0.276±0.010
CaCDC4 –/–::p6HF	0.278±0.012
CaCDC4 –/–::*CaCDC4*	0.339±0.011

[a]Mean of 3 independent experiments with standard deviation.

cells during the exponential stages of growth appeared to be similar to that of the *Cacdc4* null mutants and the rescued strains (Table 3). Although the difference was approximately 5% between BWP17 and the rest of strains, the effect was minimal.

CaCDC4 inhibits biofilm development

To assess XTT reduction, the *Cacdc4* null mutants, the auxotrophic parental strain BWP17, and the rescued strains were grown to the exponential phase. The number of colony-forming units was determined, and an equal number of cells were subjected to the XTT reduction assay, as described in the Materials and methods. The ability to reduce XTT varied among the different strains (Table 4). The XTT reduction ability was nearly identical among BWP17, the *Cacdc4* single-deletion mutant *CaCDC4* +/–, and the *CaCDC4* rescued strain *CaCDC4* –/–::*CaCDC4*. However, the XTT reduction ability of the *Cacdc4* homozygous null mutant and the strain *CaCDC4* –/–::p6HF, where *CaCDC4* –/– carried an empty plasmid p6HF-*ACT1*p, was 80% that of the parental strain BWP17, indicating that the loss of *CaCDC4* impairs the function of mitochondrial dehydrogenases.

To evaluate the abilities of the different strains to form biofilms, cells from each strain were used to establish biofilms on nonpyrogenic polystyrene, and the XTT reduction abilities of the biofilm cells were determined. The XTT reduction abilities of the biofilm cells from the test strains were divided by the growth rates and the differential XTT reduction abilities of the strains to obtain the final biofilm formation abilities. The final data were expressed as the fold difference in biofilm formation relative to that of BWP17. Importantly, the loss of *CaCDC4* in the homozygous null mutant *CaCDC4* –/– and the strain *CaCDC4* –/–::p6HF appeared to enhance biofilm development significantly, up to 2.6-fold relative to BWP17, the *Cacdc4* single-deletion mutant *CaCDC4* +/–, and the *CaCDC4* rescued strain *CaCDC4* –/–::*CaCDC4* (Fig. 4). These results strongly suggest that *CaCDC4* has a role in negatively regulating biofilm development. Similar results from homozygous null mutant (see Supplementary Figs. 1 and 2[1]) based on the wild-type strain SC5314 that was created by the use of *SAT1*-flipper approach (Reuss et al. 2004) have also indicated a 5-fold increase of biofilm formation in cells lacking *CaCDC4* compared with those carrying *CaCDC4* (Tseng et al. 2014). While filamentation is known to be associated with biofilm formation (Baillie and Douglas 1999; Ramage et al. 2002*a*, 2002*b*; Garcia-Sanchez et al. 2004; Cao et al. 2005), decoupling of the 2 has also been found in at least one case where cells with loss of *BCR1* gene function were unable to form biofilm were still able form filaments normally (Nobile and Mitchell 2005).

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2014-0526.

Conclusion

Our results clearly demonstrated that *CaCDC4* has a role in negatively regulating biofilm formation, which is associated with filamentation. Further studies to understand the mechanism underlying *CaCDC4*-dependent filamentation and biofilm formation may provide a new way of therapeutic intervention in *C. albicans* infection.

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Candida albicans DBF4 gene inducibly duplicated by the mini-Ura-blaster is involved in hypha-suppression

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ABSTRACT

The opportunistic human fungal pathogen *Candida albicans* is a natural diploid that does not have a complete sexual cycle. The ability to switch between diverse cellular forms is important to its virulence. Here, we describe the characterization of the *C. albicans DBF4* gene, a *Saccharomyces cerevisiae* homolog that encodes a regulatory subunit of Cdc7 kinase that is known to initiate DNA replication. We made a *C. albicans* strain, with one *DBF4* allele deleted by the mini-Ura-blaster and the other controlled by a repressible promoter. We also found a third *CaDBF4* copy that was later verified to be inducibly duplicated by targeted recombination with the min-Ura-blaster. Surprisingly, the strain deleted with the third *CaDBF4* copy exhibited hyphal growth under repressed conditions. We conclude that the *CaDBF4* gene is prone to being duplicated by the mini-Ura-blaster and that it suppresses hyphal growth in *C. albicans*.

1. Introduction

Candida albicans is an opportunistic human fungal pathogen that displays multimorphism. The virulence of *C. albicans* is based on its ability to alter its own morphology from the ellipsoid blastospore to various filamentous forms, including germ tubes, pseudohyphae, and true hyphae, although its morphological switching and virulence might be decoupled [1]. Switching among diverse morphological forms is influenced by environmental cues [2] and mediated by diverse signaling pathways targeting specific transcription factors that positively regulate the expression of hypha-specific genes (HSGs) [3,4]. Transcription repressors such as Tup1-Nrg1 and Tup-Rfg1 negatively regulate the expression of HSGs [5]. Cyclin-dependent kinase Cdc28 appears to be vital in the connection between signaling pathways and hyphal growth regulators [6]. Whereas cyclin Ccn1-Cdc28 is known to suppress depolarization, cyclin Hgc1-Cdc28 advances polarized growth and prevents cell separation, as recently reviewed by Sudbery [4].

We have been interested in the extent of conservation between *C. albicans* and *Saccharomyces cerevisiae* in the control of the mitotic cell cycle at G1 phase because this phase is associated with morphogenesis [7] and differentiation [8] in *S. cerevisiae*. We specifically focused on genes that are involved in the initiation of DNA replication controlled by the Dbf4-dependent kinase (DDK) [9], which is conserved throughout evolution [10]. The protein product of *S. cerevisiae CDC7* is a protein serine-threonine kinase that is periodically regulated by phosphorylation by other protein kinases [11] and by interaction with the *DBF4* gene product [12,13]. The initiation of DNA replication requires the activation of the Mcm2-7 replicative helicase by DDK [14]. To determine whether DDK is required for the initiation of DNA replication in *C. albicans*, we characterized the *C. albicans CDC7* (*CaCDC7*) gene (manuscript submitted elsewhere) and the *C. albicans DBF4* (*CaDBF4*).

2. Materials and methods

2.1. General manipulation, media and growth conditions

Escherichia coli strain DH5α was transformed with plasmid DNA by the $CaCl_2$ method as described elsewhere [15] or by electroporation [16] and was used for routine manipulation. All *C. albicans* strains (Supplementary Table 2) were derived from the auxotrophic strain BWP17 [17], CAI4 [18], or the wild-type strain SC5314 [19]. The media and routine growth conditions of *E. coli* and *C. albicans* were previously described [20]. *C. albicans* strains were trans-

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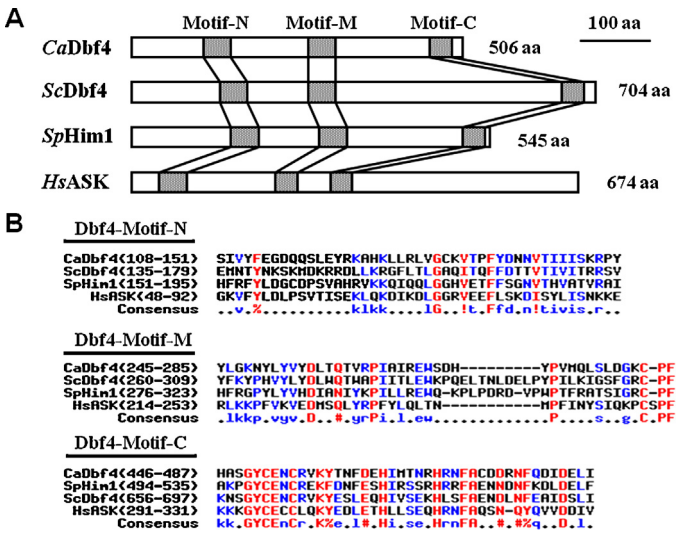

Fig. 1. The presence of three highly conserved Dbf4-motifs in *C. albicans* Dbf4 by alignment analysis. **(A)** The drawing shows the relative locations of each motif in Dbf4 from four species, represented by striped boxes. **(B)** Dbf4 motif N shows some similarity to the domain I of BRCT, which is present on a wide variety of repair and DNA damage/replication checkpoint proteins. Dbf4 motif *M* is characterized by the presence of conserved prolines and aromatic residues and promotes the association with and activation of the catalytic subunit. Dbf4 motif *C* contains a highly conserved GXCEXC(X) 9H(X) 5H(X) 2FA motif, which resembles the CCHH-type zinc finger motif. Dbf4 motif *C* is also essential for the function of Him/Dfp1 and is sufficient for interaction with the catalytic subunit. The red letters represent 100% consensus among the four species. The blue letters represent 50% consensus among the four species. *Ca*: *Candida albicans, Sc*: *Saccharomyces cerevisiae, Sp*: *Schizosaccharomyces pombe, Hs*: *Homo sapiens.* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

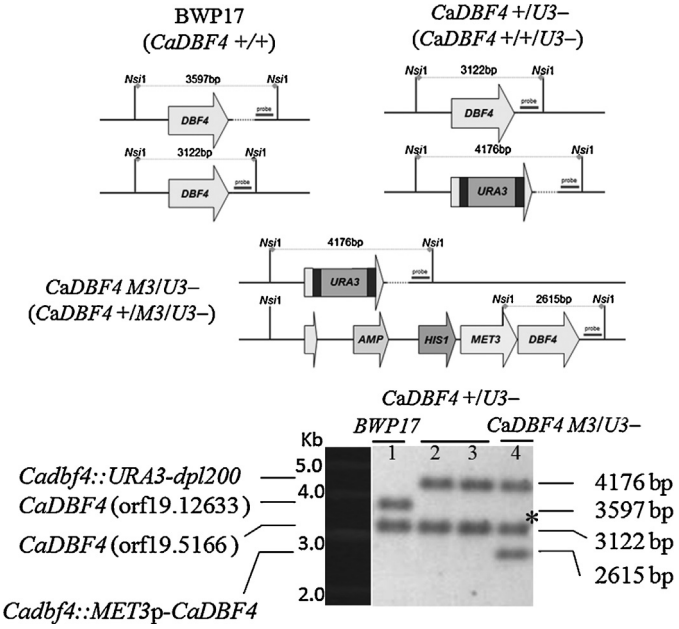

Fig. 2. Southern blotting analysis of *C. albicans CaDBF4 M3/U3–*. The blots are on the bottom panel along with the predicted structure on the upper panel. The specific alleles, with or without the insertion of the markers, and the corresponding size (in bp) are indicated. The relative positions of the probes are indicated. The auxothotrophic strain BWP17 (*CaDBF4* +/+) with two differently sized *CaDBF4* alleles was transformed using a *CaDBF4*-specific mini-Ura-blaster cassette to generate *CaDBF4 +/U3–* (later renamed *CaDBF4 +/+/U3–* due to the induced duplication of an extra copy of *CaDBF4*). Following the introduction of the linearized pFA-*HIS1-MET3*p-p*CaDBF4* cassette to *CaDBF4 +/U3–*, *CaDBF4 M3/U3–* (later renamed as *CaDBF4 +/M3/U3–*) was generated. The duplicated copy of *CaDBF4* is not shown in the diagram of both *CaDBF4 +/U3–* and *CaDBF4 M3/U3–*. In the blot, the duplicated allele of *CaDBF4* (orf19.5166), with a size of 3122 bp in lanes 2 and 3 (two independent isolates of strain *CaDBF4 +/U3–*) was masked and was revealed as fragment of 2615 bp (indicated by an asterisk) only after introduction of pFA-*HIS1-MET3*p-p*CaDBF4.*

formed by the LiAc/PEG/ssDNA method [21] or by electroporation [22].

2.2. Construction of C. albicans strains

The nucleotide sequence for *C. albicans* was obtained from the *Candida* Genome Database (http://www.candidagenome.org/). *CaDBF4* was deleted with the mini-Ura-blaster cassette *dpl200-CaURA3-dpl200*, derived from pDDB57 [23]. Briefly, the mini-Ura-blaster cassette flanked by 60 bp of upstream and downstream homology with the *CaDBF4* open reading frame was amplified by PCR using a template of genomic DNA extracted from BWP17 and the primers CaDBF4-URA3-F and CaDBF4-URA3-R (Supplementary Table 1). The PCR product was cloned into the plasmid pCR2.1-TOPO. The mini-Ura-blaster cassette flanked by the short homology regions of *CaDBF4* was then cut from the plasmid pCR2.1-TOPO using the restriction enzyme *Bst*XI and transformed into BWP17 cells. Transformants were selected for uridine prototrophism (Ura^+). To remove the *URA3* marker, cells were treated with 1 mg/ml 5-FOA. To allow for control of *CaDBF4* expression by the *MET3* promoter (*MET3*p), the partial *CaDBF4* ORF (1–292 bp) flanked by *Spe*I sites was PCR generated using template of genomic DNA extracted from BWP17 and the primers CaDBF4-*Spe*-F and CaDBF4-*Spe*-R (Supplementary Table 1). The PCR product was cloned into pFA-*HIS1-MET3*p [24] at the *Spe*I site, which yielded pFA-*HIS1-MET3*p-p*CaDBF4*. Plasmid pFA-*HIS1-MET3*p-p*CaDBF4* was linearized by digesting with a unique *Eco*RI site in *CaDBF4* and was transformed into a *C. albicans* auxotrophic strain selected for His^+ prototrophy. To repress *CaDBF4* expression, strains were grown in SD medium or on plates with 2.5 mM methionine and cysteine (Met/Cys), which turns off the expression of *MET3*p-driven downstream genes [24]. To allow the constitutive expression of *CaDBF4* in *C. albicans* cells, the protein coding sequence of *CaDBF4* was PCR-amplified and cloned into the plasmid vector p6HF-*ACT1*p with the primers CaDBF4-*Xho*I-F and CaDBF4-*Xho*I-R (Supplementary Table 1) to generate p6HF-*ACT1*p-*CaDBF4*. This plasmid, along with the empty plasmid p6HF-*ACT1*p, was linearized with *Nco*I, introduced into *C. albicans* cells, and selected for Ura+ transformants that targeted and integrated at the *RP10* locus.

