計 畫 編 號：NHRI-EX98-9808SI

國家衛生研究院整合性醫藥衛生科技研究計畫

以蛋白質體學鑑定調節白色念珠菌型態形成之主要成份

計畫名稱

98年度成果報告

執 行 機 構： 中山醫學大學

計 畫 主 持 人： 謝家慶 助教授

本年度執行期間： 98 年 1 月 1 日 至 98 年 12 月 31 日

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目錄

壹、98年度計畫研究成果摘要

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執 行 機 構：中山醫學大學

計畫主持人：謝家慶

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關 鍵 字：白色念珠菌、型態形成、二維電泳、差異性蛋白體圖譜、cDNA庫、原核生物SCF泛素E3連接酶測定、體外激酶測定、突變tRNA使CUG 密碼子調適編碼絲氨酸

成 果 分 類：
- ☐ 癌症基礎與臨床研究(可複選，最多三項)
- ☐ 分子與基因醫學研究
- ☐ 臨床研究
- ☐ 生物技術與藥物研究
- ☐ 生物統計與生物資訊研究
- ☐ 醫療保健政策研究
- ☐ 環境衛生與職業醫學研究
- ☐ 醫學工程研究
- ☐ 老年醫學研究
- ☐ 精神醫學與藥物濫用研究
- ☐ 疫苗研究
- ☐ 幹細胞研究
- ☐ 奈米醫學研究
- ☑ 其他重要疾病或醫藥衛生問題研究

（1）中文摘要

本研究計劃旨在提供一對伺機性人類真菌病原菌—白色念珠菌如何透過後轉譯修飾，尤其是經由泛素化及磷酸化之型態形成機制其相關基因有整基因組及詳盡的瞭解。我們對白色念珠菌細胞週期中 G1 時期的調控的研究持有興趣且曾研究與釀酒酵母中分別於 G1 到 S 期 及 DNA 複製所必需之 *CDC4* 及 *CDC7* 的白色念珠菌同源基因。相反於 *CDC4* 及 *CDC7* 在釀酒酵母為必要基因，其白色念珠菌的同源基因在白色念珠菌中非必要且能遏止酵母菌至菌絲形態之轉變。由於白色念珠菌的 *CDC4* (*CaCDC4*) 為能編碼一種 Skp1-cullin-1/Cdc53/F-box (SCF) 泛素 E3 連接酶複合物中之主要成員，能將標靶蛋白質經泛素化使之降解；而白色念珠菌的 *CDC7* (*CaCDC7*) 則編碼一種蛋白質激酶，可將標靶物透過磷酸化改變其活性；再者，蛋白質的泛素化常需要先被磷酸化。因此我們認為以特定時空調控依賴磷酸化及泛素化重要蛋白質的水解可能為白色念珠菌形態控制的關鍵。據此，我們提出由 SCF 進行泛素依賴的蛋白質水解及磷酸化是瞭解白色念珠菌型態形成的核心之假說而向貴院(NHRI)提出一個為期五年的研究計劃來驗證此假說，雖然此計畫最後僅核准為三年。即便如此我們仍朝研究目標獲得一些進展。此計畫可依涉及之特定技術平台分為兩大部分，因此，進度報告將據以概述如下。壹、以二維電泳建構白色念珠菌差異性泛素化或磷酸化蛋白體圖譜。貳、構築白色念珠菌 cDNA 庫及其他質體以便於原核生物內進行特定 SCF 泛素 E3 連接酶反應以得其標靶蛋白質或於體外實施特定激酶反應而得其受質。

壹、以二維電泳建構白色念珠菌差異性泛素化或磷酸化蛋白體圖譜

一、 細胞形態誘導

為了由不同白色念珠菌細胞形態分析差異性次蛋白體圖譜，首要任務是

發展有效誘導包括酵母菌、真菌絲及假菌絲特定細胞形態之方法。事實上，此過程中我們發現能否誘導白色念珠菌特定細胞形態產生有品系專一性。我們無法成功誘導原先設定使用之營養缺陷品系 BWP17(*ura3/ura3 his1/his1 arg4/arg4*)產生假菌絲，我們因而改用野生型品系 SC5314。即便如此適當誘導條件，尤其是假菌絲的誘導是在嘗試不同試劑及條件歷經一段不短時間才算建立。列於本報告第**拾項附錄**中 **Table 1(其後的圖、表及文獻皆列於附錄)** 出示了誘導不同細胞形態的試劑及條件。不同白色念珠菌細胞形態極易在顯微鏡下區別。如出芽的酵母菌 **(Figure 1A)**，延長的真菌絲細胞**(Figure 1B)**，伸長但有分支的細胞**(Figure 1C)**。我們的結論為：白色念珠菌可經培養於 YPD 培養液加 10% 胎牛血清誘導產生真菌絲，而假菌絲的產生則仰賴高磷酸的緩衝溶液。

二、 二維電泳及定蛋白體圖譜

為能確保有效萃取白色念珠菌總體蛋白質以便施用於二維電泳，細胞破裂方法在建立二維電泳步驟**(Table 2)**及合適的操作條件**(Table 3)**過程中被完善化。我們測試了包含 Merck 及 Pierce 發展的商品化試劑、快速冷凍後研磨及自備伴有玻璃珠且含蛋白水解酶抑制劑的溶解液。就產量及所需時間而言，我們發現自備具玻璃珠的溶解液**(Table 4)**是最好的選擇。然而，此方法勞力需求高，涉及重覆震盪樣品，且一次能處理之樣品有限。我們有預見此問題而於原 NHRI 研究計劃中列入可同時處理多樣品的均質機為儀器項目，惜未被同意。為能靈敏偵測並於為使之後回收二維電泳膠上之蛋白質時易於操作，我們比較了包括 silver、blue silver 及 instant blue 三種染膠方法**(Figure 2)**。由於 blue silver 高背景且有一些無法分開的蛋白質點**(Figure 3A)**，而 silver 染回收時間過長 **(Figure 3B)** 我們將此兩者排除以平衡低背景及操作所需時間。 Instant blue **(Figure 3C)** 顯然具低背景及較短

的操作時間，因而被採用為往後的分析。為了將所有操作(二維電泳)及分析系統(膠體掃描及分析) 做完整的運作， 我們以酵母菌、真菌絲及假菌絲細胞進行差異性蛋白質圖譜的分析，並以三者的混合物為正常化標準。雖然要得相同量的蛋白質需進一步完善化，從兩次獨立蛋白質製備得到一致性蛋白體圖譜是可行的。我們做了幾組分析，代表性的一次顯示細胞專一表現的蛋白質可被顯現(**Figure 4**)。然而，增加蛋白質數目至合理的數千點其方法的建立是進行泛素化或磷酸化次蛋白體圖譜之前必須完成的事項。

貳、構築白色念珠菌 cDNA 庫及其他質體便於原核生物內進行特定 SCF 泛素 E3 連接酶測定以得其標靶蛋白質或體外實施特定激酶測定而得其受質

一、 構築白色念珠菌 cDNA 庫

由於無論是於原核生物內進行特定 SCF 泛素 E3 連接酶反應以得其標靶蛋白質或是於體外實施特定激酶反應而得其受質皆仰賴一好品質的白色念珠菌 cDNA 庫，我們尋求建立能夠構築 cDNA 庫的最好方法。基於其高的初級有效量(titers)、平均 cDNA 插入片段較大、高比例的全長基因及不需要限制酶連接酶便能將 cDNA 選殖入多重終點載體的特性，我們選用了 Invitrogen CloneMiner cDNA 構築的方法。為了純化高品質的 RNA 及 mRNA (**Figure 5A & B**)，我們分別選用了 MasterPure™ yeast RNA purification 套組 (EPICENTRE Biotechnologies) 及 FastTrack MAG micro mRNA isolation 套組 (Invitrogen)。經過將白色念珠菌 mRNAs 反轉錄成 cDNAs (**Figure 6A**) 且連接 *att*B 配接子(adapter)，cDNA 便置於一含 *att*P 位點的施予(donor)載體 (DONR222) (**Figure 5C**)和 BP Clonase 的反應使帶 *att*B 位點的 cDNA 受質與帶 *att*P 位點的 DONR222 進行體外重組產生以一入口(entry)載體 pENTR 為基礎內含兩側有 *att*L 位點的 cDNA 之入口 cDNA 庫(**Figure 6B**)。在一次的嘗試中，我們得到一個入口 cDNA 庫內含 2.6×10^7

獨立選殖株的，其中 87%具 cDNA 插入片段 (**Figure 5D**)，具有足夠代表白色念珠菌 mRNA 數目的選殖株可用於標定送入終點載體。然而更多的獨立選殖株且具更高比例的插入片段仍是值得做的。帶 *att*L 位點的選殖株及帶 *att*R 位點的終點載體(pDEST)可進一步被 LR Clonase II 催化執行重組而產生一個帶 *att*B 位點的表達株(pEXPR) (**Figure 6C**)。Gateway Nova pDEST 載體用於 LR 反應以創造 pEXPR 組成質體(**Figure 6C**)。其中以 Nova pET-53-DEST (**Figure 7A**)為一終點載體用於體外實施特定激酶反應而得其受質，而 Nova pCOLA-3-DEST (**Figure 7B**) 為另一終點載體用於原核生物內進行特定 SCF 泛素 E3 連接酶反應以得其標靶蛋白質。

二、白色念珠菌 cDNA 有效率的及可適應 CUG 密碼子表達於 *E. coli* 中之考慮事項

由於白色念珠菌是一種真核生物具備能編碼絲氨酸而非白氨酸的非通用密碼子 CUG，我們尋求引進一種 *E. coli* 品系 Rosseta 2(DE3)pLysS (Novagen) (**Figure 8**)。此品系帶有染色體上的一份在 *lacUV5* 啟動子控制下的 T7 RNA 聚合酶基因，適合用於標靶基因選殖入由 T7-驅動的表達載體產生蛋白質。此品系帶有具備一 P15A 複製子能抗氯黴素之質體(pLysS)，此質體能編碼一種可抑制 T7 RNA 聚合酶的 T7 溶菌酶用以在 IPTG 誘導前遏止 T7 RNA 聚合酶的基礎表達因此使影響細胞生長及生存力的重組蛋白質穩定。重要的是，pLysS 質體可以表達七種 *E. coli* 罕見 tRNAs 以便於表達真核生物的基因。

未了使白色念珠菌 cDNA 表達於 *E. coli* 時其 CUG 密碼子能調適編碼絲氨酸而非白氨酸，我們尋求發展一種以載體 pACSE3 (**Figure 9**) [1, 2]為基礎選殖入能編碼絲氨酸之突變 $tRNA_{CGA\rightarrow CAG}$ 基因(**Table 5**)[3]且能被 IPTG 誘導表達的質體。帶有 CUG 密碼子的白色念珠菌其 cDNA 於 *E. coli* 表達