2.3. Isolation of genomic DNA and southern blotting

Genomic DNA from the *C. albicans* strains was isolated by the MasterPure™ Yeast DNA Purification Kit (EPICENTRE, Madison, WI, USA). Southern blotting was performed according to standard protocols with the aid of the Rapid Downward Transfer System (TURBOBLOTTER™) using 10 μg of restriction enzyme-digested genomic DNA. A probe was generated using the PCR DIG probe synthesis kit (Roche), with the template being genomic DNA extracted from BWP17 using the primers CaDBF4-Probe(S)-F and CaDBF4-Probe(S)-R (Supplementary Table 1). The DNA on the blot was hybridized with the probe using DIG Easy Hyb (Roche). To reveal the gene deletion, the DIG Luminescent Detection Kit (Roche) was used after hybridization, and the blot was exposed to X-ray film for up to 24 h, as appropriate.

2.4. RT-PCR analysis

Cells were grown to mid-log phase and total RNA was extracted using the MasterPure™ Yeast RNA Purification Kit (EPICENTRE, Madison, WI, USA) per the manufacturer's instructions. Five micrograms of total RNA was converted into cDNA using the SuperScript III Reverse Transcriptase Kit (Invitrogen) per the manufacturer's

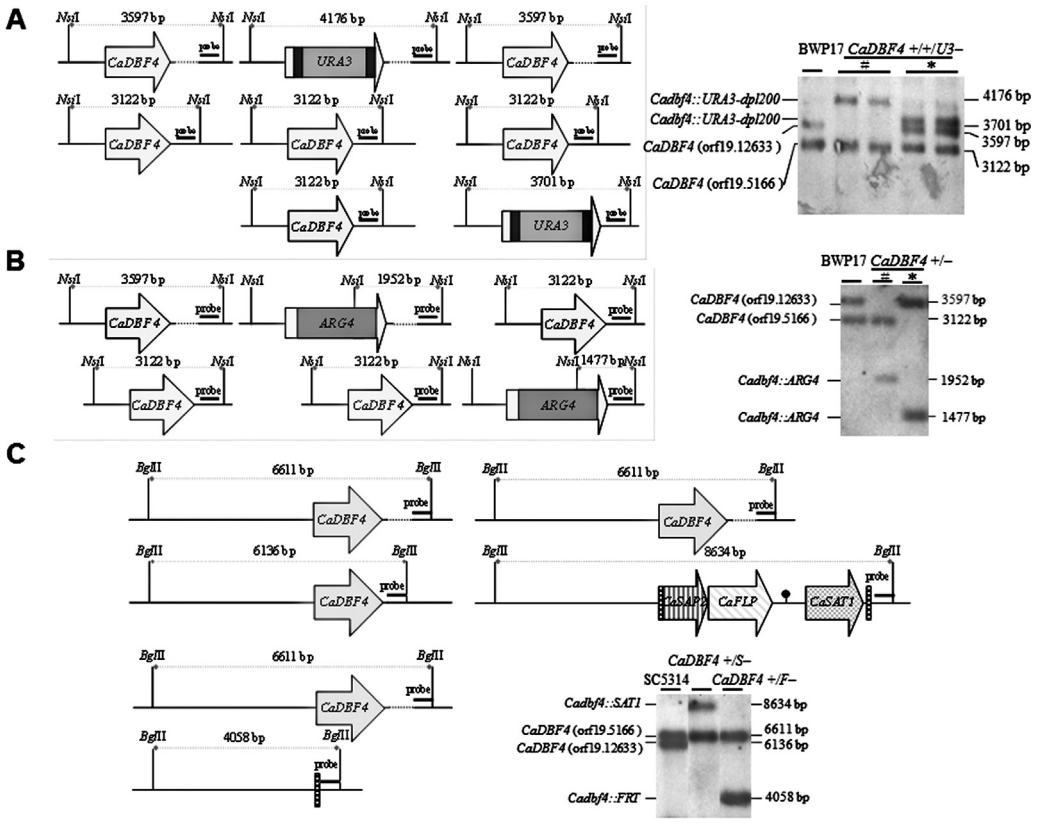

Fig. 3. *CaDBF4* deletion with the mini-Ura-blaster method induces duplication of *CaDBF4* as assessed by Southern blotting. Genomic DNA from each constructed strain was digested with specific restriction enzymes and Southern blot analysis was performed. (**A**) *Nsi*I-digested genomic DNA from the strain with *CaDBF4* deleted by the mini-Ura-blaster approach. Two independent isolates of strain *CaDBF4* +/+/*U3*– (**#** and *) resulted from the mini-Ura-blaster targeting of alleles orf19.12633 and orf19.5166. (**B**) *Nsi*I-digested genomic DNA from the strain with *CaDBF4* deleted by the *ARG4* marker. One isolate of strain *CaDBF4* +/– (**#** and *) resulted from the *ARG4* marker targeting of alleles orf19.12633 and orf19.5166. (**C**) *Bgl*II-digested genomic DNA from the strain with *CaDBF4* deleted by the *STA1* flipper approach. The blots are in the right-hand panel, and the predicted structure is in the left-hand panel. The specific alleles with or without the insertion of markers, and their corresponding sizes (in bp) are indicated. Relative positions of the probes are shown. The genotypes and names of all relevant strains are listed in Supplementary Table 2.

instructions. The cDNA was then subjected to PCR with a pair of the *CaDBF4*-specific primers CaDBF4-F and CaDBF4-R (Supplementary Table 1), thereby targeting downstream of the coding sequence and generating a product of 366 bp.

2.5. Immunoblot analysis

The total protein was extracted from cultured cells as previously described [25] and was resolved by 10% SDS-PAGE. The protein was partially purified from cells carrying the plasmid p6HF-*ACT1*p, which contains the open reading frame of the gene integrated at *RP10* with a 6×His and FLAG tag. Tagged protein was purified using Ni^{++}-NTA agarose as previously described [25]. Precipitated proteins were resolved by SDS-PAGE, transferred electrophoretically to PVDF membranes (PerkinElmer, Boston, USA) and probed with a polyclonal antibody to FLAG (Sigma) at a 1:2000 dilution. Blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (PIERCE).

2.6. Cellular image observation and recording

Colonies were photographed with a MEIJI stereoscopic microscope EMZ5 at 40× magnification. Cells in liquid culture were visualized and recorded with a Nikon 50i microscope at 400× magnification. The green fluorescence images were assessed using a ZEISS Axioskop 2 microscope (Zeiss, Jena, Germany). Digital images were acquired using a MicroFirr digital camera (Olympus, Melville, NY, USA) and processed using the Optronics PictureFrame 2.1 imaging software (Optronics, Goleta, CA, USA). The micrographs were digitized and processed using the Adobe Photoshop software.

3. Results and discussion

3.1. C. albicans DBF4 *is a structural homologue of* S. cerevisiae DBF4

We first examined the structural identity of *C. albicans* Dbf4 (*Ca*Dbf4) protein. The protein sequence of *Ca*Dbf4, orf19.5166, was obtained from the *Candida* Genome Database (CGD) and subjected to Pfam for known functional domains. Three highly conserved regions were identified [26,27] (Fig. 1A) and matched the BRCA1C terminus (BRCT) domain, the Dfp1/Him1 central region, and the DBF zinc finger domain. These domains are similar to the Dbf4 protein (Fig. 1B), which suggests that *Ca*Dbf4 is a structural homologue of Dbf4. Using the protein sequence of *Ca*Dbf4, we conducted a blast search against six frames of translated DNA sequences of CGD and were only able to identify a single *CaDBF4* sequence with significant homology, thus confirming the absence of structurally and functionally redundant *CaDBF4* genes.

3.2. DBF4 *alleles are heterologous and are induced to duplicate by the mini-Ura-blaster in* C. albicans

To establish the function of *CaDBF4* in *C. albicans*, we constructed a *Cadbf4* deletion mutant. Because *CaDBF4* encodes a protein with structural homology to known Dbf4 proteins whose function in

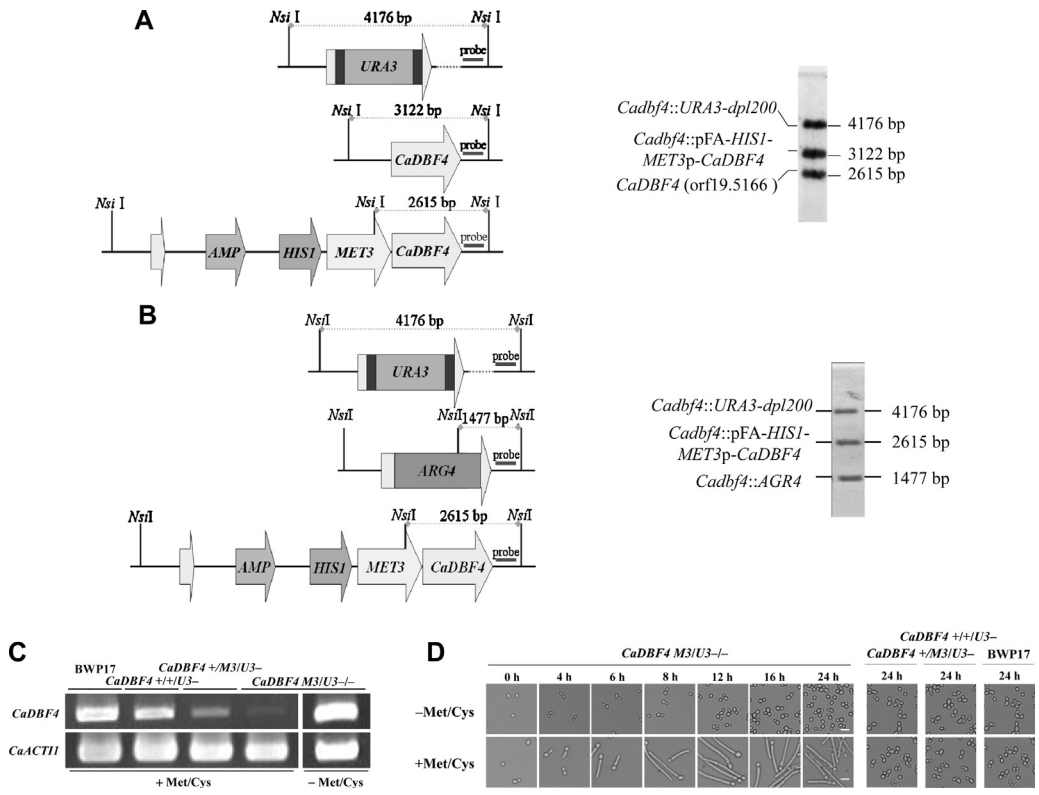

Fig. 4. *C. albicans* strain *CaDBF4 M3/U3–/–*, whose *CaDBF4* expression is controlled by *MET3*p, exhibits hyphal growth under the repressed condition. (**A**) Southern blotting analysis of strain *CaDBF4 +/M3/U3–*. *Nsi*I-digested genomic DNA from the strain *CaDBF4 +/M3/U3–* with the duplicated *CaDBF4* copy. One *CaDBF4* allele was deleted by the mini-Ura-blaster, and the other *CaDBF4* allele was placed under *MET3*p control. (**B**) Southern blotting analysis of strain *CaDBF4 M3/U3–/–*. *Nsi*I-digested genomic DNA from strain *CaDBF4 M3/U3–/–* with one *CaDBF4* allele under *MET3*p control, the other *CaDBF4* allele deleted by the mini-Ura-blaster, and the duplicated *CaDBF4* copy deleted by *ARG4*. The blots are shown in the right-hand panel, and the predicted structure is in the left-hand panel. The specific alleles with or without the insertion of the markers and their corresponding size (in bp) are indicated. Relative positions of the probes are shown. (**C**) Assessment of the expression of *CaDBF4* by RT-PCR. Cells of the strain *CaDBF4 M3/U3–/–*, together with BWP17, *CaDBF4 +/+/U3–*, and *CaDBF4 +/M3/U3–* were grown in SD medium with the required supplements in the absence (−Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for 16 h prior to collection for RT-PCR. *C. albicans ACT1* (*CaACT1*) was used as a loading control. (**D**) Microscopic assessment of cells of the strains as in (**C**). Cells were grown in SD medium with the required supplements in the absence (−Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for the indicated times prior to observation under a microscope. Bars represent 10 μm.

DNA replication is essential, we predicted that the *dbf4* homozygous null mutant would be lethal. Therefore, we first made a strain with one *CaDBF4* allele deleted and the other under the control of the *MET3* promoter (*MET3*p), which is methionine and cysteine repressible [24]. To delete one *CaDBF4* allele, we used the mini-Ura-blaster approach [23]. The plasmid pDDB57 [23], with the *dpl200* region flanked by *URA3*, and primers (Supplementary Table 1) with sequences homologous to *URA3-dpl200* and the up- and down-stream sequences of *CaDBF4* were PCR amplified and introduced into the *C. albicans* auxotrophic strain BWP17 (*ura3 arg4 his1*) [17] (Supplementary Table 2). Ura$^+$ cells were *CaDBF4 +/U3–* (Supplementary Table 2; Supplementary Fig. 2), which was confirmed by Southern blot (Fig. 2). Additionally, Southern blotting of the auxotrophic strain BWP17 revealed that the two *CaDBF4* alleles are heterologous, with one containing an extra 375 bp downstream sequence (Fig. 2), which is consistent with the sequence shown in the CGD. To make a strain that was capable of conditionally expressing *CaDBF4* under *MET3* control, we PCR-amplified the partial *CaDBF4* sequence and cloned it into the plasmid vector pFA-*HIS1-MET3*p [28]. After linearizing at a unique restriction site within the partial *CaDBF4* sequence, we introduced the plasmid into strain *CaDBF4 +/U3–* and selected for His$^+$ to obtain the strain *CaDBF4 M3/U3–* (Supplementary Table 2; Supplementary Fig. 2). Surprisingly, even with the integration of the pFA-*HIS1-MET3*p-based plasmid at the shorter *CaDBF4* allele, the uninterrupted shorter *CaDBF4* allele remained (Fig. 2), which suggests the presence of three copies of the *CaDBF4* gene, one longer and two shorter alleles, in the diploid genome of *C. albicans*.

To validate the observation of three copies of the *CaDBF4* gene and to determine whether the three *CaDBF4* alleles in the *C. albicans* genome are strain-specific, we introduced the *URA3-dpl200* cassette with the up- and down-stream sequences of *CaDBF4* into strains BWP17 and CAI4 (*ura3*) [18] (Supplementary Table 2) and selected for Ura$^+$ cells. We randomly selected and assessed fifteen (BWP17 as a parental) to thirty (CAI4 as a parental) Ura$^+$ transformants by yeast colony PCR. It appeared that the mini-Ura-blaster cassette targeted the longer or the shorter *CaDBF4* allele with equal frequency (data not shown). Two Ura$^+$ isolates of BWP17 origin with the longer or shorter *CaDBF4* allele targeted, together with the BWP17 parental strain, were subjected to Southern blot analysis. The size of the *Nsi*I-fragment enclosing *CaDBF4* shifted from 3597 bp to 4176 bp and from 3122 bp to 3701 bp, which demonstrated that the longer and shorter *CaDBF4* genes were disrupted by the mini-Ura-blaster cassette but that the third copy of *CaDBF4* remained uninterrupted (Fig. 3A). Similar results were found in Ura$^+$ isolates derived from CAI4. These results reinforced the idea that the *C. albicans* genome has three *CaDBF4* copies. This organization in the genome is likely general among *C. albicans* strains.

To exclude the possibility that the three *CaDBF4* copies were restricted to the auxotrophic *C. albicans* strains, we introduced the *STA1* flipper cassette [29] carrying a *CaSTA1* selection marker conferring nouseothrecin resistance (Nou$^+$) and the up- and down-stream sequences of *CaDBF4* into the wild-type *C. albicans* strain

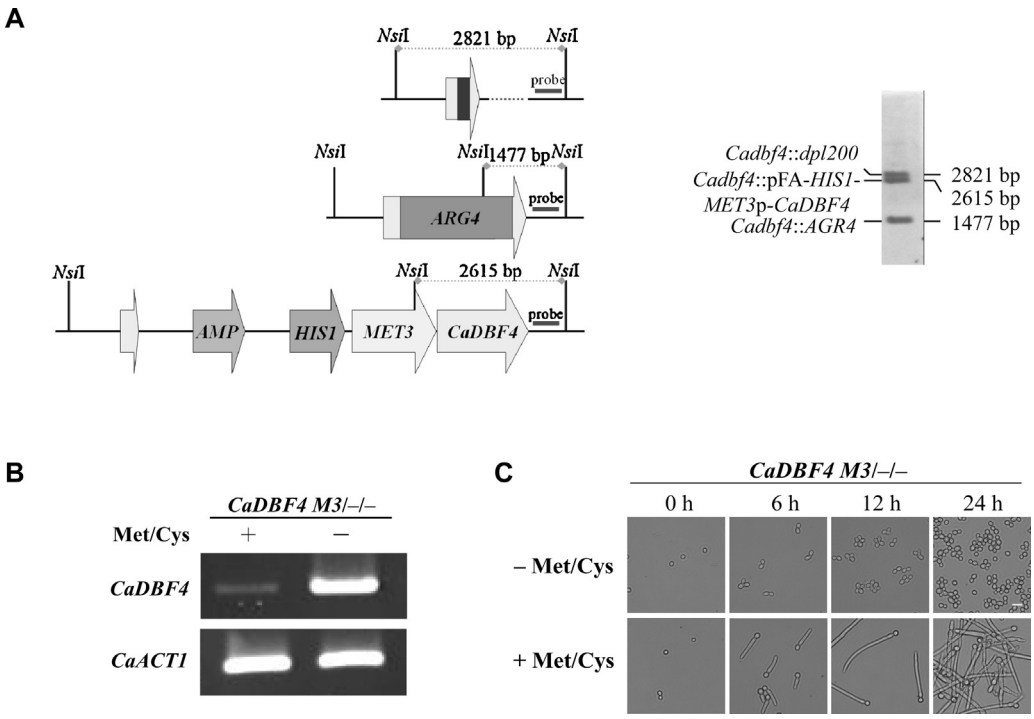

Fig. 5. *URA3*-popped out *C. albicans* strain *CaDBF4 M3/–/–*, whose *CaDBF4* expression controlled by *MET3*p, shows hyphal growth under the repressible condition. (**A**). Southern blotting analysis of strain *CaDBF4 M3/–/–*. *Nsi*I-digested genomic DNA from strain *CaDBF4 M3/–/–*, with one *CaDBF4* allele under *MET3*p control, the other *CaDBF4* allele deleted by the mini-Ura-blaster cassette with the *URA3* popped out afterwards, and the duplicated *CaDBF4* copy deleted by *ARG4*. The blot is shown in the right-hand panel, and the predicted structure is in the left-hand panel. The specific alleles with or without the insertion of the markers and their corresponding sizes (in bp) are indicated. Relative positions of the probes are shown. (**B**). Expression of *CaDBF4* by RT-PCR. Cells of the strain *CaDBF4 M3/–/–* were grown in SD medium with the required supplements in the absence (−Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for 16 h prior to collecting for RT-PCR. *CaACT1* was used as a loading control. (**C**). Microscopic assessment of cells Cells were grown in SD medium with the required supplements in the absence (−Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for the indicated times prior to observation under a microscope. Bars represent 10 μm.

SC5314 [19] (Supplementary Table 2) and selected for Nou$^+$ cells to obtain strain *CaDBF4 +/S–*. The selection cassette was then popped out to obtain *CaDBF4 +/F–* (Supplementary Table 2; Fig. 3C). Although, the longer and shorter *CaDBF4* alleles were present in the genome of the SC5314 strain, the third allele was not detected (Fig. 3C), which suggested that either the triple allele is unique to auxotrophic strains, or there are only two alleles in the *C. albicans* genome and the third copy of *CaDBF4* was induced by the mini-Ura-blaster cassette. Although, we could not introduce the mini-Ura-blaster cassette into the wild-type strain SC5314 because the resulting transformants were non-selectable, we detected only two alleles in BWP17 or CAI4 when we introduced the *STA1*-flipper cassette (data not shown). The use of an *ARG4* marker to delete *CaDBF4* also led to only two alleles in either BWP17 (Fig. 3B) or CAI4 strains (data not shown). Similar results were found using *HIS1* selection (data not shown). Hence, we conclude that two heterologous *CaDBF4* alleles are present in the *C. albicans* genome and that both alleles of *CaDBF4* can be inducibly duplicated by the mini-Ura-blaster cassette. At least two similar results of genes potentiallybeing inducibly duplicated by the Ura-blaster cassette with *URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* have been reported, although the genes were thought to have three copies in the genome [30,31]. Nevertheless, in our hands, among the five genes that we used for strain construction with the mini-Ura-blaster cassette, *CaDBF4* is the only gene that was inducibly duplicated. These results imply that mini-Ura-blaster induced duplication is DNA sequence specific. It would be important to see whether DNA sequences flanked with repeats also have duplication potential when introduced in genomes other than *C. albicans* genome because duplication alters genome, which might lead to undesired consequences.

3.3. DBF4 *is an essential gene and depletion of* DBF4 *leads to hyphal growth in* C. albicans

To determine the phenotypic consequence of loss of *CaDBF4* function in *C. albicans*, strain *CaDBF4 +/M3/U3–* (Supplementary Table 2; Fig. 4A), in which one *CaDBF4* allele was deleted and the other was placed under the control of *MET3*p, was used to introduce a cassette in which the *AGR4* marker gene had replaced the majority of the *CaDBF4* gene such that the third duplicated *CaDBF4* could be deleted to obtain strain *CaDBF4 M3/U3–/–* (Supplementary Table 2; Fig. 4B). To assess the repressibility of *CaDBF4* under the control of *MET3*p, we extracted RNA for RT-PCR analysis from strain *CaDBF4 M3/U3–/–* and from the parental strains grown in the presence and absence of 2.5 mM methionine and cysteine (Met/Cys). The expression of *CaDBF4* was repressed in strain *CaDBF4 M3/U3–/–* but not in the parental strains (Fig. 4C). We next wanted to determine the necessity of *CaDBF4* in *C. albicans* by growing these strains on plates with selective media in the presence or absence of Met/Cys. Surprisingly, strain *CaDBF4 M3/U3–/–* was able to form colonies in the presence of Met/Cys with wrinkled surfaces (Supplementary Fig. 2). The depletion of *CaDBF4* did not impair the proliferation of the cells because the doubling time was similar between strain *CaDBF4 M3/U3–/–* and the parental strains when grown in the presence of Met/Cys (data not shown). These results suggest that *CaDBF4* inhibits filamentous development and may not be essential. To verify the necessity of *CaDBF4* in *C. albicans*, we established a *Cadbf4* homozygous null mutant. However, though the *Cadbf4* heterozygous null mutants were very easy to produce, no *Cadbf4* homozygous null mutants were obtained. To conclusively determine if *CaDBF4* is essential to *C. albicans*, we created *CaDBF4 +/S–*, where one *CaDBF4* allele was deleted by the *STA1*-flipper cassette,