蛋白質時會編碼為絲氨酸，這對進行體外激酶或 SCF 泛素 E3 連接酶測定可能會是關鍵。

三、選擇載體系統容納白色念珠菌基因編碼 SCF 泛素 E3 連接酶之各個成份及相關蛋白質

由於以原核生物為基礎的 SCF 泛素 E3 連接酶測定需要表達 SCF 各個成份及相關蛋白質(**Figure 10**) [4] 於 *E. coli*，能容納相關成分的所屬基因之載體系統極具關鍵性。我們採行了 pQLink 載體系統 [5] 可將每個基因選殖入此載體依序將之結合使多個基因都加入單一的 pQLink 載體 (**Figure 11**)。由於複製子及抗生素篩選基因相容性的原因，為數五個載體能表達七個罕見之 *E. coli* tRNA 且具誘導性同時表達一個白色念珠菌調適 $tRNA_{CAG}$ 編碼絲氨酸及八個蛋白質用於此測試 (**Table 6**)。而在體外激酶測定中則需要三個載體能表達七個罕見之 *E. coli* tRNA 且具誘導性同時表達一個白色念珠菌調適 $tRNA_{CAG}$ 編碼絲氨酸及一個蛋白質 (**Table 7**)。

叁、其它相關研究：與此 NHRI 計畫有關研究成果之草稿

在執行 NHRI 計劃過程中，有些與之有關或有部分關係之研究也同時實施。其中有關 *CaCDC4* 的功能研究結果得一題為「Dissecting the *Ca*Cdc4 domains reveals instability nature of CaCdc4 and its involvement in cell flocculation」之完稿手稿(**MS1**)已可投稿，列入**本報告第拾壹項**(98 年度之著作影本或手稿中)即將要投稿發表。為使泛素編碼序列引進白色念珠菌 *ADH1* 基因座同時利用誘導產生一個 6×HisFLAG 標記泛素使此被誘導之 6×HisFLAG 標記泛素因過量表現與內生性無標記泛素競爭，我們發展一種 Tet-on 載體。一個草稿版本的手稿 (**MS2**)， 題為「Construction of *Candida albicans* Tet-on tagging vectors with an Ura-blaster cassette」已列入**本報告第拾壹項**中。作為以原核生物為基礎測定 SCF^{CaCdc4} 泛素 E3 連接酶的標靶蛋

白質的一個替代研究，結果得到一初期版的手稿(**MS3**)，題為「Affinity purification of *Candida albicans Ca*Cdc4 associated proteins reveals presence of novel proteins for morphogenesis」，其草稿附於**本報告第拾壹項**中。

（2）英文摘要

The main research objective of this proposal is to provide a genome-wide and comprehensive view on genes responsible for morphogenesis by the mechanisms of post-tranlational modifications (PTMs), focusing on ubiqitination and phosphorylation, in the opportunistic human fungal pathogen *Candida albicans*. We have been interested in study the control of G1-phase of the cell cycle in *C. albicans*. We have analyzed two *C. albicans* genes, *CDC4* and *CDC7*, whose *S. cerevisiae* counterparts are required for G1-to-S transition and DNA replication, respectively. Contrasting to the *CDC4* and *CDC7* of *S. cerevisiae* being essential, *C. albicans* equivalents appear to be nonessential and suppresses yeast-to-filament transition. The fact that *C. albicans CDC4* (*CaCDC4*) encodes a key member of the Skp1-cullin-1/Cdc53/F-box (SCF) ubiquitin E3 ligase complex, involved in ubiquitinating target proteins for degradation, that *C. albicans CDC7* (*CaCDC7*) encodes a protein kinase for alteration of activity by phosphorylation of targets, and that prior phosphorylation is common as pre-requisite for ubiquitination led us to think that temporally and spatially controlled phospho-ubiquitin-dependent proteolysis of key proteins may play a pivotal role in morphological control of *C. albicans*. Hence, we have postulated that ubiquitin-dependent proteolysis by SCF and phosphorylation is center to our understanding the morphogenesis of *C. albicans* and initiated a five-year proposal from NHRI to validate this hypothesis, although the proposal had been approved as a three-year one. Nevertheless, we have made some progress towards the aims that we set out to do. The aims can be categorized as two parts, each involved in a specific platform technology; hence, this progress report will be summarized accordingly as firstly, differential *C. albicans* proteome profiling by 2-D gel electrophoresis for ubiquitinated or phosphorylated proteins, and secondly, construction of *C. albicans* cDNA library and constructs used in a prokaryote-based assay for targets of specific SCF

ubiquitin E3 ligases or *in vitro* assay for substrates of specific kinases.

I. Differential *C. albicans* proteome profiling by 2-D gel electrophoresis for ubiquitinated or phosphorylated proteins

Ia. Induction of cell types

To allow analyze the differential subproteome profile among different cell types of *C. albicans*, our initial task was to develop effective methods that can induce specific cell types, including yeast, hyphae, and pseudohyphae. During the process of developing the methodology, we have actually discovered that the ability to induce different cell types is strain-dependent. We were unable to induce the production of pseudohypal development on the auxotrophic strain (*ura3/ura3 his1/his1 arg4/arg4*), our original strain of choice; instead, we managed to induce the pseudohypal growth on the wild-type strain SC5314, although optimal inductions, especially the pseudohyphal form, have been established for some period of time with a different reagents and conditions. **Table 1** listed in the **appendix of item 10 of this report** (the following figures, tables, and references are also shown in the appendix) shows the conditions and reagents used for the induction of different cell types. The distinct cell types can be easily distinguished under microscope as shown in **Figure 1A**, the rapidly growing yeast with budded cells, **Figure 1B**, the extended elongated hyphal cells, or **Figure 1C**, the elongated cells with branching. We conclude that *C. albicans* cells can be induced into hyphal or pseudohyphal cells with either YPD medium plus 10% of fetal calf serum or a high phosphate buffer.

Ib. 2-D gel electrophoresis and differential proteome profiling

To ensure efficient extraction of total protein for 2-D gel electrophoresis, methods of cell disruption were optimized, together with establishing steps for 2-D gel electrophoresis (**Table 2**) and optimal operating conditions (**Table 3**). We have tested several commercialized cell lysis reagents for yeast (Merck and Pierce), snap freezing followed by grinding, and home-made protease

inhibitor-containing lysis buffer with glass beads. We found that the home-made lysis buffer (**Table 4**) with glass beads was the best choice in terms of yield and time required. However, as the method is labor-intensive, requiring vortexing repeated times, number of samples can be dealt with at a given time is limited. We have foreseen this problem and have actually listed a homogenizer in our original NHRI grant proposal but were unable to get approval. To allow detection and later recovering of proteins on the 2-D gel with high sensitivity and ease of manipulation, we have compared the three different methods of gel staining, including silver staining, blue silver, and instant blue (**Figure 2**). To balance between low background and time required for processing, we have excluded blue silver due to its higher background with many non-separable proteins spots (**Figure 3A**), and silver staining owing to its lengthy time for recovering proteins (**Figure 3B**). Instant blue (**Figure 3C**) appeared to be low background and shorter time of processing was therefore selected for the following analysis. To run through all the operating (2-D gel electrophoresis) and analytical systems (gel scanning and analysis), we have performed differential proteome profiling on yeast, hyphal, and pseudohyphal cells, with the mixture of the three as a normalized standard. It appeared that consistent proteome profiles between two independent protein preparations were obtainable, although further optimization is required to obtain constant amount of proteins. We have completed sets of analysis and a representative set was shown in **Figure 4** in which cell type-specific expressed proteins can be revealed. Nonetheless, methods to increase the number of protein spots to a reasonable scale of thousands are required before enrichment of subproteome of either phosphoproteome or ubiqitinated proteome can be performed.

II. Construction *C. albicans* cDNA library and other constructs used in a prokaryote-based assay for targets of specific SCF ubiquitin E3 ligases or *in vitro* assay for substrates of specific kinases

IIa. Construction of *C. albicans* cDNA library

As both a prokaryote-based assay for targets of specific SCF ubiquitin E3 ligases and *in vitro* assay for substrates of specific kinases rely on a good quality of *C. albicans* cDNA library, we have sought to establish a best possible method for cDNA library construction. The approach of CloneMiner cDNA construction developed by Invitrogen with characteristics of high primary titers, large average insert sizes, a high percentage of full-length genes, highly efficient cloning of cDNA to multiple destination vectors without the need for restriction enzyme digestion and ligation was chosen. To enable purification of high quality of RNA and mRNA (**Figure 5A & B**), the MasterPure^{TM} yeast RNA purification kit (EPICENTRE Biotechnologies) and FastTrack MAG micro mRNA isolation kit (Invitrogen) were used, respectively. After reverse transcribing purified *C. albicans* mRNAs into cDNAs (**Figure 6A**) and ligating with *att*B adapter, the cDNA was then put into a reaction containing a donor vector (DONR222) (**Figure 5C**) with *att*P sites and BP Clonase such that an *in vitro* recombination between a substrate (cDNA) with *att*B sites and the DONR222 with *att*P sites occurs to generate an entry cDNA library based on an entry vector pENTR with *att*L sites flanking the cDNA (**Figure 6B**). From one attempt, we obtained an entry cDNA library with 2.6 $\times 10^7$ independent clones, 87% of which contain cDNA inserts (**Figure 5D**), which has sufficient number of clones representing *C. albicans* mRNA for targeting the destination vectors, although it is desirable to have more independent clones with higher percentage of insert-containing clones. The entry clone with *att*L sites and a destination vector (pDEST) with *att*R sites can be further catalyzed by LR Clonase II for recombination to generate an expression clone (pEXPR) (**Figure 6C**) with *att*B sites. Gateway Nova pDEST vectors are used in the LR reaction to create pEXPR constructs. The Nova pET-53-DEST (**Figure 7A**) or Nova pCOLA-3-DEST (**Figure 7B**) were adopted as destination vectors in the *in vitro* assay for kinase substrates

and the prokaryote-based assay for targets of specific SCF ubiquitin E3 ligases, respectively.