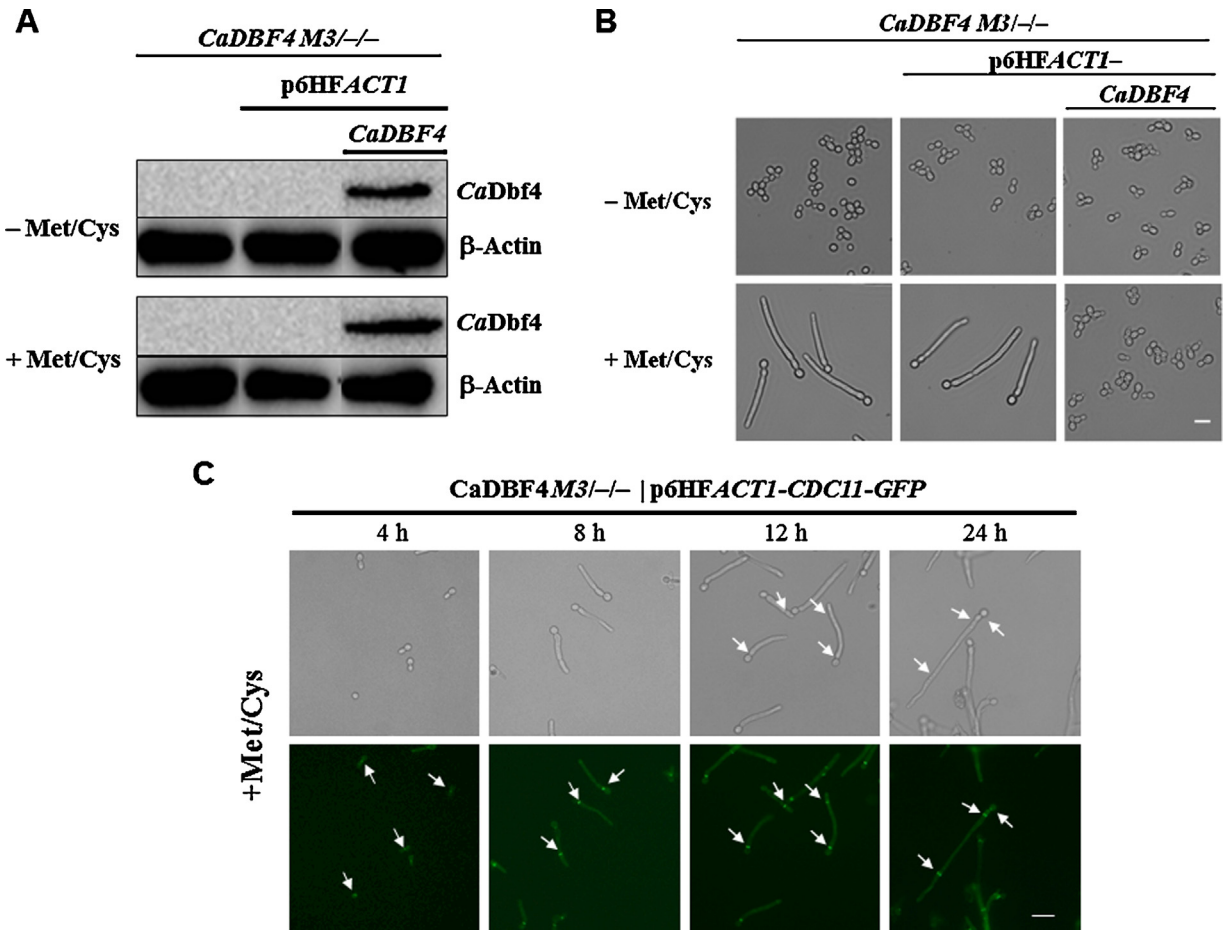

Fig. 6. *C. albicans* cells with *CaDBF4* expression repressed grow as hypha but are reversed to yeast by the introduction of a constitutively expressed copy of *CaDBF4*. Cells of the strain *CaDBF4 M3*/–/– were transformed with the restriction-linearized plasmid p6HF-*ACT1*p-*CaDBF4* or the empty plasmid p6HF-*ACT1*p targeted to the *RP10* locus to obtain the strains *CaDBF4 M3*/ – / – | *CaDBF4* and *CaDBF4 M3*/ – / – |p6HF-*ACT1*p, respectively. Cells of those two strains, together with *CaDBF4 M3*/–/–, were grown in SD medium with the required supplements in the absence (–Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for 12 h prior to assessment. (**A**). Verification of the expression of *Ca*Dbf4 from strain *CaDBF4 M3*/ – / – |*CaDBF4* by western blot. β-Actin was used as a loading control. (**B**). Microscopic observation. (**C**). Verification of the hyphal form of *CaDBF4 M3*/–/– when grown under repressed condition. Cells of the strain *CaDBF4 M3*/–/– were transformed by the restriction-linearized plasmid p6HF-*ACT1*p-*CDC11-GFP* to generate *CaDBF4 M3*/ – / – |*CaDBF4 CDC11-GFP*. Cells were then grown in SD medium with the required supplements in the absence (–Met/Cys) or presence (+Met/Cys) of 2.5 mM methionine and cysteine for the indicated times prior to observation under a fluorescent microscope. Bars represent 10 μm.

and then removed the cassette to obtain *CaDBF4* +/*F*– (Supplementary Table 2; Supplementary Fig. 3). A cassette with *ACT1* promoter (*ACT1*p)-driven *CaDBF4*, which was capable of constitutively expressing *CaDBF4*, was incorporated into the *SAT1*-flipper cassette (*SAT1*-flipper-*ACT1*p-*CaDBF4*) and introduced into *CaDBF4* +/*F*– to generate *CaDBF4* +/*F* – |*SAT1*-flipper-*ACT1*p-*CaDBF4*. However, this cassette was unable to be removed by BSA-induction. Taken together, our data suggest that *CaDBF4* is likely an essential gene in *C. albicans*.

Because depletion of *CaDBF4* resulted in growth on plates as colonies with possibly filamentous forms (Supplementary Fig. 2), we attempted to verify this observation. Cells of strain *CaDBF4 M3*/*U3*–/– and parental strains were grown in the presence or absence of Met/Cys for up to 24 h and observed microscopically. Cells of all strains, including *CaDBF4 M3*/*U3*–/– in SD medium without Met/Cys, grew in the yeast form (Fig. 4D). In contrast, cells of strain *CaDBF4 M3*/*U3*–/– in medium with Met/Cys grew in the filamentous form and showed progressive development in a time-dependent manner compared with the parental strains (Fig. 4D). These data suggest that *CaDBF4* is required to suppress the yeast-to-filament transition.

3.4. *Neither* URA3 *nor its flanking gene* IRO1 *interferes with deletion of* DBF4 *for the function of hypha-suppression in* C. albicans

To discount the possibility of the *C. albicans URA3* gene residing in the *CaDBF4* locus and, consequently, causing the morphological changes that we observed [32,33], we treated strain *CaDBF4 M3*/*U3*–/– with 5-FOA to induce the loss of *URA3* to obtain strain *CaDBF4 M3*/–/– (Supplementary Table 2; Fig. 5A). The correct structure was verified by Southern blot analysis (Fig. 5A), and the repressible expression of *CaDBF4* was confirmed by RT-PCR (Fig. 5B). Whereas cells of strain *CaDBF4 M3*/–/– grew in the yeast form in medium without Met/Cys, cells with Met/Cys grew in the filamentous form (Fig. 5C), which suggests that *CaDBF4* rather than *URA3* is involved in the morphological transition. Strain *CaDBF4 M3*/–/– is derived from strain BWP17 whose *URA3* flanking gene *IRO1* being partially deleted [34] and is altered in the *CaDBF4* locus.To refute the possibility of filamentous mode of growth is resulted from partially deleted *IRO1* and alteration of *CaDBF4* locus in strain *CaDBF4 M3*/–/–, we introduced an *ACT1*p-driven *CaDBF4* into the *RP10* locus (Supplementary Fig. 4) before deleting each of the two alleles of *CaDBF4* with *HIS1* and *ARG4* selection mark-

ers. Cells of this strain grew in the yeast form, confirming that the *IRO1* gene plays no role in the morphological alteration caused by *CaDBF4* (Supplementary Fig. 5). To verify that the hyphal growth of strain *CaDBF4 M3*/−/− is a consequence of the depletion of *CaDBF4*, we constructed strain *CaDBF4 M3*/ − / − |*CaDBF4* (Supplementary Table 2) with a functional copy of *CaDBF4* driven by *ACT1*p (Supplementary Fig. 4). . *Ca*Dbf4 protein was generated by strain *M3*/ − / − |*CaDBF4*, regardless of the presence of Met/Cys (Fig. 6A). Cells of strain *CaDBF4 M3*/ − / − |*CaDBF4* even under the *MET3*p-repressed condition grew as yeast forms, equivalent to those under the de-repressed condition (Fig. 6B), which suggested that *CaDBF4* is indeed required for suppressing hyphae from yeast. To distinguish that the filamentous forms resulted from the depletion of *CaDBF4* are hypha, pseudohypha, or both, we introduced an *ACT1*p-driven *CDC11-GFP* into the *RP10* locus of strain *CaDBF4 M3*/−/− to generate *CaDBF4 M3*/ − / − |*CaCDC11-GFP* (Supplementary Table 2; Fig. 6C). Cells of this strain produced a Cdc11-GFP fusion protein that localizes at the septa between cells. Indeed, in the presence of Met/Cys, cells of this strain clearly grew in the hyphal mode, with extended filaments separated by septa. Thus, we conclude that cells lacking *CaDBF4* use the hyphal mode of growth.

We noted that *C. albicans DBF4* has been examined previously [35]. However, the previous data came from a microarray analyses that were summarized in a table; in that table, *C. albicans DBF4* was only listed as a down-regulated gene under pseudohypa-induced conditions. Additionally, a specific position upstream of *CaDBF4* having a potential binding site of transcription factor *CPH1*, which is known to be important in the morphological transition, is listed in the table. In contrast, our data are the first to demonstrate directly by strain construction that *CaDBF4* is associated with the inhibition of hyphal growth in *C. albicans*.

Both DNA-replication stress and DNA damage can cause constitutive filamentous growth in *C. albicans* [36,37]. *Ca*Dbf4 is a regulatory subunit of *C. albicans* Cdc7 and may also play a role in the coordination of DNA replication checkpoints and hyphal regulatory networks. We propose that the target of *C. albicans* Cdc7-Dbf4 suppresses the yeast-to-hypha transition and that the depletion of *C. albicans* Cdc7-Dbf4 inhibits the target, ultimately leading to hyphal development.

4. Conclusion

In this study, we found that *CaDBF4* exists as two heterologous alleles in the diploid genome of *C. albicans*. Both alleles of *CaDBF4* were capable of being inducibly duplicated by the mini-Ura-blaster cassette. Moreover, we discovered that *CaDBF4* is a hyphae-suppressor, which suggests that the *CaCDC7* is also a hyphae-suppressor and that DDK plays a role in morphogenesis; these hypotheses have been verified recently (manuscript submitted). This novel role of a DDK regulatory subunit encoded by *CaDBF4* appears to be unusual across the evolutionary spectrum and may be associated with *C. albicans* ability to interact with its host.

Conflict of interest

The authors declare that they have no conflicts of interest

Authors contributions

J.C.S. conceived and designed the study. J.C.S. and T.J supervised the project. T.J., T.L.T., J.Y.W., and Y.T.S. constructed the strains. T.J. performed the Southern blotting analysis and microscopic observations. J.Y.W performed the western blotting analysis. T.H.L. edited the manuscript. J.C.S wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2015.06.013

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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

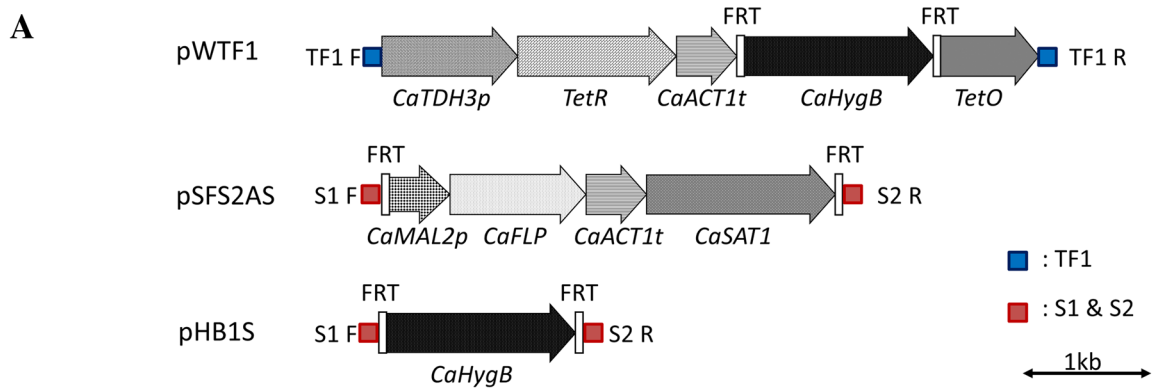
All strains were obtained via electroporation (De Backer et al. 1999). To make competent cells, cells from a colony of approximately 2 mm in diameter were inoculated into 2 ml of YPD and grown at 30 °C overnight. The next day, the culture was diluted into 50 ml of fresh YPD at 1:100 ratio and grown overnight 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
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CaHygB
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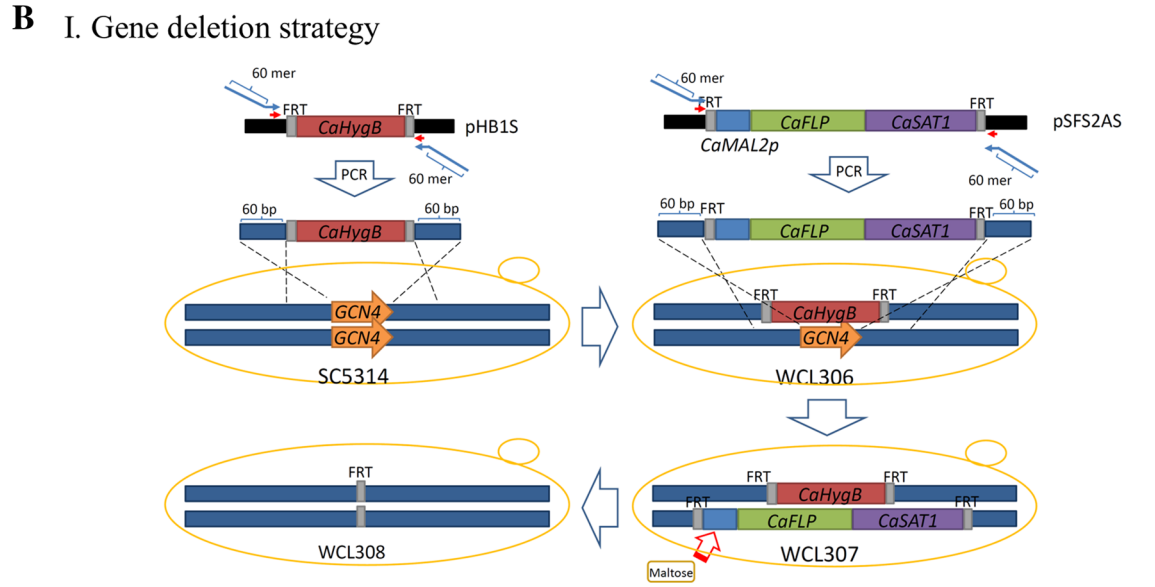
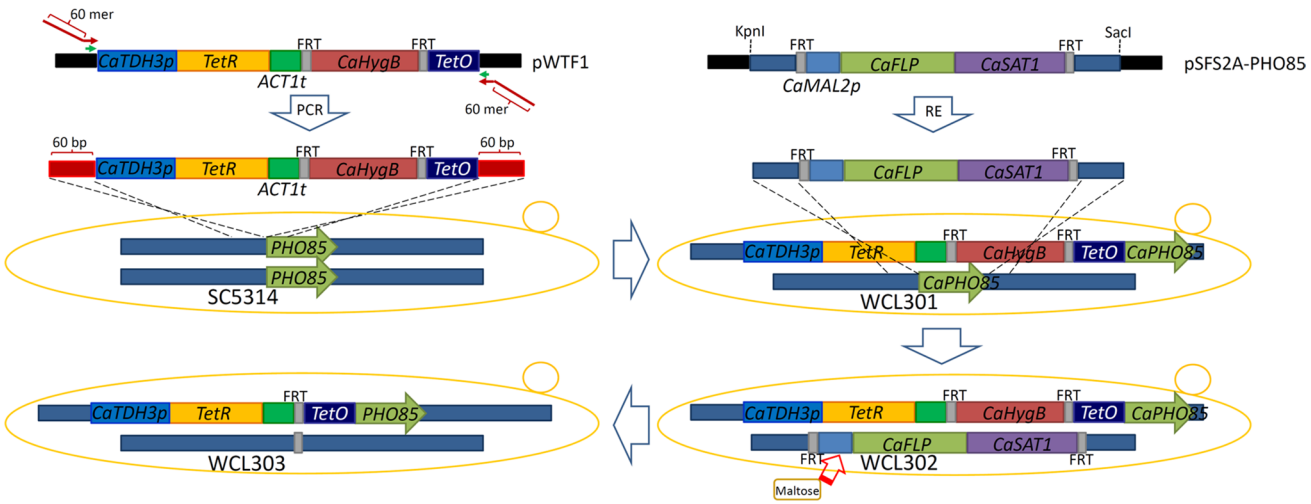
S2 R
: TF1
: S1 & S2
1kb


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).

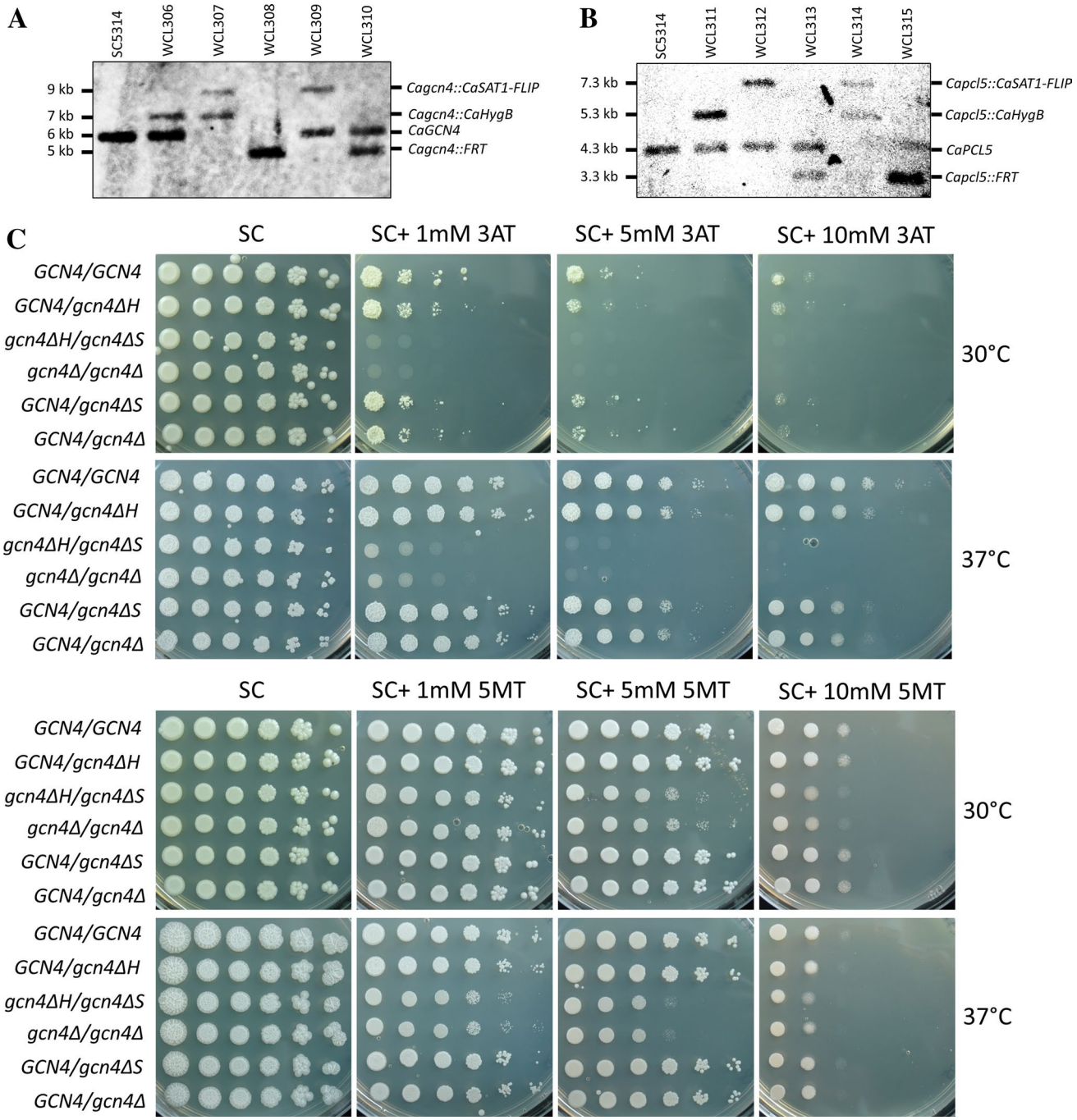
Strategies of gene deletion and conditional gene expression