IIb. Consideration of efficient and CUG codon-adapted expression of *C. albicans* cDNA in *E. coli*

Given that *C. albicans* is a eukaryote with a non-universal codon usage of CUG encoding serine instead of leucine, we have sought to introduce an *E. coli* strain Rosseta 2(DE3)pLysS (Novagen) (**Figure 8**), carrying a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter, suitable for protein production of target genes cloned in T7-driven expression vectors. The strain also carries a chloramphenicol-resistant plasmid (pLysS) with a P15A replicon that encodes T7 lysozyme, an inhibitor of T7 RNA polymerase, which suppresses basal expression of T7 polymerase prior to IPTG-induction and hence stabilizes recombinant proteins that affect cell growth and viability. Importantly, plasmid pLysS is capable of expressing seven rare *E. coli* tRNAs that facilitate expression of eukaryotic genes.

To allow CUG codon of *C. albicans* adapting to encoding serine rather than leucine during the *C. albicans* cDNA expression in *E. coli*, a pACSE3-based plasmid (**Figure 9**) [1, 2] carrying gene of mutant $tRNA_{CGA \rightarrow CAG}$ for serine (**Table 5**) [3] capable of being IPTG-inducibly expressed was sought to develop such that *C. albicans* proteins expressed in *E. coli* from cDNA with CTG codon encodes serine residues, which could be critical in the *in vitro* kinase or prokaryote –based SCF ubiquitin E3 ligase assays.

IIc. Selection of vector systems to accommodate genes encoding components of *C. albicans* SCF ubiqutin E3 ligase and related proteins

Since the prokaryote-based SCF ubiquitin E3 ligase assay requires expressing SCF components and related proteins (**Figure 10**) [4] in *E. coli*, the vector systems that allow accommodating genes for those components are critical. We have adopted the pQLink vector system [5] to allow cloning each of

genes into the vector, followed by step-wise joining those genes into a single pQLink vector (**Figure 11**). Due to compatibility of replicons and antibiotic selecting genes, a total of five vectors capable of expressing seven rare *E. coli* tRNAs, and simultaneously and inducibly expressing one *C. albicans*-adapted $tRNA_{CAG}$ for serine and eight proteins were used in this assay (**Table 6**) as compared to those of three vectors capable of expressing seven rare *E. coli* tRNAs, and simultaneously and inducibly expressing one *C. albicans*-adapted $tRNA_{CAG}$ for serine and one protein in the *in vitro* kinase assay (**Table 7**).

III. Other related studies: Drafts associated with the NHRI proposal

During the process of conducting the NHRI proposal, several studies directly relevant to or partly associated with the proposal have also been carried out. One of them regarding the functional study of *CaCDC4* resulted in a manuscript entitled "Dissecting the *Ca*Cdc4 domains reveals instability nature of CaCdc4 and its involvement in cell flocculation", which is ready to submit for publication as attached (**MS1**) in **the item 11** (98 fiscal year publications) **of this report**. To allow ubiquitin encoding sequence introducing into *C. albicans ADH1* locus and is expressed as a 6×HisFLAG tagged ubiquitin in a doxycycline induced manner in which the induced ubiquitin-6×HisFLAG can compete with the endogenous non-tagged ubiquitin due to being overexpressed, a Tet-on vector has been developed. A draft version of manuscript (**MS2**) entitled "Construction of *Candida albicans* Tet-on tagging vectors with an Ura-blaster cassette" is attached in the **in the item 11**. As an alternative of prokaryote-based assay for targets of SCF^{CaCdc4} ubiquitin E3 ligase, the study resulted in an early draft of manuscript (**MS3**) entitled "Affinity purification of *Candida albicans Ca*Cdc4 associated proteins reveals presence of novel proteins for morphogenesis" and is attached in the **in the item 11**.

貳、98年度計畫著作一覽表

Journal

序號	計畫產出名稱	產出型式	Impact factor	致謝對象
1	Ping Chang Lai, Wei-Chung Lai, Tzu-Ling Tseng, Chun-Wen Cheng, and Jia-Ching Shieh Dissecting domains of Candida albicans Cdc4 reveals its instability nature and involvement of cell flocculation. Biochem Bioph Res Co 2009;Supported by 98NHRI-EX98-9808SI (SCI) Submitted	Foreign	2.648	NHRI
2	Wei-Chung Lai, Tzu-Ling Tseng, Ting Jian, Tai-Lin Lee, and Jia-Ching Shieh Construction of Candida albicans Tet-on tagging vectors with an Ura-blaster cassette. Yeast 2009;Supported by 98NHRI-EX98-9808SI (SCI) in Preparation	Foreign	2.622	NHRI
3	Tzu-Ling Tseng, Wei-Chung Lai, Chuan Li, Hsiao-Fang Sunny Sun, Tzong-Der Way, and Jia-Ching Shieh Affinity purification of Candida albicans CaCdc4 associated proteins reveals presence of novel proteins for morphogenesis. Biochem Bioph Res Co 2009;Supported by 98NHRI-EX98-9808SI (SCI) in Preparation	Foreign	2.648	NHRI

Patent

序號	計畫產出名稱
	無

Book

序號	計畫產出名稱
	無

Conference Paper

序號	計畫產出名稱

	無

Technical Report

序號	計畫產出名稱
	無

參、98年度計畫重要研究成果產出統計表

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

(係指執行98年度計畫之所有研究產出結果)

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〔註〕：

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部份，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專　　著：為對某項學術進行專門性探討之純學術性作品。

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者。

技術服務：凡有關各項研究計畫之規劃與評審、技術督察與指導及專業技術服務事項等。

肆、98年度計畫重要研究成果

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

計畫之新發現、新發明或對學術界、產業界具衝擊性(impact)之研究成果，請依性質勾選下列項目。

☐1. 研發或改良國人重要疾病及癌症的早期診斷方式及治療技術

☐2. 發展新的臨床治療方式

☐3. 發展新生物製劑、篩檢試劑及新藥品

☐4. 瞭解常見疾病及癌症之分子遺傳機轉

☐5. 瞭解抗癌藥劑對癌細胞之作用機制

☐6. 提供有效的疾病預防策略

☐7. 利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展

☐8. 醫療保健政策相關研究

☐9. 瞭解環境毒理機制及重金屬對人體健康的影響

☐10. 研發適合臨床使用的人造器官及生醫材料

☐11. 縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用

☐12. 改進現有醫療器材的功能或增加檢驗影像的解析能力

☑13. 其他重要疾病或醫藥衛生問題研究

白色念珠菌型態形成的機制能進一步了解以期未來對此真菌感染能有效控制

一、 計畫之新發現、新發明或對學術界、產業界具衝擊性（impact）之研究成果，請敘述其執行情形。

本計畫為建構測定之技術平台以瞭解後轉譯修飾中之泛素化或磷酸化是否為白色念珠菌型態形成扮演關鍵角色。發展兩大方法，其一為不同白色念珠菌形態是否有差異性泛素化或磷酸化次蛋白體圖譜之產生，由此尋求差異性蛋白質以分析其扮演形態形成之功能。另一部分則為於原核生物測定真核生物特有之 SCF 泛素 E3 連接酶，避免於真核系統中標靶蛋白質因 SCF 泛素 E3 連接酶的作用而被降解難以被鑑定出來，或於體外系統鑑定激酶受質。

由於白色念珠菌為二倍體且無完全有性生殖無法如釀酒酵母利用遺傳方法分析，任何非遺傳法且具基因組層次或如本計劃次蛋白質體層次分析相關蛋白質功能皆具非凡意義，即便蛋白體圖譜建構本身不具特殊技術新穎性。尤其本計劃之標的為調控型態形成之蛋白質，更顯現其未來治療白色念珠菌感染的價值，因型態形成能力與白色念珠菌致病力有關。

至於第二部分之以原核生物測定真核生物特有之 SCF 泛素 E3 連接酶則具技術新穎性，因牽涉成份極多，所需載體數目亦屬極限，加以需引進原核生物罕用之 tRNA 基因，極具挑戰性，另外加入適應白色念珠菌非通用之 CUG 密碼子之 tRNA 基因則屬白色念珠菌特有之需求。目前，第一項技術平台仍處於建構白色念珠菌差異性蛋白體圖譜及其完善化階段，而第二項技術平台則處於構築白色念珠菌 cDNA 庫及取得不同載體及特定 *E. coli* 品系並完成部分 SCF 泛素 E3 連接酶相關成份基因的選殖，一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因及相關載體也已取得用於適應白色念珠菌非通用之 CUG 密碼子。

二、 計畫對民眾具教育宣導之研究成果（此部份將為規劃對一般民眾教育

或宣導研究成果之依據，請以淺顯易懂之文字簡述研究成果，內容以不超過300字為原則）

白色念珠菌是一種人類伺機性的真菌病原菌，能造成全身性感染而致死，常見於免疫不全的病人。雖有藥物治療但它發展抗藥性的可能及藥物的副作用都讓我們需對其有更進一步了解以期有效控制其感染。由於它的致病力與其型態形成能力相關，我們致力於研究其型態形成的調控機制，而牽涉此調控機制的基因及其蛋白質產物便是我我們積極要尋找的對象。我們利用兩個技術平台試圖尋找相關蛋白質。第一種方法是較廣泛的尋找是否在白色念珠菌的酵母菌形、真菌絲形及假菌絲形之間有不同蛋白質透過改變其特性執行功能。另外一種則是用已知型態形成的蛋白質在一種測定系統中收尋更多與其活性調節有關的蛋白質，而此類蛋白質的功能一定也與控制型態形成有關。

三、 簡述年度計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

一為白色念珠菌細胞形態可順利誘導；其二為可實施二維電泳並建立差異性蛋白體圖譜；其三為以 Invitrogen CloneMiner cDNA 方法構築白色念珠菌 cDNA 庫中之入口 cDNA 庫；其四為考量完備白色念珠菌 cDNA 有效率的及可適應 CUG 密碼子表達於 *E. coli* 中，其中包含引進原核生物罕用之 tRNA 基因及一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用之 CUG 密碼子於 *E. coli* 表達；其五為選擇載體系統容納白色念珠菌基因編碼 SCF 泛素 E3 連接酶之各個成份及相關蛋白質，其中獲得載體 pQLink 系列可同時容納多個基因極具價值。

以 Invitrogen CloneMiner cDNA 方法構築白色念珠菌 cDNA 庫中之入口 cDNA 庫其優勢為可有多重終點載體選擇而能執行不同用途，如本實驗目的

外可構築酵母菌雙雜核 cDNA 庫；由三、四及五項完成後可以原核生物測定真核生物特有之 SCF 泛素 E3 連接酶，建立後具廣泛應用性有技轉潛力；引進原核生物罕用之 tRNA 基因及一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用之 CUG 密碼子於 *E. coli* 表達，尤其後者可誘導產生，雖僅適用於解決白色念珠菌基因於 *E. coli* 表達時其非通用之 CUG 密碼子如何可編碼絲氨酸的問題，此白色念珠菌重組基因如其他物種表達於 *E. coli* 的方法雖屬白色念珠菌專一仍具有技轉潛力。

四、 成效評估（技術面、經濟面、社會面、整合綜效）

就技術面而言，建構差異性泛素化或磷酸化白色念珠菌次蛋白體圖譜的能力為往後其他轉譯修飾次蛋白體圖譜的分析奠定基礎；而以原核生物測定真核生物特有之 SCF 泛素 E3 連接酶同時需解決多項問題，建立完備可有個研究面向衍生；如其他真核生物複合體酵素可否引入原核生物測定；白色念珠菌以外物種具非通用密碼子其重組基因可用相似方法於 *E. coli* 表達。就經濟面而言，一個技術平台解決多個問題本身便是極具成本效益；加上具技術轉移的可能性；再者，本計劃技術層面非涉高成本儀器之運作；固其實施極具經濟效益。就社會面而言，透過本計劃之實施，人員訓練後之發展具多重可能性，可擴大未來就業競爭力；若運作得宜更可能有更多人員參與，代表更多的就業人員及社會的穩定。

本計劃整合綜效而言，技術上有延展性，經濟上能商品化，社會上提供好的就業條件能實現社會正義，實在需要更長時間的計畫支持。

五、 下年度工作構想及重點之妥適性

下年度目標訂在發展有效豐富化被泛素化或磷酸化之白色念珠菌次蛋白質體的方法方能妥適地建構包括泛素化或磷酸化之差異性次蛋白質體定

量圖譜，此部分訂於計劃目標之第二及第三項。另外，預計完成所有質體建構，包括白色念珠菌 cDNA 庫，才能於第三年執行包括計劃目標之第四及第五項：鑑定白色念珠菌 SCF 泛素 E3 連接酶之特定標靶蛋白質及鑑定色念珠菌 *Ca*Cdc7 激酶之受質。

六、 檢討與展望

本計劃之執行，我們是持積極的態度。然而有些實驗設計即便參考文獻及初步的數據顯然有時仍有不足。最令我們訝異的莫過於白色念珠菌細胞形態之轉變與否具品系依賴性，使得原先結合特定品系構築而有效純化泛素化蛋白質之構想不得作不依賴特定白色念珠菌品系方向設計，此為待檢討之處。然而獲得本計劃並執行時，我們仍本著精益求精的態度持續思考設定之目標如何有效完成及技術平台如何更臻完備。數項原先並未完善考慮之事項：引進原核生物罕用之 tRNA 基因及一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用之 CUG 密碼子於 *E. coli* 表達；為容納白色念珠菌基因編碼 SCF 泛素 E3 連接酶之各個成份及相關蛋白質，思考多種可能性卻仍可能受限，之後於尋得可同時容納多個基因於同一 pQLink 載體下方成為最可能的選擇。

我們對能有機會發展分屬兩類技術平台的本計劃持有極大的期待。一方面引進原核生物罕用之 tRNA 基因及一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用 CUG 密碼子於 *E. coli* 表達雖為平凡概念，若能建立對研究白色念珠菌具不凡的貢獻；以原核生物測定真核生物特有之 SCF 泛素 E3 連接酶，建立後則具廣泛應用性，我們也有大期待；能順利建構差異性泛素化或磷酸化白色念珠菌次蛋白體圖譜則對爾後我們發展其他轉譯修飾次蛋白體圖譜為一基石，對白色念珠菌研究又可向前邁一大步。

伍、98年度計畫所培訓之研究人員

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

種類				人數	備註
專任人員	1.	博士後研究人員	訓練中	0	
			已結訓	0	
	2.	碩士級研究人員	訓練中	2	二維電泳及電泳膠蛋白體圖譜分析
			已結訓	0	
	3.	學士級研究人員	訓練中	0	
			已結訓	0	
	4.	其他	訓練中	0	
			已結訓	0	
兼任人員	1.	博士班研究生	訓練中	1	建構細菌行念珠菌SCF汎素連接酵素反應
			已結訓	0	
	2.	碩士班研究生	訓練中	1	建立念珠菌cDNA庫
			已結訓	0	
醫師			訓練中	0	
			已結訓	0	

特殊訓練課程（請於備註欄說明所訓練課程名稱）

陸、參與98年度計畫所有人力之職級分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	2人
第二級	副研究員、副教授、總醫師、助教授	1人
第三級	助理研究員、講師、住院醫師	1人
第四級	研究助理、助教、實習醫師	3人
第五級	技術人員	0人
第六級	支援人員	0人
合計		7人

〔註〕：

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事、會計、秘書、事務人員及維修、機電人員等。

柒、參與98年度計畫所有人力之學歷分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

類別	學　　歷　　別	參與人次
1	博士	3 人
2	碩士	2 人
3	學士	0 人
4	專科	0 人
5	博士班研究生	1 人
6	碩士班研究生	1 人
7	其他	2 人
合計		9 人

捌、參與98年度計畫所有協同合作之研究室

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

機構	研究室名稱	研究室負責人
成功大學	分子醫學研究所	孫孝芳
中山醫學大學	生物醫學科學學系	李娟
中山醫學大學	生物醫學科學學系	謝家慶

玖、九十八年度計畫執行情形

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

一、 請簡述原計畫書中，九十八年預計達成之研究內容

Our original aims shown below were designed for five years. We have set our focus on aims #1, especially on recruiting and training, establishing methodology, and essential equipment purchasing. An essential work on *C. albicans* cDNA library construction has also been included in which the cDNA library can be used in specific aim #3 and #4. We have designed to carry out the rest of work in parallel in the next four years.

As the situation changed, we have re-scheduled the aims. **In the first year**, we have aimed to establish quantitative proteome profiling (2-D gel electrophoresis, gel staining, scanning, and analysis) of *C. albicans* among cells of yeast, hyphae, and pseudohyphae. In parallel, a *C. albicans* strain expressing 6His-FLAG-ubiquitin in replace with ubiquitin in the original aim#1has been set to carry out. Also, *C. albicans* cDNA libraries used in the original aim#4 and #5 have been included. Most importantly, recruiting and training personnel, establishing methodology, and essential equipment purchasing have been central to our progress. In the second year, we have aimed to establish efficient methods for enrichment of subproteome (ubquitinated and phosphorylated proteins) that can be used in aim#2 and #3. All the constructs including the most appropriate *C. albicans* cDNA libraries have set to completed for used in the aim#4 and #5. In the final year, we have aimed to identify targets by differential subproteome profiling as in aim#2 and #3 and by prokaryote-based SCF ubiquitin E3 ligase and *in vitro* kinase assay as in aim#2 and #3, respectively.

1. To construct *C. albicans* strain expressing 6His-FLAG-ubiquitin in replace with ubiquitin
2. To differentiate *C. albicans* ubiquitin-proteome during morphogenesis by

quantitative profiling

3. To differentiate *C. albicans* phosphoproteome during morphogenesis by quantitative profiling
4. To identify specific targets of *C. albicans* SCF E3 ligases
5. To identify specific targets of *Ca*Cdc7 kinase

二、請詳述九十八年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

As shown in the table below, we have achieved nearly all the aims that we have set out to do with some requiring further optimization for perfection, except to construct a *C. albicans* strain expressing 6×HisFLAG-ubiquitin. This is due to the fact that the cell-type induction in *C. albicans* is strain-dependent as described in the summary. The strain we originally used was an auxotrophic BWP17 (*ura/ura his/his arg/arg*), suitable for introducing sequence encoding 6×HisFLAG with ubiquitin gene by PCR using a vector system with *C. albicans URA3* for selection. The alternative Tet-on system where ubiquitin-encoding gene is introduced into *C. albicans ADH1* locus and inducibly expressed as a 6×HisFLAG tagged ubiquitin by doxycycline for competing with the endogenous non-tagged ubiquitin is also *URA3*-based. Hence, we will focus our work on establishing methods for enrichment of ubiquitinated proteins from a wild-type SC5314 strain devoid of strain construction for conducting differential subproteome profiling.

In fact, we have done more works than we have set out to do by making constructs required for a prokaryote-based SCF ubiuitin E3 ligase assay besides construction of *C. albicans* cDNA library. Due to multi-members of SCF complex and associated proteins for the assay, and tRNAs adapt for eukaryotic cells and *C. albicans* non-universal CUG codon, multiple vectors with compatible replicons and antibiotic resistant genes were introduced, which have

been thoroughly thought through.

	Month	1	2	3	4	5	6	7	8	9	10	11	12	%*	Note
1	Induction of cell types (yeat, hyphae, and pesudohypae)				×	×	×	×	×					90	Further optimization is still needed
2	Establishment quantitative proteome profiling								×	×	×	×	×	80	Not optimal
3	Construction of a *C. albicans* strain expressing 6His-FLAG-ubiquitin				×	×	×	×	×					30	Closed due to strain used not being able to induce pseudohyphae
4	Construction of *C. albicans* cDNA libraries						×	×	×	×	×	×	×	90	Need further optimization
5	Other constructs for SCF ubiuitin E3 ligase assay					×	×	×	×	×	×	×	×	80	Not being listed as this year's work[§]
6	Recruiting personnel	×	×	×										100	
7	Training personnel			×	×	×	×	×	×	×	×	×	×	100	Ongoing, sufficient for this year
8	Establishing methodology	×	×	×	×	×	×	×	×	×	×	×	×	100	Ongoing, sufficient for this year
9	Purchasing the essential equipments		×	×	×	×	×							100	
10	Operation and optimization of new Equipments						×	×	×	×	×	×	×	80	Further optimization is still needed

* represents % of achievement; [§] These have not been scheduled on this year's work

拾、附錄

The tables and figures for the report summary of the proposal are on pages 10-1~10-6 and 10-7~10-19, respectively.

References are on page 10-20.