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

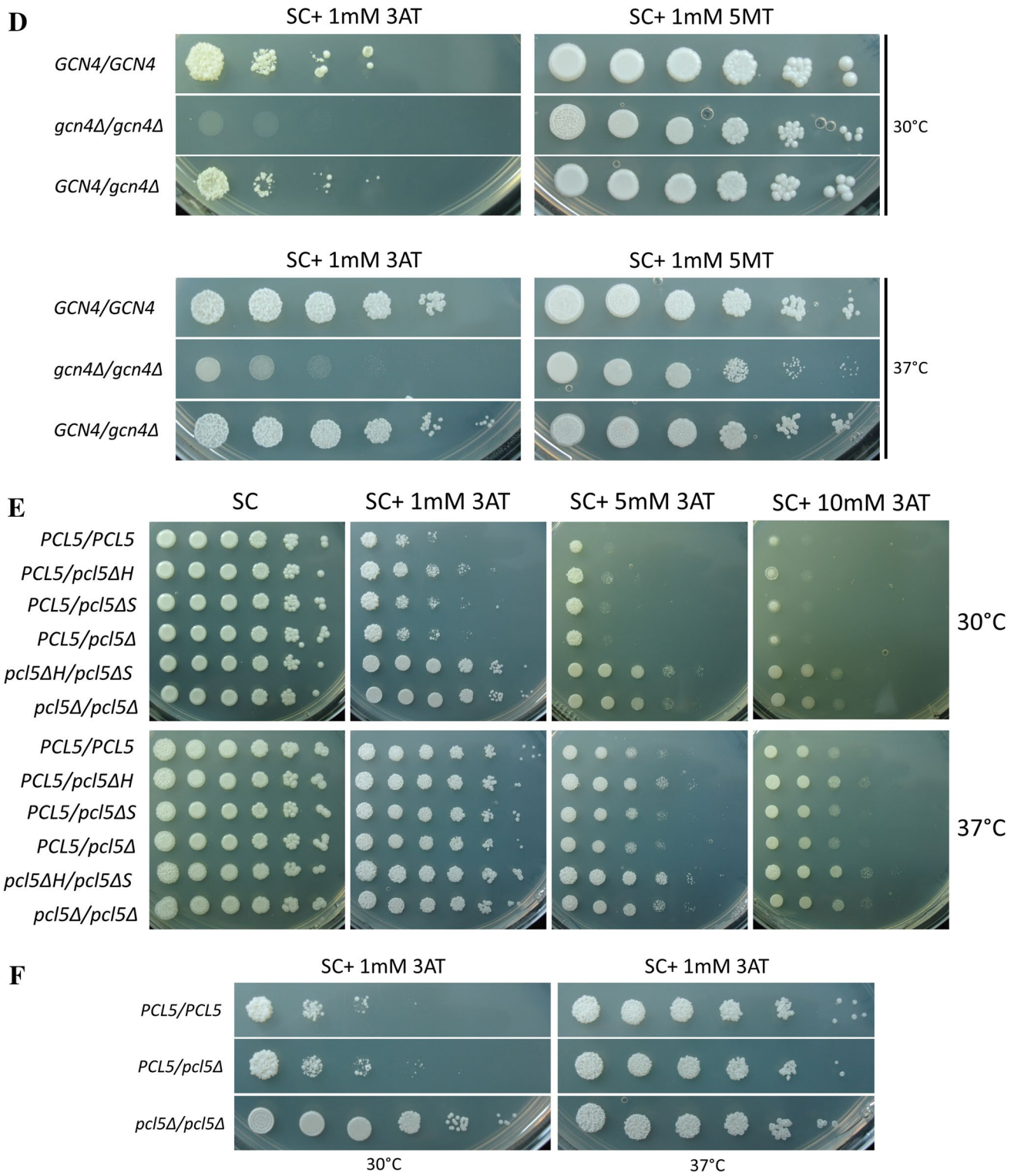


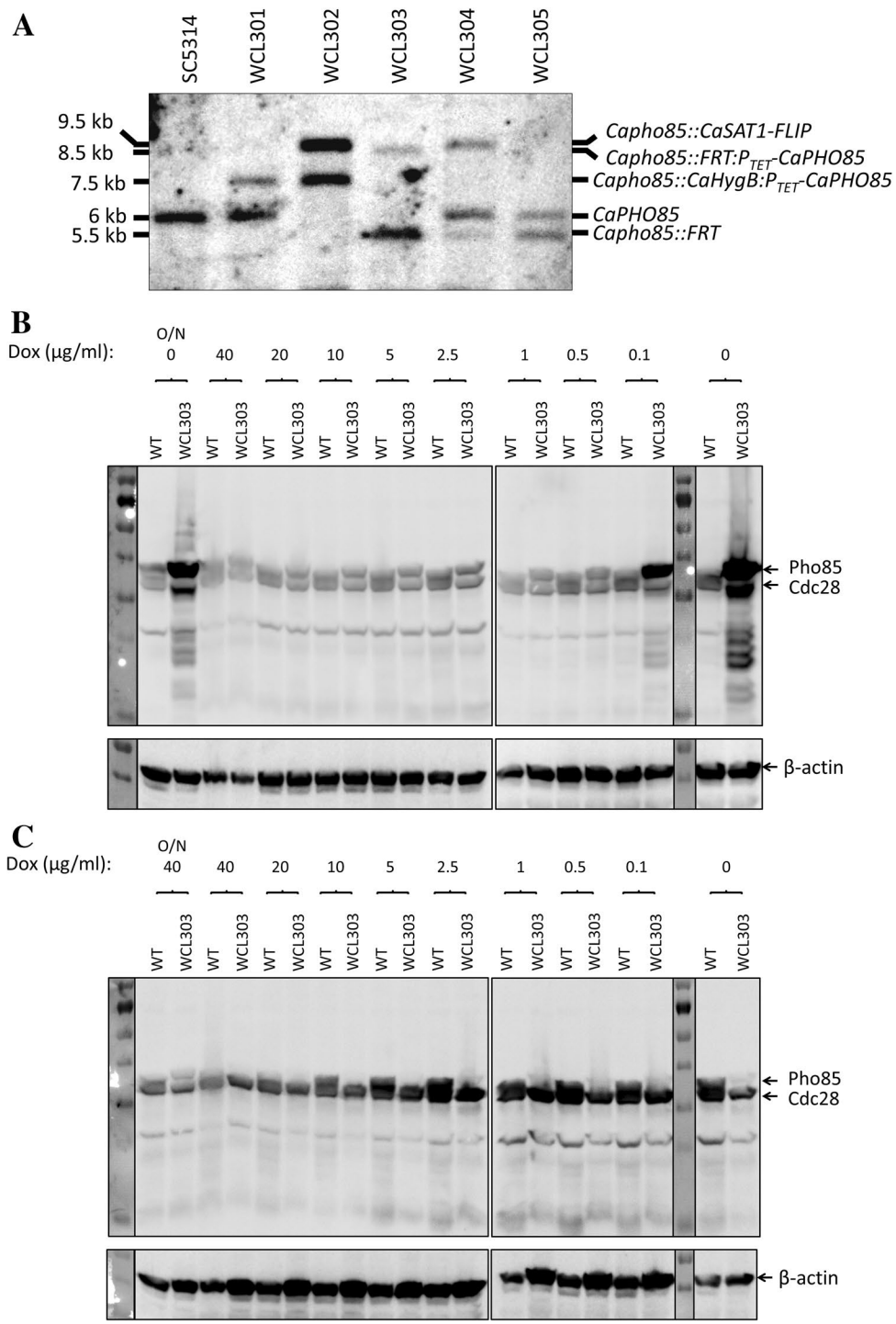
Fig. 2 continued

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

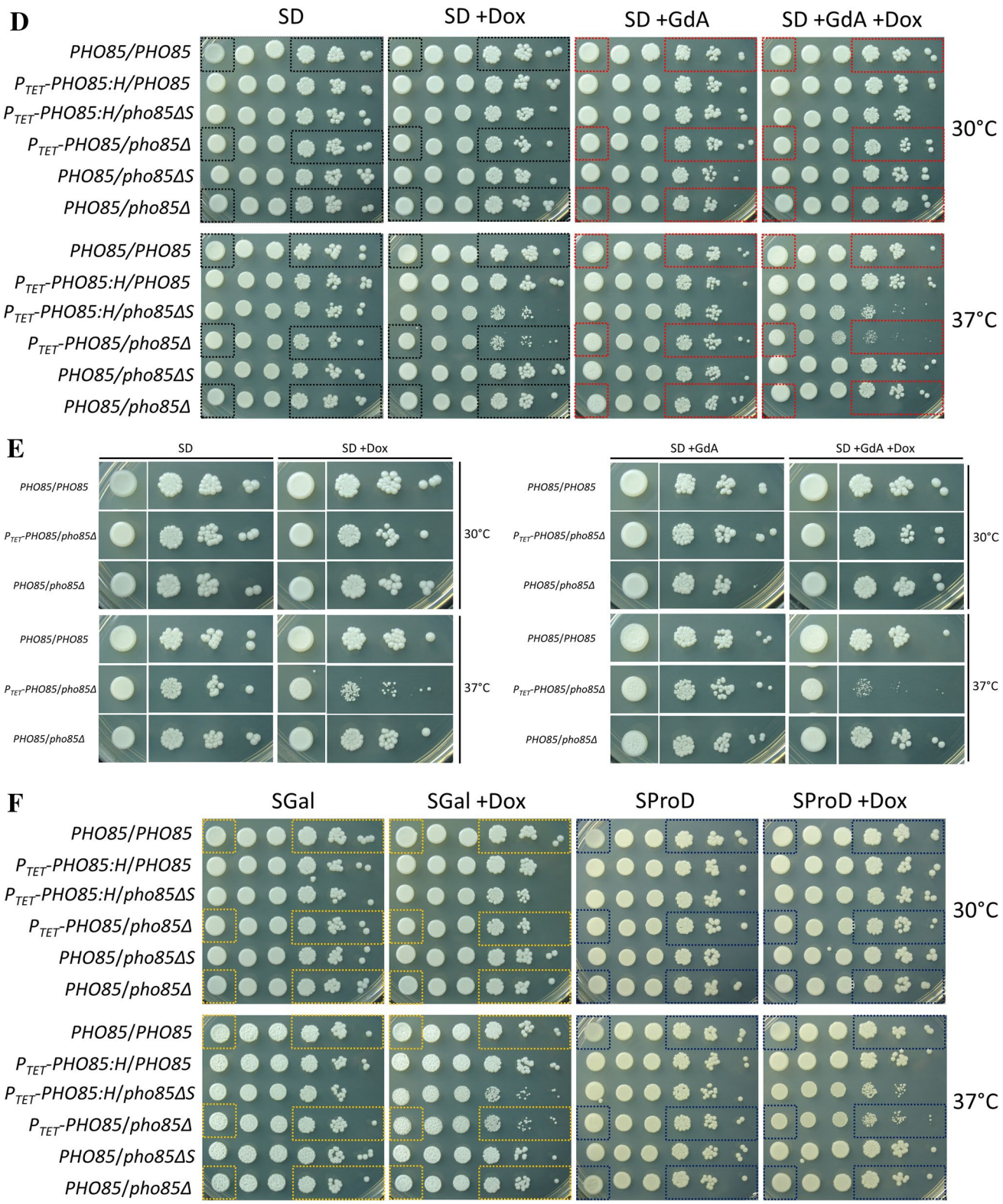


Fig. 3 continued

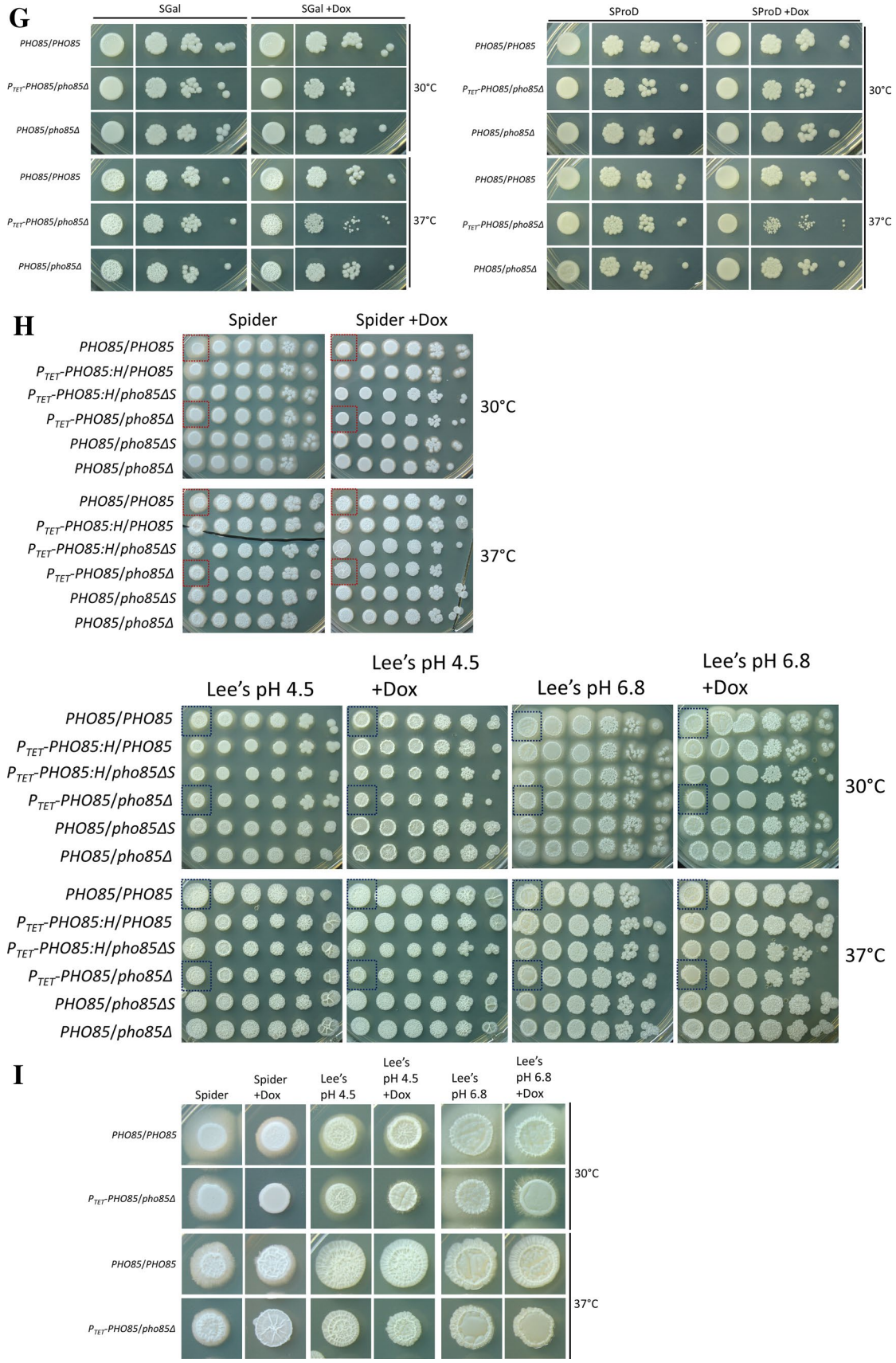


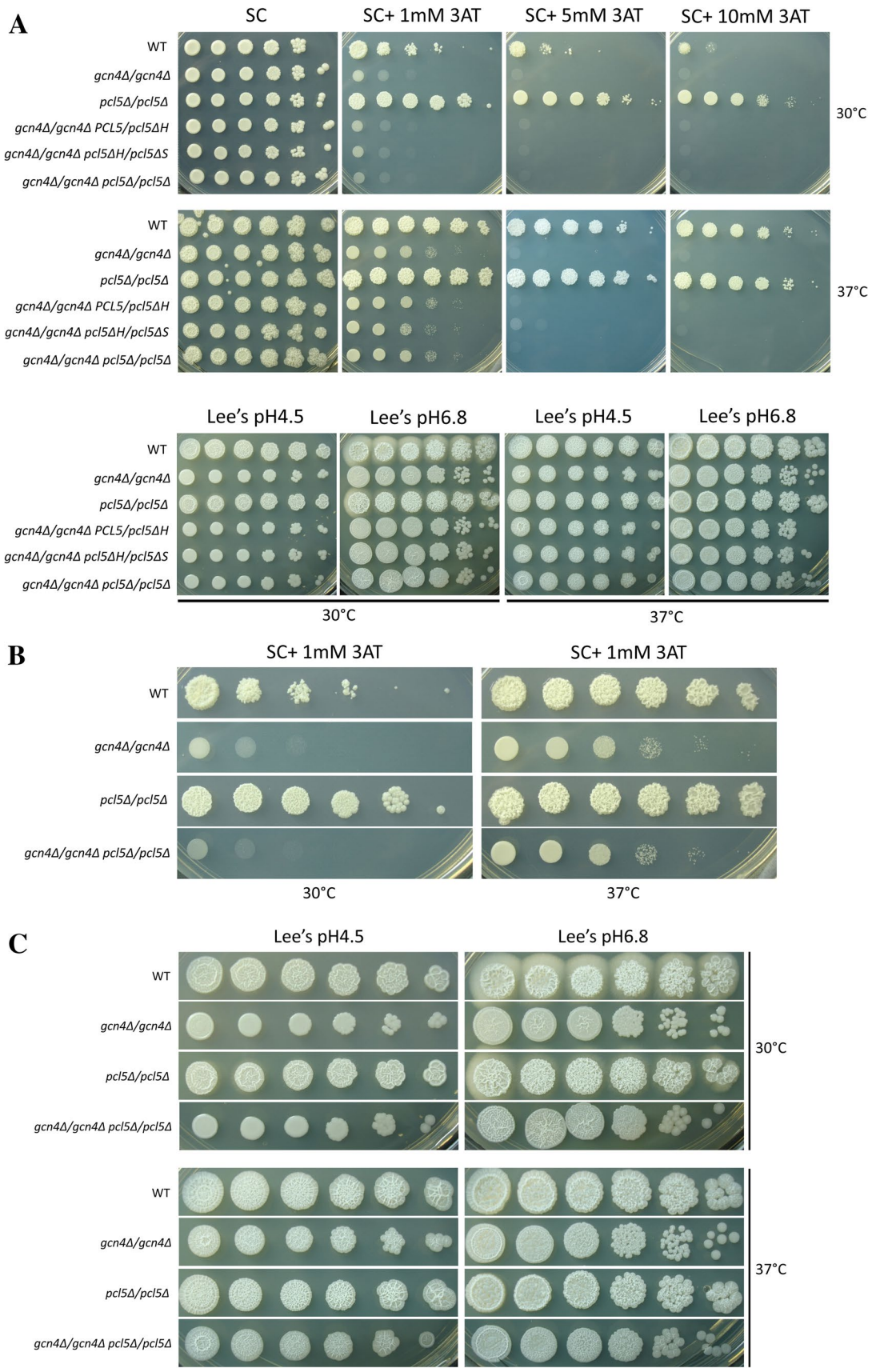
Fig. 3 continued

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

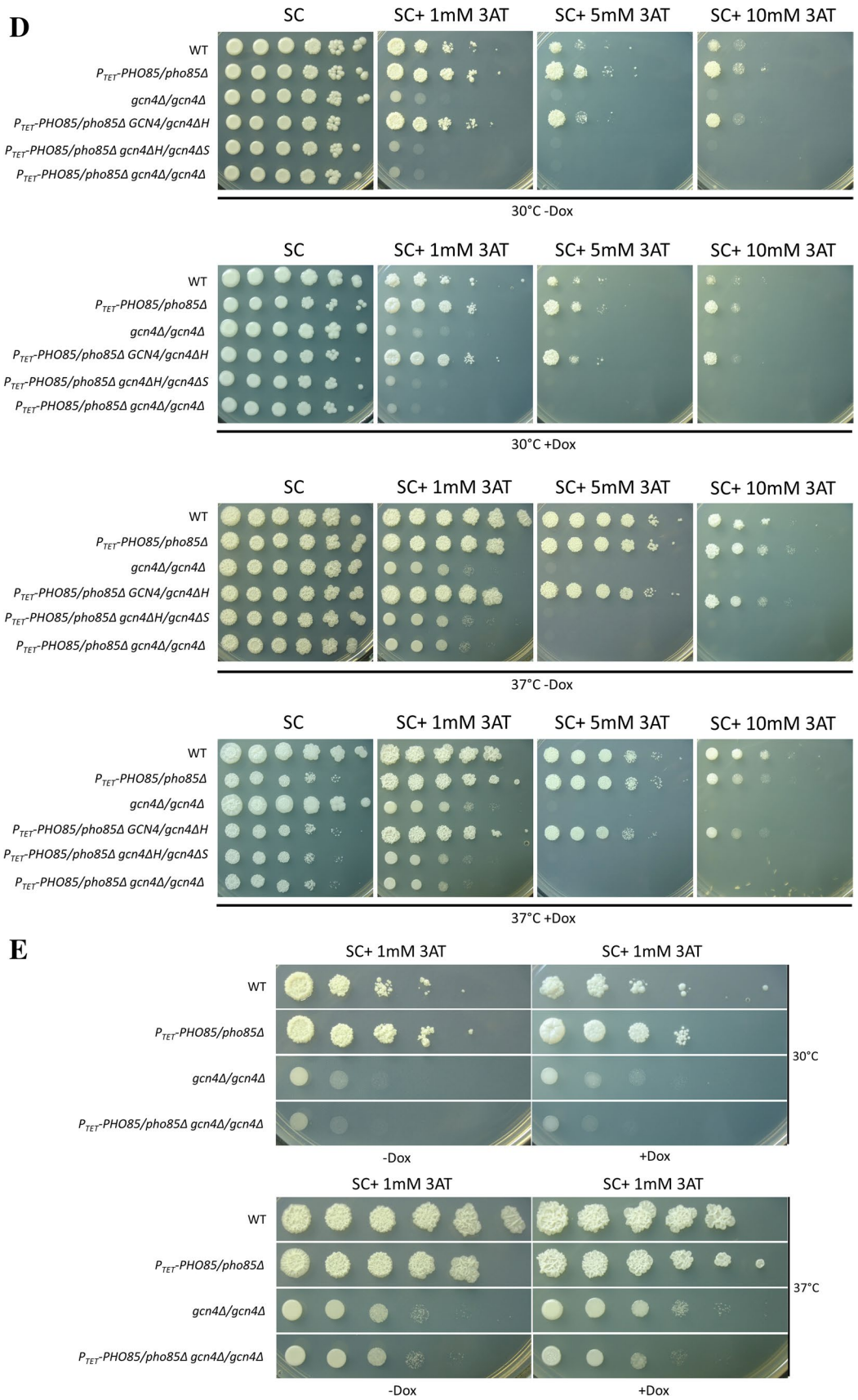


Fig. 4 continued

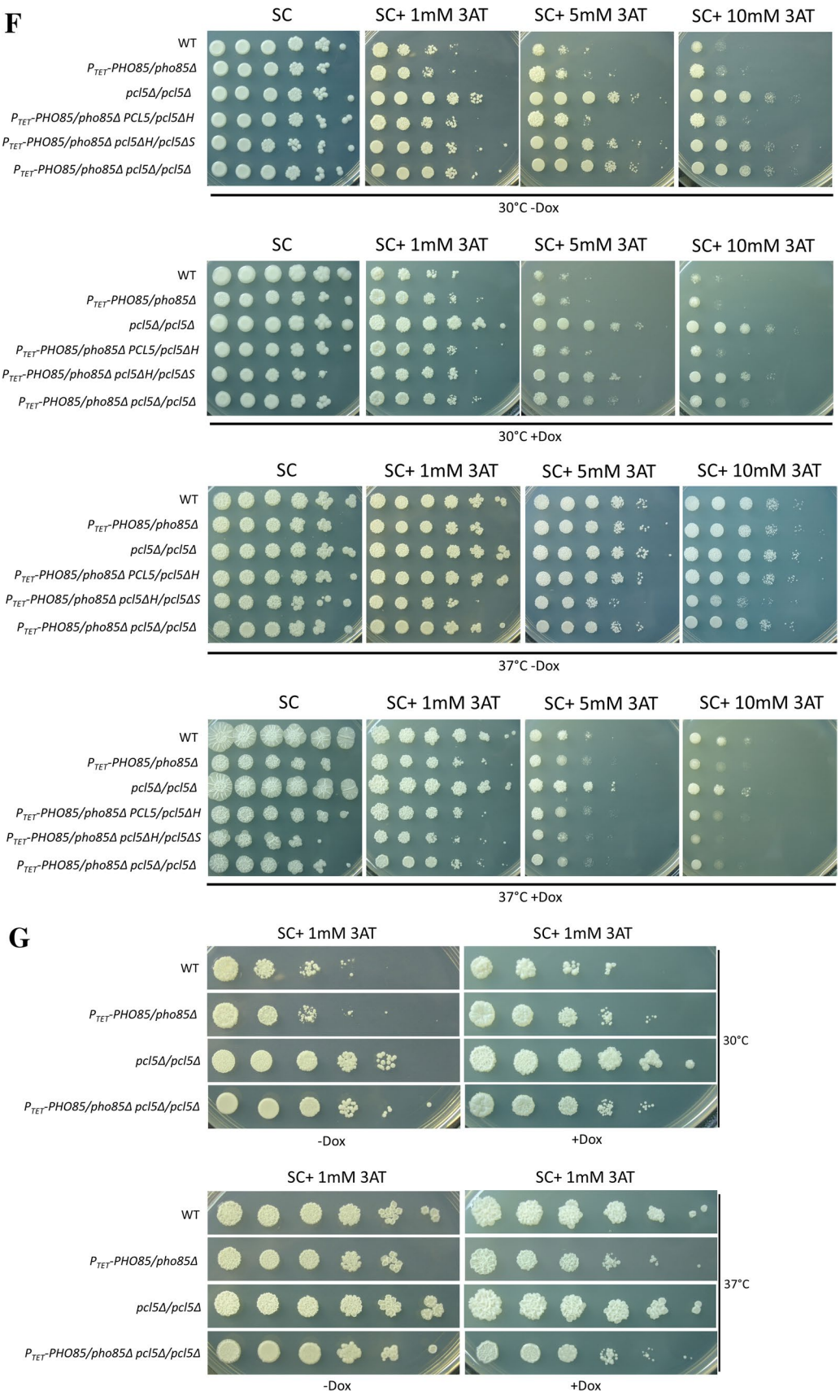


Fig. 4 continued

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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附件五

國科會補助專題研究計畫出席國際學術會議心得報告

日期：104 年 5 月 25 日

計畫編號	NSC 101－2629－B－040－001－MY3		
計畫名稱	釐清白色念珠菌 CDC4 在型態生成及逆境反應之對話所扮演的角色		
出國人員 姓名	賴威仲	服務機構 及職稱	國立中興大學生科系 及 中山醫學大學生醫系　　博士生
會議時間	104 年 5 月 7 日 至 104 年 5 月 9 日	會議地點	京都, 日本
會議名稱	(中文) (英文) 2015 International Congress on Chemical, Biological and Environmental Sciences (ICCBES 2015)		
發表題目	(中文) (英文) Tetracycline-regulated *Candida albicans* Pho85p Expression in Responses of Environmental Changes		

一、參加會議經過

這個會議是由「Higher Education Forum」所主辦，主要目的是提供不同的科學研究領域、政府單位、和公司行號可以有一個交流的平台，舉辦的地點大多選擇亞洲地區，如日本、韓國、泰國和中國，參與者多以亞洲的從業人員和研究人員為主，也有許多從亞洲以外的地區的人參與。我在偶然的機會下，看到這個會議的資訊，便積極地爭取參加這個機會的可行性。再與謝老師討論之後，決定以“Tetracycline-regulated *Candida albicans* Pho85p Expression in Responses of Environmental Changes”為題目，

投稿到這個會議。此次會議的地點是在日本京都市的京都研究園區(Kyoto Research Park)，這是個緊鄰京都市的一個研究園區，著重在資訊、生技、電子和機械產業創新發展。交通上相當便利，可以搭乘 JR 地鐵至丹波口站步行前往，或是搭乘公車前往都很方便。

5 月 7 日先行報到，下午已經開始了幾個議程，議題包括了管理、資訊科學、教育、數學等，可說是包羅萬象。總共有三個小型討論室及一個大型演講廳，可以依照題材選擇感興趣的演講。

5 月 8 日有兩個 Keynote Speech，一個是 Jui-ichi Kadokawa 教授的演講，題目為“Precision Synthesis of Polysaccharides and Application in Self-assembled Supramolecular Materials”；另一個則是 Feili Tu-Keefner 教授的演講，題目為“The Role of Multiple Literacies in Today’s Ever-Changing, High-Tech Society”。可能是因為自己所學背景的關係，覺得 Jui-ichi Kadokawa 教授的研究相當有趣，他所提及的多醣類的應用，以及目前在合成製造上的不易，確實是一個很需要突破的部分，特別是在工業發展上。

5 月 9 日這天，我被安排在上午的海報時段，會議秘書很熱心的協助大家交流研究議題，避免大家不好意思互動。席間有泰國的研究學者分享他們致力於篩檢當地常見的遺傳疾病，也印尼的學者分享當地農業改良的策略，相當有趣。

二、與會心得