Table 1. Induction of different cell types of *C. albicans*

Strain (SC5314)	Conditions
Yeast form	1. YPD medium (pH5.2)+2% glucose, 30℃ O/N 2. YPD medium (pH5.2)+2% glucose, refreshed at 30℃ 4hr
Hyphae form	1. YPD medium (pH5.2)+2% glucose, 30℃ O/N 2. YPD medium (pH7.2)+2% glucose+10% fetal calf serum, induced at 37℃ % for 1.5hr
Pseudohyphae	1. YPD medium (pH5.2)+2% glucose, 30℃ O/N 2. 1st induction : 2.5mM KH2PO4 (pH6.5) 10.2mM L-proline 2.6mM N-acetyl-D-glucosamine 3mM MgSO4 • 7H2O 2% Glucose at 37℃ for 4hr 3. 2nd induction : 200/250/300/350mM Potassium phosphate buffer (pH6.5) 10.2mM L-proline 2.6mM N-acetyl-D-glucosamine 3mM MgSO4 • 7H2O 2% Glucose at 37℃ for 4hr

Table 2. Steps involved in 2D-gel electrophoresis

1. Sample preparation: as in Table 1
   SC5314 (yeast form)
   SC5314 (hyphae form)
   SC5314 (300mM GPP mediumàpseudohyphae)
2. Cell washing (with ddH2O)
3. Cell disruption:
   Lysis buffer (see Table 3) + glass beads (425-600 μm, acid-washed)
4. 2-D clean up kit
5. Quantitating protein samples: 2-D Quant Kit
6. Sample loads for InstantBlue TM staining : 125μg
7. Strips: pH3-10, 7cm
8. IEF: Conditions (see Table 4)
9. Equilibration:
   SDS equilibration buffer (+ iodoacetamide): 25min
   SDS equilibration buffer (+ iodoacetamide): 25min
10. SDS-PAGE: 12%
11. Protein staining :
    InstantBlueTM, containing Coomassie dye (G-250), ethanol, phosphoric acid and solubilizing agents in water
    O/N at room temperature

Table 3. Content of lysis buffer for protein extraction from *C. albicans*

Voltage mode	Voltage (V)	Time	kVh
Passive Rehydration	0V	2:00	
Active Rehydration	60V	14:00	
Step and hold	300V	0:30	0.2
Gradient	1000V	0:30	0.3
Gradient	5000V	1:20	4.0
Step and hold	5000V	0:25	2.0

Table 4. Content of lysis buffer for protein extraction from *C. albicans*

Lysis buffer (pH 7.0)
250mM Tris-HCl pH 8.0
250mM NaCl
50mM NaF
0.5% Triton x-100
0.1% tween 20
0.5% NP40
10% Glycerol
2mM Sodium ortho-vanadate
2mM PMSF
1X Protease inhibitor
10mM β-mercaptoethanol

Table 5. tDNA operons in the E. coli K12 Genome

Name	Isoacceptors (with Unmodified Anticodons)	Coordinate[a]	Length	Angle[b]	Strand
rrnC	**Glu2**(UUC) **Asp1**(GUC) **Trp**(CCA)	3944496	3,599	1.5	leading
argX	**Arg3**(CCG) **His**(GUG) **Leu1**(CAG) **Pro3**(UGG)	3979988	436	4.6	leading
rrnA	**Ile1**(GAU)[c] **Ala1B**(UGC)	4034730	194	8.8	leading
rrnB	**Glu2**	4165951	75	19.0	leading
tufB	**Thr4**(UGU) **Tyr2**(GUA) **Gly2** (UCC)[c] **Thr3**(GGU)	4172967	441	19.6	leading
rrnE	**Glu2**	4207352	75	22.2	leading
pheU	**Phe** (GAA)	−4360204	75	34.1	lagging
glyV	**Gly3** (GCC) **Gly3 Gly3**	4389938	298	36.4	leading
leuX	**Leu4** (CAA)	4493973	84	44.4	leading
leuV	**Leu1 Leu1 Leu1**	−4603970	322	53.0	lagging
rrnH	**Ile1**[c] **Ala1B Asp1**	225381	3,623	73.2	leading
aspV	**Asp1**	236931	76	74.1	leading
ThrW	***Thr2***(CGU)	262095	75	76.1	leading
thrX[d]	**Thr2**	296402	76	78.7	leading
argU	**Arg4**(UCU)	563946	76	99.5	leading
metT	**Metm**(CAU) **Leu3**(UAG) **Gln1**(UUG) **Gln1 Metm Gln2**(CUG) **Gln2**	−696356	703	109.7	lagging
lysT	**Lys**(UUU) **Val1**(UAC) **Lys Val1 Lys Lys Lys**	779777	1,098	116.3	leading
serW	**Ser5**(GGA)	−925194	87	127.5	lagging
serT	**Ser1**(UGA)	−1030935	87	135.7	lagging
serX	**Ser5**	−1096875	87	140.8	lagging
tyrT	**Tyr1**(GUA) **Tyr1**	−1286845	378	155.6	lagging
valV	**Val2A**(GAC) **Val2B**(GAC)	1744459	157	191.1	lagging
glyW	**Gly3 Cys**(GCA) **Leu5**(UAA)	−1990140	302	210.1	leading
serU	**Ser2**(CGA)	−2041579	89	214.1	leading
asnT	**Asn**(GUU)	2042571	75	214.2	lagging
asnW	**Asn**	−2056124	75	215.3	leading
asnU	**Asn**	2057873	75	215.4	lagging
asnV	**Asn**	2060282	75	215.6	lagging
proL	**Pro2**(GGG)	2284231	76	233.0	lagging
argW	**Arg5**(CCU)	2464329	74	246.9	lagging
alaW	**Ala2**(GGC) **Ala2**	−2516251	190	251.0	leading
valU	**Val1**(UAC) **Val1 Val1 Lys**	2518951	397	251.2	lagging
rrnG	**Glu2**	−2727464	75	267.4	leading
ileY	**Ile2**(CAU)[c]	−2783857	75	271.7	leading
serV	**Ser3**(GCU) **Arg2**(ACG) **Arg2 Arg2 Arg2**	−2816667	861	274.3	leading
metZ	**Metf1**(CAU) **Metf1 Metf1**	2945409	296	284.3	lagging
glyU	**Gly1** (CCC)[c]	−2997079	73	288.3	leading
pheV	**Phe**	3108383	75	296.9	lagging
ileX	**Ile2**[c]	3213239	75	305.1	lagging
metY	**Metf2** (CAU)	−3315930	76	313.0	leading
leuU	**Leu2** (GAG)	−3319799	86	313.3	leading
rrnD	**Ile1**[c] **Ala1B Thr1**(GGU)	−3424789	3,572	321.5	leading
proK	**Pro1**(CGG)	−3706321	76	343.3	leading
selC	**Sel-Cys**(UCA)	3833849	90	353.2	lagging

Note that *SerU* tRNA gene shaded in yellow is used to generate a tRNA mutant (CGA→CAG) encoding serine for codon CUG

Table 6. Genes and into which vectors they cloned for the prokaryote-based SCF ubiquitin E3 ligase assay

Vector	Replicon	MCS1	MCS2	MCS3	MCS4	MCS5	Selection marker gene
pACSE3	P15A	tRNA gene *serU* mutant (TCG→ CTG) of *E. coli* strain DH10B for tRNA (CGA →CAG)					Tet^R, tetracyclin resistant
pCDFDuet-1	CloDF13	*CaUBA1* (E1-encoding gene) (orf19.7438)	*CaUBC4* (*CaCDC34*) (E2-encoding gene) (orf19.6529)				Sm^R, streptomycin/spectinomycin resistant
Gateway Nova pCOLA-3-DEST	ColA	*C. albicans* cDNA (substrates)					Kan^R, kanamycin resistant
pQLinkN	ColE1	*CaCDC4* (F-box protein-encoding gene) (orf19.2559)	*CaCDC53* (Cul1-encoding gene) (orf19.1674)	*CaSKP1* (orf19.4427)	*CaHRT1*(*CaROC1*, *CaRBX1*) (orf19.233.1)	*CaUBI3* (ubiquitin-encoding gene)	Amp^R, ampicillin/carbenicillin resistant
pQLinkH	ColE1	*CaCDC4* (F-box protein-encoding gene) (orf19.2559)	*CaCDC53* (Cul1-encoding gene) (orf19.1674)	*CaSKP1* (orf19.4427)	*CaHRT1*(*CaROC1*, *CaRBX1*) (orf19.233.1)	*CaUBI3* (ubiquitin-encoding gene)	Amp^R, ampicillin/carbenicillin resistant
pQLinkG	ColE1	*CaCDC4* (F-box protein-encoding gene) (orf19.2559)	*CaCDC53* (Cul1-encoding gene) (orf19.1674)	*CaSKP1* (orf19.4427)	*CaHRT1*(*CaROC1*, *CaRBX1*) (orf19.233.1)	*CaUBI3* (ubiquitin-encoding gene)	Amp^R, ampicillin/carbenicillin resistant
pLysS (carried by *E. coli* strain	P15A	Seven tRNA genes for the					Cam^R, chloramphenicol resistant

Rosseta 2)		codon AUA, AGG/AGA, AGA, CUA, CCC, GGA, and CGA/CGG					
Note that pQLink vectors allow genes on three different pQLink vectors joining to have combination of gene products of non-tagged version (from pQLinkN, $(His)_7$-tagged version(from pQLinkH), and GST-tagged version(from pQLinkG) in *E. coli* (see also **Figure 11**).							

Table 7. Genes and into which vectors they cloned for the *in vitro* kinase assay

Vector	Replicon	MCS	Selection marker gene
pACSE3	P15A	tRNA gene *serU* mutant (TCG→CTG) of *E. coli* strain DH10B for tRNA (CGA→CAG)	Tet^R, tetracyclin resistant
Gateway Nova pET-53-DEST	ColA	*C. albicans* cDNA (substrates)	AmpR, ampicillin/carbenicillin resistant
pLysS (carried by *E. coli* strain Rosseta 2)	P15A	Seven tRNA genes for the codon AUA, AGG/AGA, AGA, CUA, CCC, GGA, and CGA/CGG	Cam^R, chloramphenicol resistant