這個會議主要是幫助不同領域的研究交流，在會場中相當熱鬧。會議人員不會單單使用英語接待，考量到來自亞洲不同國家的人，會親切地以不同母語接待，算是蠻特別的地方。因為京都是日本很重要的文化古都，所以還安排了和服體驗的活動，增添了會議上輕鬆的氛圍。

三、發表論文全文或摘要

Tetracycline-regulated *Candida albicans* Pho85p Expression in Responses of Environmental Changes

Wei-Chung Lai[1,2], Hsiao-Fang Sunny Sun[3], Ho Lin[1] and Jia-Ching Shieh[2]
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Candida albicans is an important human fungal pathogen. Due to its diploid nature and incomplete sexual cycle, development of a system coupled a tetracycline-repressible (Tet-off) feature with a dominant selectable marker is critical to study the essential genes directly in wild-type *C. albicans*. Pho85, a cyclin-dependent kinase, is known to participate in environmental adaptation. We adopted *PHO85* to prove the feasibility of such a system as homozygous null *pho85* mutant of *C. albicans* is unobtainable due to its essentiality. In this study, a Tet-off plasmid pWTF1, containing a *CaTDH3* promoter driven tetracycline-regulative activator (tTA), a hygromycin B resistance gene (*CaHygB*) flanked with *frt* sequences, and a seven-copies of tetracycline response element fused to a minimal *CaOP4* promoter, was established. To obtain strain WCL301 with one *C. albicans PHO85* (*CaPHO85*) allele being Tet-off regulated, the cassette of pWTF1 carrying *CaPHO85* was PCR-amplified and transformed into wild-type SC5314 for hygomycin resistance (Hyg^+). Next, to obtain strain WCL302 with the other *CaPHO85* allele being deleted, the PCR-amplified *SAT1*-flipper cassette was transformed into WCL301 for Hyg^+ and nouseothricin resistance (Nou^+). Further, by removing both *CaHygB* and *CaSAT1* through FLP/*frt* recombination, strain WCL303 was obtained and assessed functionally. The doxycycline (Dox)-dependent repression of *CaPHO85* in *C. albicans* was confirmed by western blotting. The growth defect of *C. albicans* lacking *CaPHO85* in response to nutrient deficiency was verified by spotting assays with various semi-solid media in the presence of Dox. We conclude that *Ca*Pho85 plays an important role for survival in nutrition depleted conditions.