Figures and legends

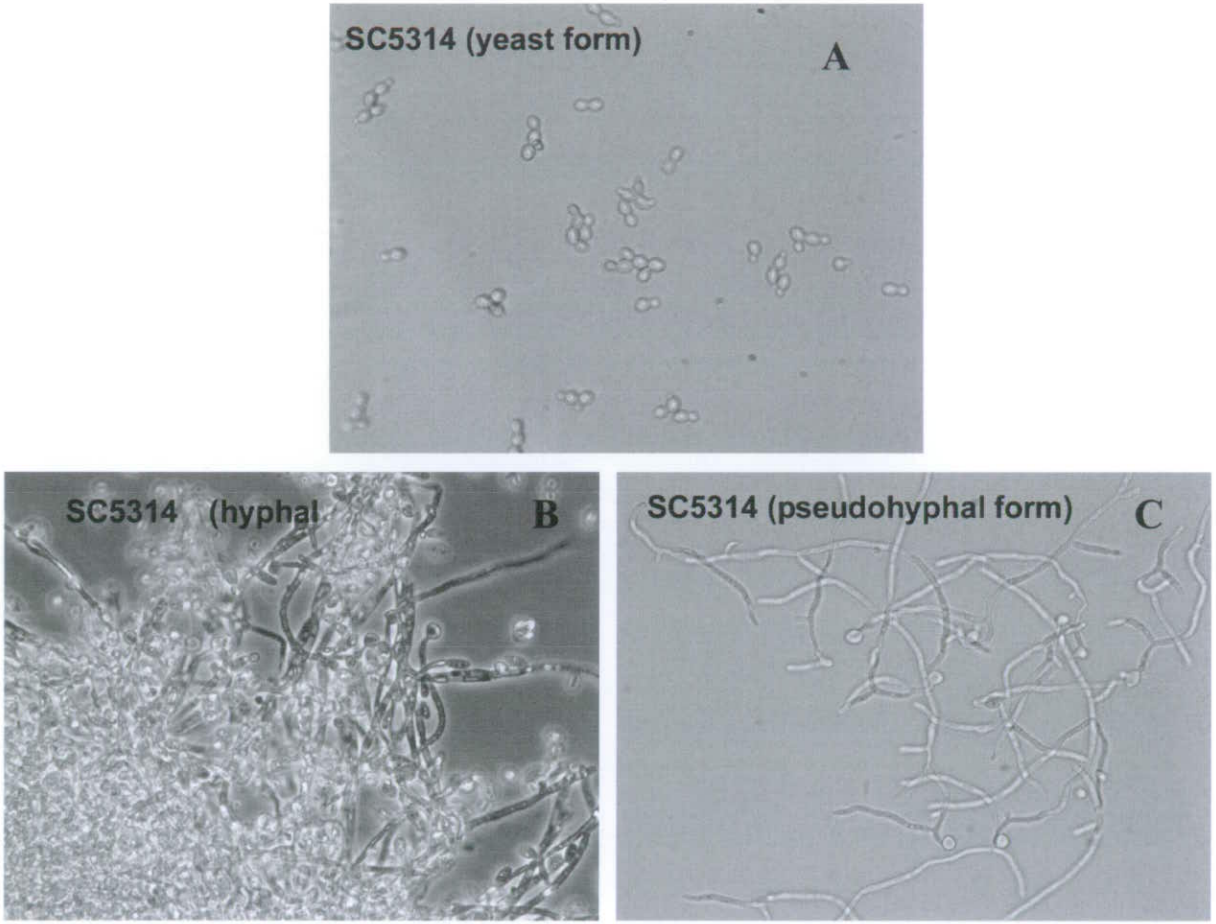


Figure 1. Induction of different cell types of *C. albicans*. The wild-type strain SC5314 is used. (**A**) Yeast form. (**B**) Hyphal form. **C.** pseudohyphal form.

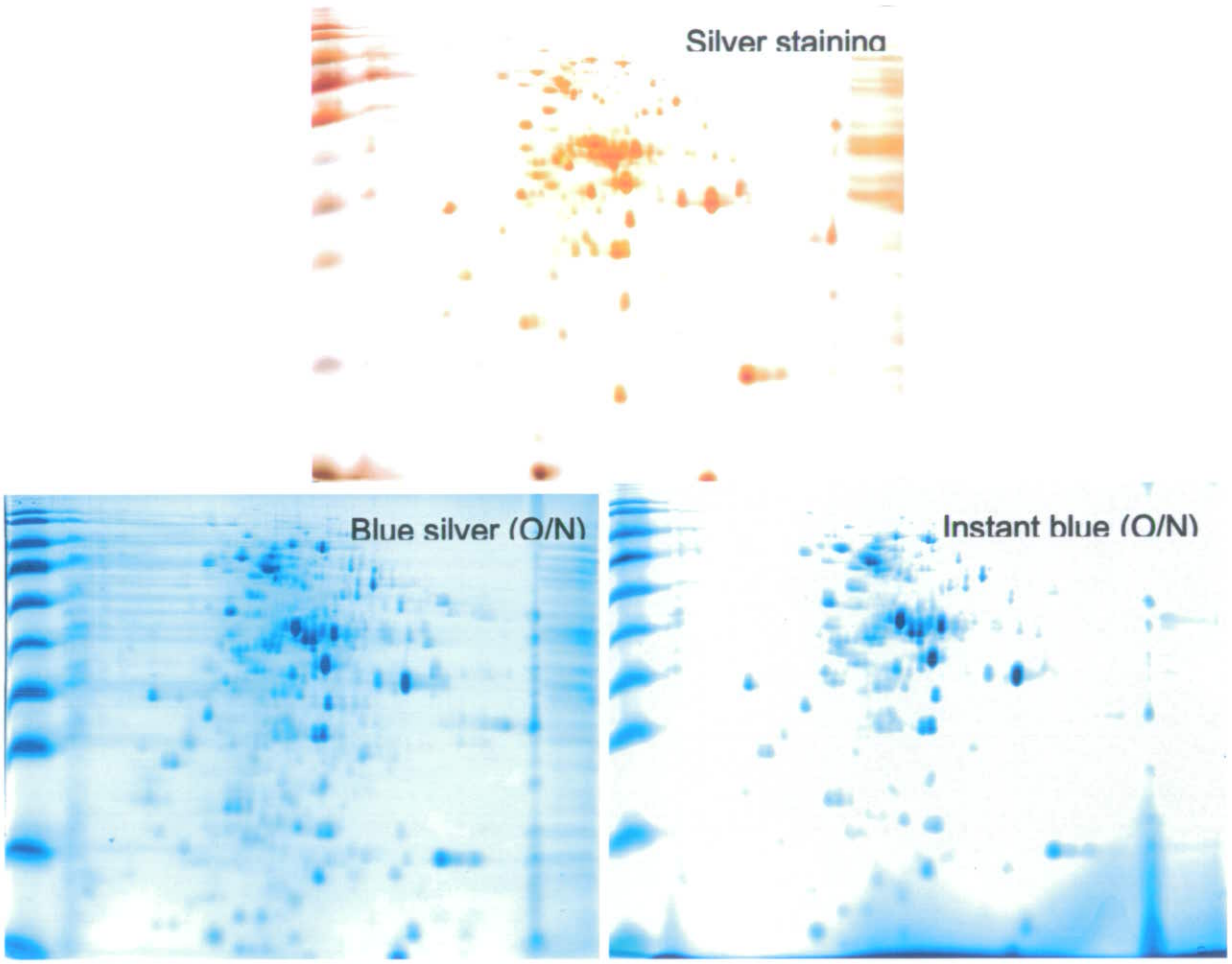


Figure 2. Scanned images of 2D-gel stained with diverse agents. The gels stained with different reagents are indicated.

Figure 3

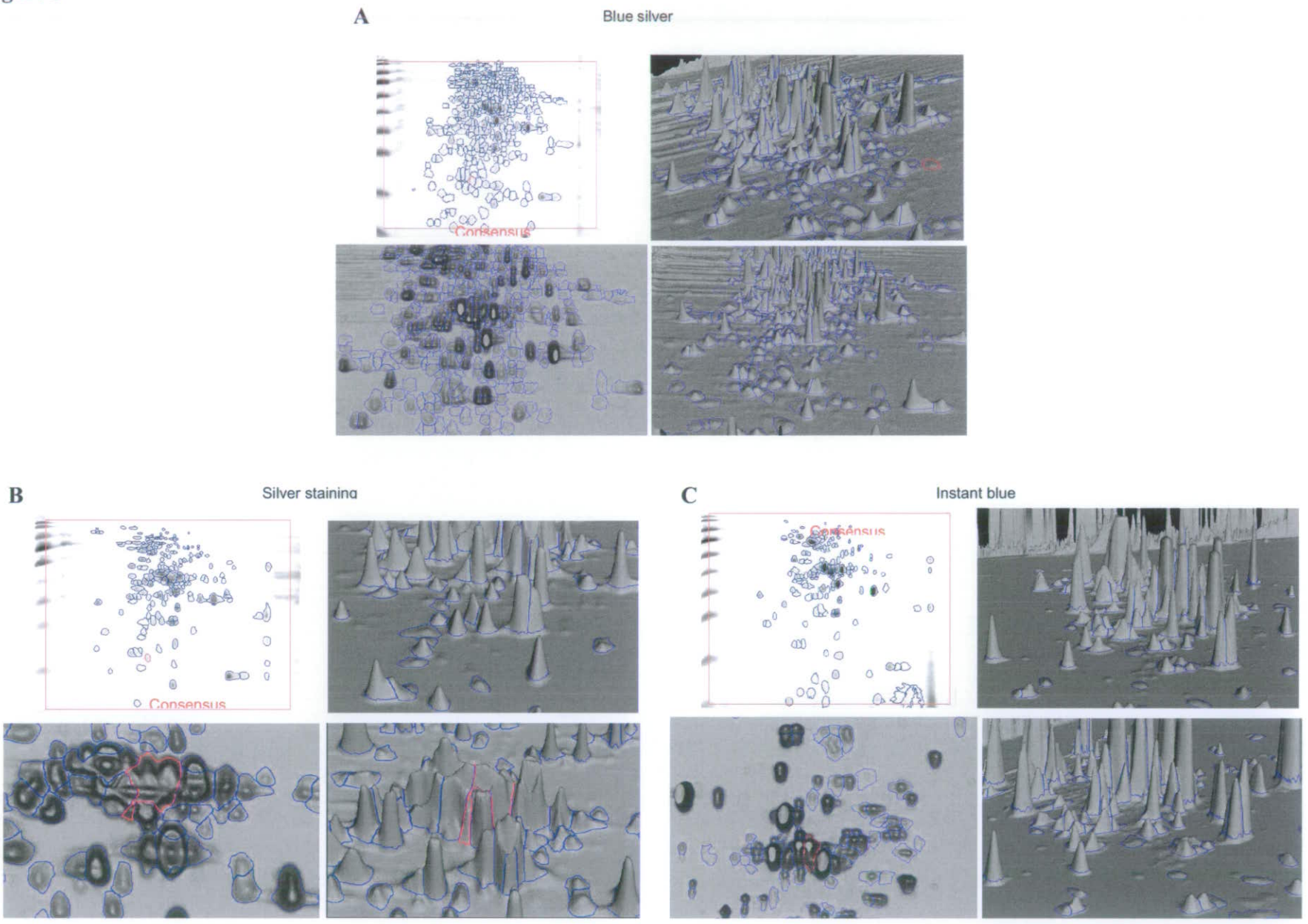
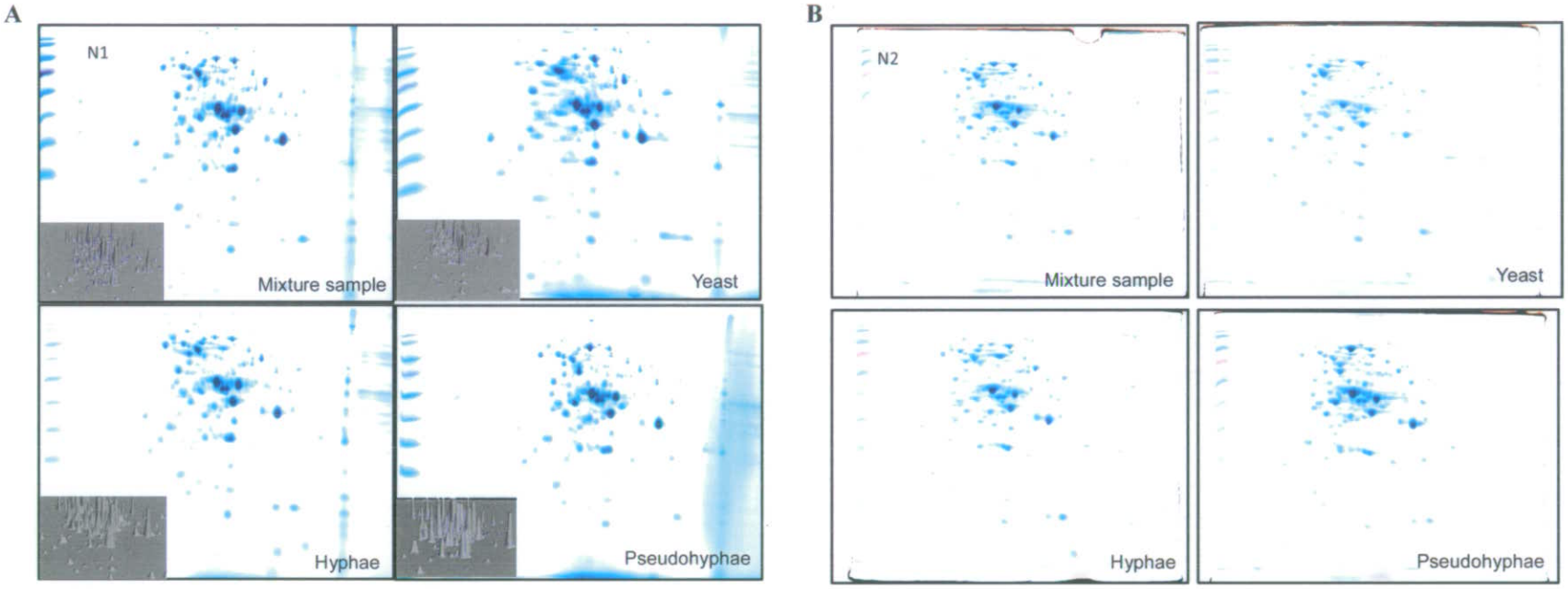
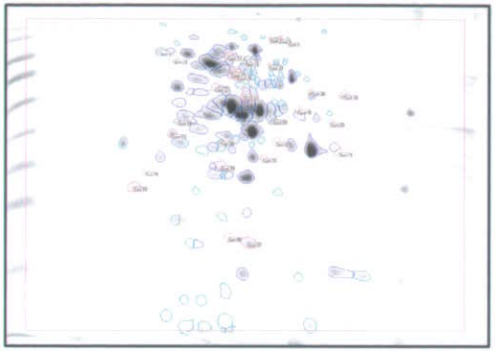

A
Blue silver
Consensus
B
Silver staining
Consensus
C
Instant blue
Consensus

Figure 3. Analysis of proteome profiles of 2D-gel among different staining agents. (**A**) Blue silver. (**B**) Silver staining. (**C**) Instant blue. Global and localized 1D images are on the top-left and bottom-left panels, respectively. Global and localized 3D images are on the top-right and bottom-right panels, respectively. Individual proteins clustered together may be difficult to determine presence of specific proteins within a particular region, as indicated on bottom panels in (**B**) or on bottom-left in (**C**).

Figure 4

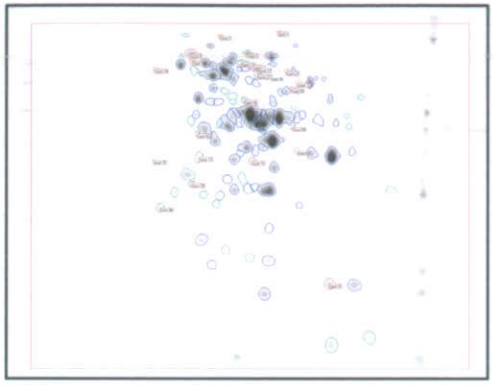

A
N1
Mixture sample
Yeast
Hyphae
Pseudohyphae
B
N2
Mixture sample
Yeast
Hyphae
Pseudohyphae

C



Complete Matches = 53	mixture sample		yeast form	
Match	Spot No.	Norm. Vol. Ratio (Discontinuous)	Spot No.	Norm. Vol. Ratio (Discontinuous)
18	17	1	18	-8.263
69	66	1	58	-3.608
82	78	1	66	-3.389
71	68	1	59	-3.075
15	14	1	9	-2.238
100	96	1	83	2.021
10	9	1	12	2.163
43	42	1	41	2.5
83	80	1	63	2.541
1	1	1	3	2.626
30	29	1	25	2.67
32	31	1	26	2.691
105	99	1	87	3.031
90	88	1	78	4.17

D



Complete Matches = 53	mixture sample		hyphae form	
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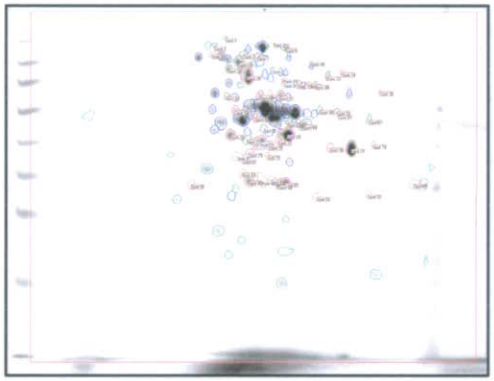
69	66	1	59	-12.198
89	86	1	79	-5.267
42	41	1	41	-3.876
5	4	1	5	-3.355
71	68	1	62	-2.866
83	80	1	73	-2.85
84	81	1	75	-2.582
19	18	1	11	-2.373
100	96	1	86	-2.336
23	22	1	23	-2.115
27	26	1	25	-2.066
108	102	1	91	2.312
86	83	1	76	2.681
34	33	1	35	3.018
18	17	1	17	3.382
9	8	1	6	5.492

E



Complete Matches = 53	mixture sample		pseudohyphae form	
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81	79	1	61	-22.636
18	17	1	21	-21.588
70	67	1	63	-15.208
78	75	1	65	-14.587
73	70	1	64	-5.753
85	82	1	71	-5.697
8	7	1	7	-4.645
3	2	1	1	-4.091
59	57	1	51	-3.069
22	20	1	18	-3.061
37	36	1	34	-2.933
24	23	1	20	-2.661
58	55	1	49	-2.41
6	5	1	5	-2.385
10	9	1	9	-2.283
31	30	1	27	-2.163
45	43	1	42	-2.116
29	28	1	26	2.304
43	42	1	41	2.481
39	38	1	33	2.49
94	90	1	78	2.578
93	89	1	75	2.752
42	41	1	40	3.219
34	33	1	32	3.59
36	34	1	31	4.258
26	25	1	24	5.046
4	3	1	2	5.783
54	51	1	50	5.797
88	85	1	69	6.794
99	95	1	76	7.653
61	60	1	58	8.488
105	99	1	87	13.055
90	88	1	73	50.347
87	84	1	70	66.194

Figure 4. Establisment of differential proteome profiling among cell types of *C. albicans*. (**A**) and (**B**) proteome profiles of two independently prepared protein samples (N1 and N2) from *C. albicans* cells of yeast, hyphae, and pseudohyphae. An equal amount of proteins from each of the three cell types was mixed and used for normalization. (**C**), (**D**), and (**E**) show analyzed profiles of differential proteome of yeast, hyphae, and pseudohyphae against the mixed samples, respectively. Protein spots are numbered for their individual identities on the gels, accompanied by the tables with the corresponding identities. Proteins up-regulated with more than two-fold (+2) and down-regulated with less than two-fold (-2) are given values in the table with red and green, respectively. Consensus and non-consensus proteins are defined in blue and light on the gels, respectively. Cell type-specific proteins are defined in orange, representative ones on each of the gels are indicated in arrows with orange.