四、建議

這個會議相當適合初次參與國際研討會的人，因為會議人員除了講英文，還會使用其他語言，包括國語。在投稿的題材上，因為其主旨是整合不同領域的研究以提供交流的平台，所以適合各種領域的研究人員參加。

五、攜回資料名稱及內容

這個會議給每位參加人員一本會議的議程和一個隨身碟集結了所有投稿文章，另外還贈送了抹茶點心。

六、其他

附件五

國科會補助專題研究計畫出席國際學術會議心得報告

日期：104 年 8 月 15 日

計畫編號	NSC 101－2629－B－040－001－MY3 (GM3)		
計畫名稱	釐清白色念珠菌 *CDC4* 在形態生成及逆境反應之對話所扮演的角色 Deciphering the role of CaCDC4 on crosstalk between morphogenesis and stress response in *Candida albicans* (GM3)		
出國人員姓名	謝家慶	服務機構及職稱	中山醫學大學生物醫學科學學副教授
會議時間	104 年 7 月 31 日至 104 年 8 月 2 日	會議地點	中國上海
會議名稱	(中文) 2015 第五屆世界微生物大會 (英文) BIT's 5th Annual World Congress of Microbes-2015		
發表題目	(中文) (英文) *Candida albicans DBF4* Gene is inducibly Duplicated by the Mini-Ura-Blaster and Involved in a Novel Role of Hyphae-Suppression		

一、參加會議經過

This was a three-day's conference covering all aspects of microbial research. The conference actually constituted several symposia including Virus and Infection, Bacteriology and Infection Mycology. After the first day morning's opening ceremony and keynote forum, the rest of two-and-a-half days were directed to the specialized session according to participants' expertize. The participants were free to attend any session even outside their research interest.

二、與會心得

Based on my previous experience in 2013 as a speaker and a session moderator, I attend this similar conference. The conference appeared to be the effort made by the Chinese to embrace science research, particularly the communication of science, including successful in organizing an international conference. Therefore, members of the organizing team (all young fellows) were quite actively involved in all aspects of the conference, which indicates China's ambition to become the world center of life science. However, this conference seems to be less funded by the Chinese Government according to message release from the conference staff. Nevertheless, I can still witness the good talks among some of the bad ones. Importantly, there were several renounced scholars from academia and industry, particularly on vaccine

development and mechanism and epidemiology of viral infection addressed in the keynote speech. Some of the talks were outstanding, whereas a few required more effort to improve. But the local speakers in most cases were excellent in delivering their ideas. I was particularly impressive by the active attitude of the young students who have tried to raise questions during and after the speech and made conversation with the speakers. Perhaps the coverage of the conference was comprenensive, in some cases; audience might not be unable to grasp the importance of research from what the speakers' talk. In general, this is an excellent conference worth of attending in the future.

三、發表論文全文或摘要

The Dbf4-dependent protein kinase encoded gene *CDC7* is conserved in initiating DNA replication. We made a *Candida albicans* strain, with one *C. albicans CDC7* allele deleted and the other's expression repressible, whose cells grew as hyphae under the repressed condition, albeit *Cacdc7* homozygous null was unobtainable. *C. albicans* cells formed hypha when expressed either the catalytically inactive *Ca*Cdc7 (K232R) or the phophoacceptor deficient *Ca*Cdc7 (T437A). While *Ca*Cdc7 interacted with *Ca*Dbf4, neither cells of the strain repressing *CaCDC7* were rescued by constitutively expressing *C. albicans DBF4* nor vice versa. We conclude that *CaDBF4*-dependent *CaCDC7* being an essential gene suppresses yeast-to-hypha transition. .

四、建議

Conference like this covering rather diverse topics that allow the participants to attend only limited talks or topics of their choice. But one should always bear in mind that in many cases we get ideas from outside of our research discipline more than from within our own research scope. I would suggest that we ought to organize international conference of this kind from time to time. Also, local students should actively participate.

五、攜回資料名稱及內容

All the participants were given a booklet covering program with details of with topics of talks or posters and their associated abstracts and biographies of all speakers. The booklet entitled "2015 第三屆微生物大會 (or BIT's 5th Annual World Congress of Microbes-2015)"

六、其他

科技部補助計畫衍生研發成果推廣資料表

日期:2016/03/26

科技部補助計畫	計畫名稱: 釐清白色念珠菌CDC4在形態生成及逆境反應之對話所扮演的角色（GM3）
	計畫主持人: 謝家慶
	計畫編號: 101-2629-B-040-001-MY3　　學門領域: 性別主流科技計畫

無研發成果推廣資料

101年度專題研究計畫研究成果彙整表

計畫主持人：謝家慶			計畫編號：101-2629-B-040-001-MY3				
計畫名稱：釐清白色念珠菌CDC4在形態生成及逆境反應之對話所扮演的角色（GM3）							
成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	無
		研究報告/技術報告	0	0	100%		無
		研討會論文	7	4	100%		為一博士生、兩碩士生及數名大學部學生成果
		專書	0	0	100%	章/本	無
	專利	申請中件數	0	0	100%	件	無
		已獲得件數	0	0	100%		無
	技術移轉	件數	0	0	100%	件	無
		權利金	0	0	100%	千元	無
	參與計畫人力（本國籍）	碩士生	4	4	100%	人次	已畢業且已就業
		博士生	1	1	100%		已畢業且已就業（博士後）
		博士後研究員	0	0	100%		無
		專任助理	0	0	100%		無
國外	論文著作	期刊論文	4	2	90%	篇	#2 為前後兩科技部計劃支持下之成果 1. Lai WC, Sun HFS, Lin H, Shieh JC (2016) A new rapid and efficient system with dominant selection developed to inactivate and conditionally express genes in Candida albicans. Current Genetics 62(1)213-235. (*corresponding author) (NSC

							101-2629-B-040-001- MY3) 2. Chien T, Tseng TL, Wang JY, Shen YT, Lin TH, Shieh JC (2015) Candida albicans DBF4 gene inducibly duplicated by the mini-Ura-blaster is involved in hypha-suppression. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 779:78-85. (*corresponding author) (NSC 97-2320-B-040-014-MY3 & NSC 101-2629-B-040-001-MY3) 3. Tseng TL, Lai WC, Lee TL, Hsu WH, Sun YW, Li WC, Cheng CW, Shieh JC (2015) A role of Candida albicans CDC4 in the negative regulation of biofilm formation. Canadian Journal of Microbiology 61(4):247-55. (*corresponding author) (NSC 101-2629-B-040-001-MY3) 4. Chin C, Lai

							WC, Lee TL, Tseng TL, Shieh JC (2013). Dissection of the Candida albicans Cdc4 protein reveals the involvement of domains in morphogenesis and cell flocculation. Journal of Biomedical Science, 20(1):97, 1-11 (*corresponding author) (NSC 101-2629-B-040-001-MY3)
		研究報告/技術報告	0	0	100%		無
		研討會論文	4	2	90%		#1 and 3 為前後兩科技部計劃支持下之成果 1. Shieh JC, Jian T, Tseng TL, Wang JY, Shen YT, Lee TL, Cheng CW, Chuan Li (2015, Aug.). Candida albicans Dbf4-dependent kinase plays a novel role in suppression of yeast-to-hypha transition. BIT's 5th Annual World Congress of Microbes 2015. Shanghai, China. NSC 97-2320-B-040-014-MY3 & NSC 101-2629-B-040-001-MY3. 2. Lai WC, Sun

							HFS, Lin H, Shieh JC (2015, May). Tetracycline-regulated Candida Albicans Pho85p Expression in Responses of Environmental Changes. International Conference on Chemical, Biology and Environmental Sciences (ICCBES), Kyoto, Japan. NSC 101-2629-B-040-001-MY3. 3. Jia-Ching Shieh (2013, Aug). Candida albicans DBF4 Gene is inducibly Duplicated by the Mini-Ura Blaster and Involved in a Novel Role of Hyphae-Suppression. The 3rd Annual International Symposia of Mycology (ISM-2013) under BIT's 3rd Annual World Congress of Microbes-2013, Shangri-La Hotel, Wuhan, China. NSC 97-2320-B-040-014-MY3 & NSC 101-2629-B-040-001-MY3. Invited

							Speaker and Session Chair 4. Lai WC, Sun HFS, Shieh JC (2013, Mar). Development of a Tet-on Based BiFC System to Explore Protein-protein Interaction in Candida albicans. 3rd Annual International Conference on Advances on Biotechnology (BIOTECH 2013), Singapore. Lai WC had been awarded one of the two best student papers. http://www.advbiotech.org/. NSC 101-2629-B-040-001-MY3.
		專書	0	0	100%	章/本	無
	專利	申請中件數	0	0	100%	件	無
		已獲得件數	0	0	100%		無
	技術移轉	件數	0	0	100%	件	無
		權利金	0	0	100%	千元	無
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	無
		博士生	0	0	100%		無
		博士後研究員	0	0	100%		無
		專任助理	0	0	100%		無

其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）	1. 博士生順利畢業並以順利取得博士後工作 2. 兩碩士生以畢業並順利就業 2. 其中成果中已於國際會議發表 [Lai WC, Sun HFS, Shieh JC (2013, Mar). Development of a Tet-on Based BiFC System to Explore Protein-protein Interaction in Candida albicans. 3rd Annual International Conference on Advances on Biotechnology (BIOTECH 2013), Singapore. Lai WC had been awarded one of the two best student papers. http://www.advbiotech.org/. NSC 101-2629-B-040-001-MY3.]發展之蛋白交互作用平台，報告博士生有獲獎。此平台材料也受過內兩實驗室使用，國外(新

	加坡Yue Wang)亦要求取得相關材料，若有成果將共同發表。此平台並受比利時團隊關注，有持續信件往來。相關論文刻正增修成果準備投稿 3. 已於國際期刊發表[Lai WC, Sun HFS, Lin H, Shieh JC (2016) A new rapid and efficient system with dominant selection developed to inactivate and conditionally express genes in Candida albicans. Current Genetics 62(1)213-235. (*corresponding author) (NSC 101-2629-B-040-001- MY3)]乃發展另一平台，相關材料已被要求取得(法國Robert Alan Arkowitz)，此平台前身[14.Lai WC, Tseng TL, Ting J, Lee TL, Cheng CW, Shieh JC (2011) Construction of Candida albicans Tet-on tagging vectors with a Ura-blaster cassette. Yeast 28(3):253-63. (*corresponding author)] 有更得要求取得此平台材料(美、英、法、中國大陸等)。 4. 此計劃意協助完成前一計劃尚未完成之成果且已發表一篇論文[2. Chien T, Tseng TL, Wang JY, Shen YT, Lin TH, Shieh JC (2015) Candida albicans DBF4 gene inducibly duplicated by the mini-Ura-blaster is involved in hypha-suppression. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 779:78-85. (*corresponding author) (NSC 97-2320-B-040-014-MY3 & NSC 101-2629-B-040-001-MY3)]；另依相關論文刻正投稿中 (Chang TW, Wu CH, Yang SY, Li WC, Chien T, Lai WC, Cheng YC, Lee TL, Shieh JCCandida albicans Dbf4-dependent Cdc7 kinase plays a novel role in the inhibition of yeast-to-hypha transition. (*corresponding author) (NSC 97-2320-B-040-014-MY3 & NSC 101-2629-B-040-001-MY3)]

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   □達成目標
   ■未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      ■其他原因
   說明：
   就論文發表量言超出預期，但仍有癥結點因計劃時程結束，在未能接續有新計畫下，論文發表可能停頓或延後。
   主要發現為： CaCDC4功能域是菌絲生成及凝絮所需（論文已發表)能負調節生物膜形成（論文已發表);CaCDC4與其親合力純化所得Thr1與Gph1與逆境HOG及TOR 路徑有關。THR1及CaCDC4與抗氧化壓力有關及生物膜形成有關；GPH1可能是附著及Germ tube生長的負控制者(此部分還待完被結果以便發表)。也發現另一CaCDC7激酶(論文正投稿)及其活性調節者CaDBF4(論文已發表)具新穎菌絲生成負調節能力。

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）
   論文
   已發表4篇
   尚有未發表之文稿至少2篇
   撰寫中文稿2篇

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   白色念珠菌形態生及逆境反應之交互作用為本計畫研究企圖解答之問題，計劃結束已釐清CaCDC4確實在白色念珠菌扮演形態生及逆境反應的橋梁
   ，CaCDC7/CaDBF4除也被發現新穎菌絲生成負調節者，也可能與菌絲誘導環境因子及基因毒性有關。釐清CaCDC4在白色念珠菌扮演形態生及逆境反應的橋梁之現象在生物學上是新發現，醫學上可作為抗真菌可能的標靶。
   此計劃也讓先前計劃尚未完成的成果得以接續(有中斷一年)並有成果
   ，CaCDC7激酶(論文正投稿)及其活性調節者CaDBF4(論文已發表)具新穎菌絲生成負調節能力，後續CaCDC7/CaDBF4可能與菌絲誘導條件及基因毒性有關，已提出新計劃申請希望有機會由性別計劃資助。此外與計劃有關的技術平台已完

成並發表一篇論文，有助後續研究。其他相關平台發展完成能讓白色念珠菌研就更完備，這方面有賴新計畫支持。
新穎基因功能及其與其它已知訊息路徑交互作用的釐清有助了解白色念珠菌生物特性，有力於發展新的抗真菌藥物，技術平台得發展則力於白色念珠菌基因功能的研究