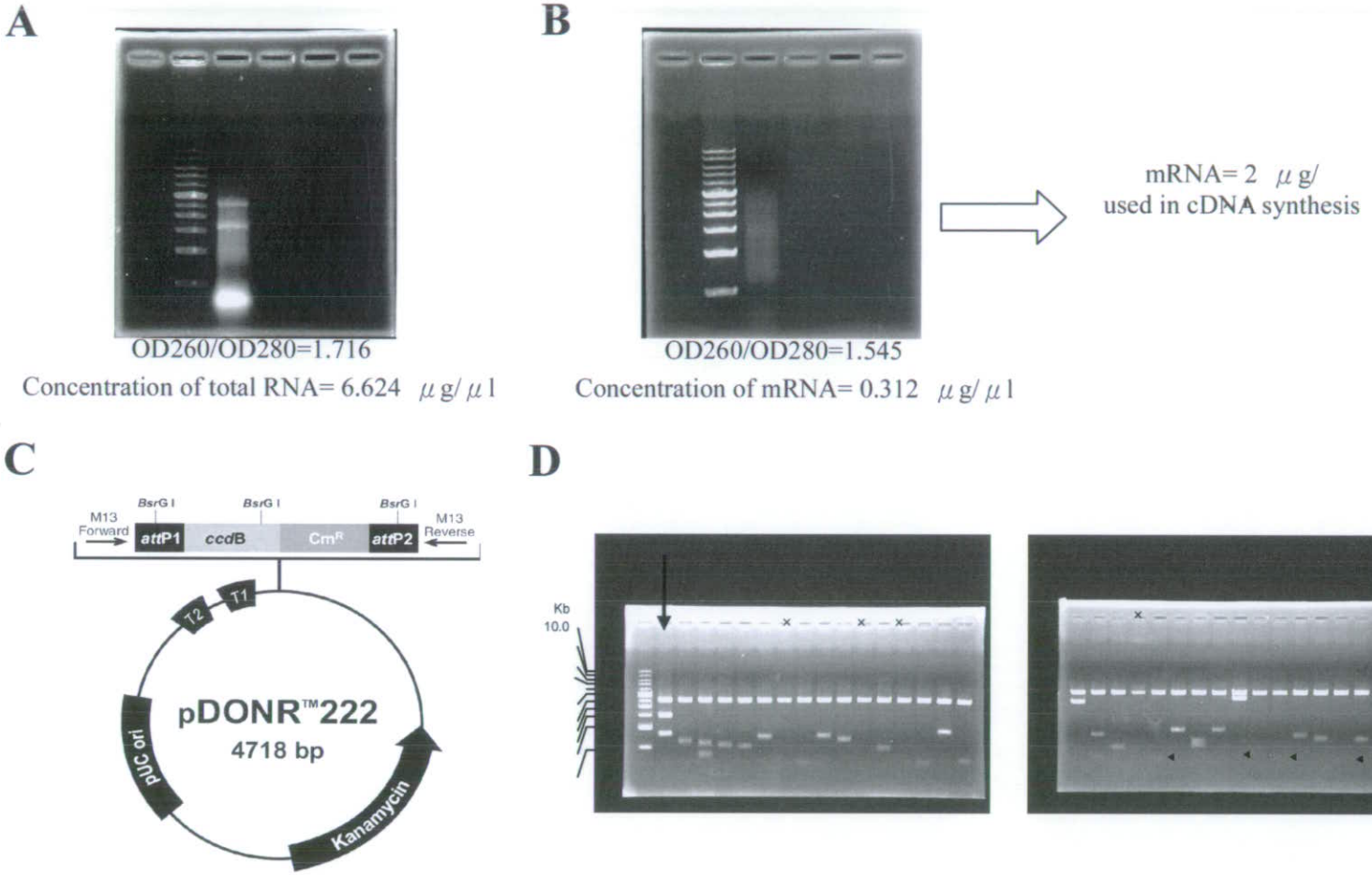

Figure 5. Verification of the process of cDNA library construction. (**A**) Purified *C. albicans* total RNA. (**B**) Purified *C. albicans* mRNA. (**C**) A map of entry clone-based vector pDONR 222. (**D**) Thirty-one randomly selected clones from the entry cDNA library for presence of cDNA. The clones were purified and digested with *Bsr*GI prior to resolving on 1% agarose gel and revealing by EtBr staining. Note that pDONR 222 gives a digestion pattern of 2.5 kb, 1.4 kb, and 790 bp when digested with *Bsr*GI, indicated by arrow. Entry clones with cDNA inserts give a common vector DNA of 2.5 kb and other DNA fragments with various sizes when digested with *Bsr*GI. Arrow heads are used to indicate cDNA inserts with DNA fragments barely seen due to their small size. The crosses indicate those clones without cDNA inserts.

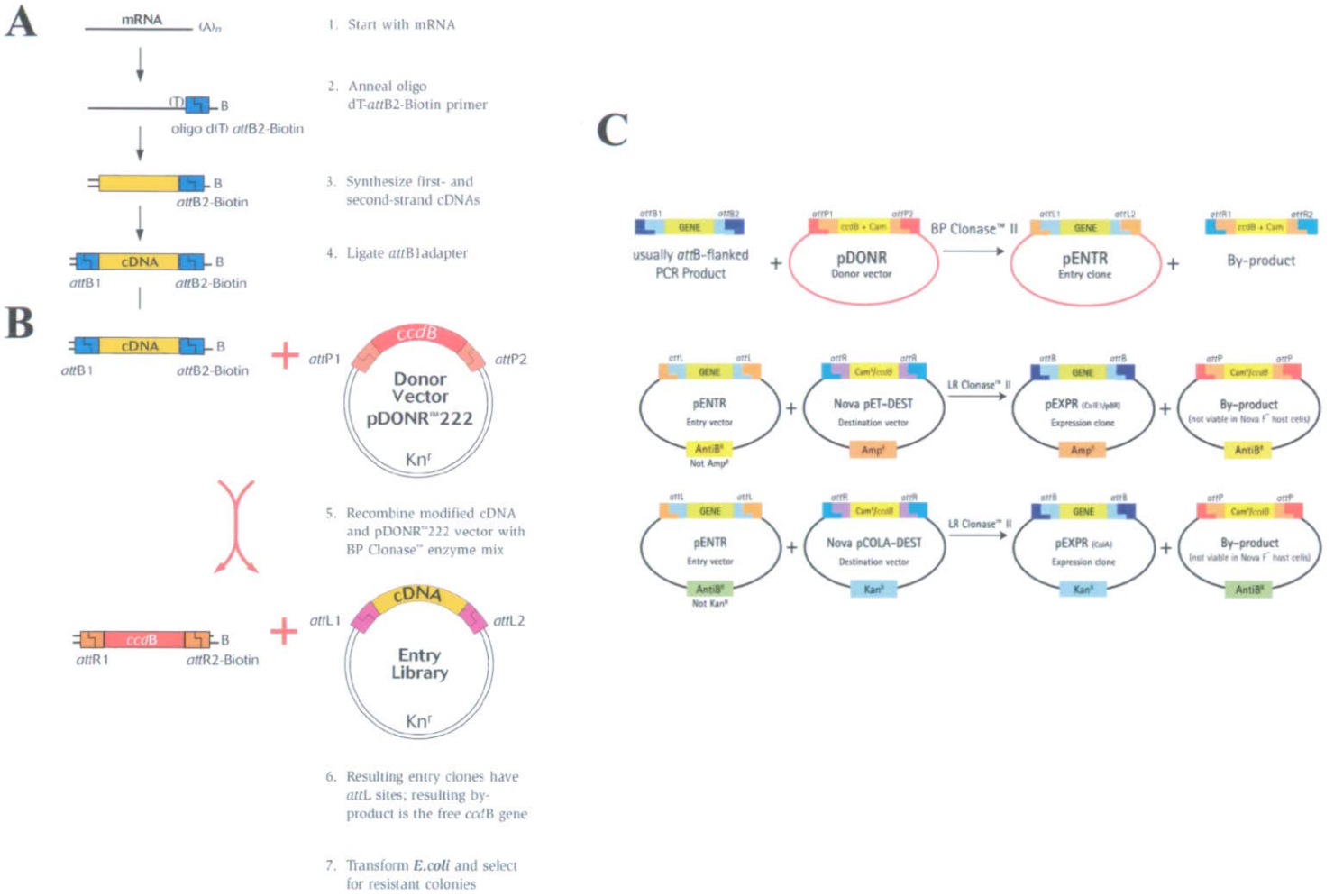

Figure 6. Diagram showing the steps of the CloneMiner™ (Invitrogen) cDNA library construction. (**A**) synthesis of cDNA. (**B**) Construction of the entry cDNA library. (**C**) Construction of the destination cDNA library.

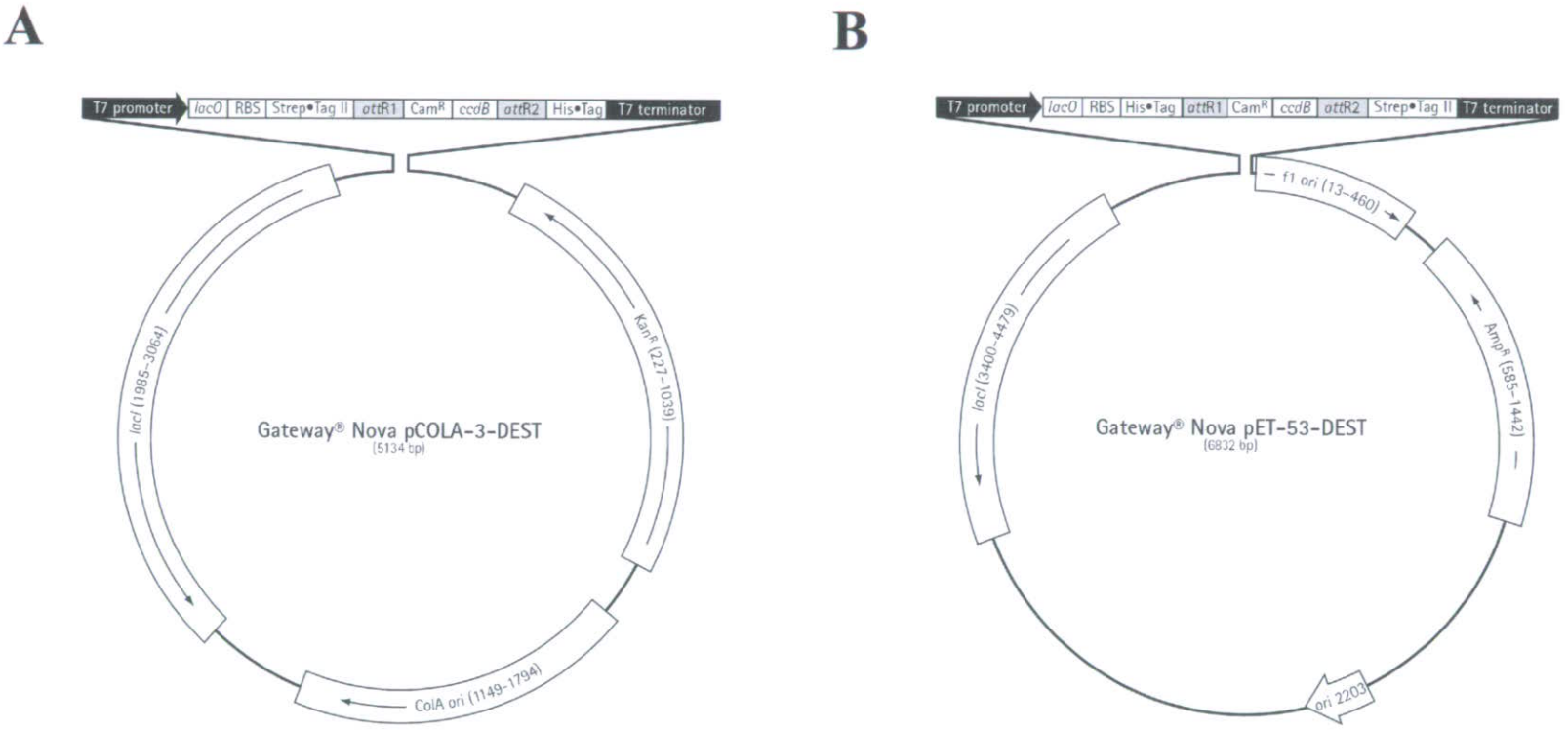

Figure 7. Maps of vectors for destination *C. albicans* cDNA libraries. (**A**) Nova pCOLA-3-DEST. (**B**) Nova pET-53-DEST.

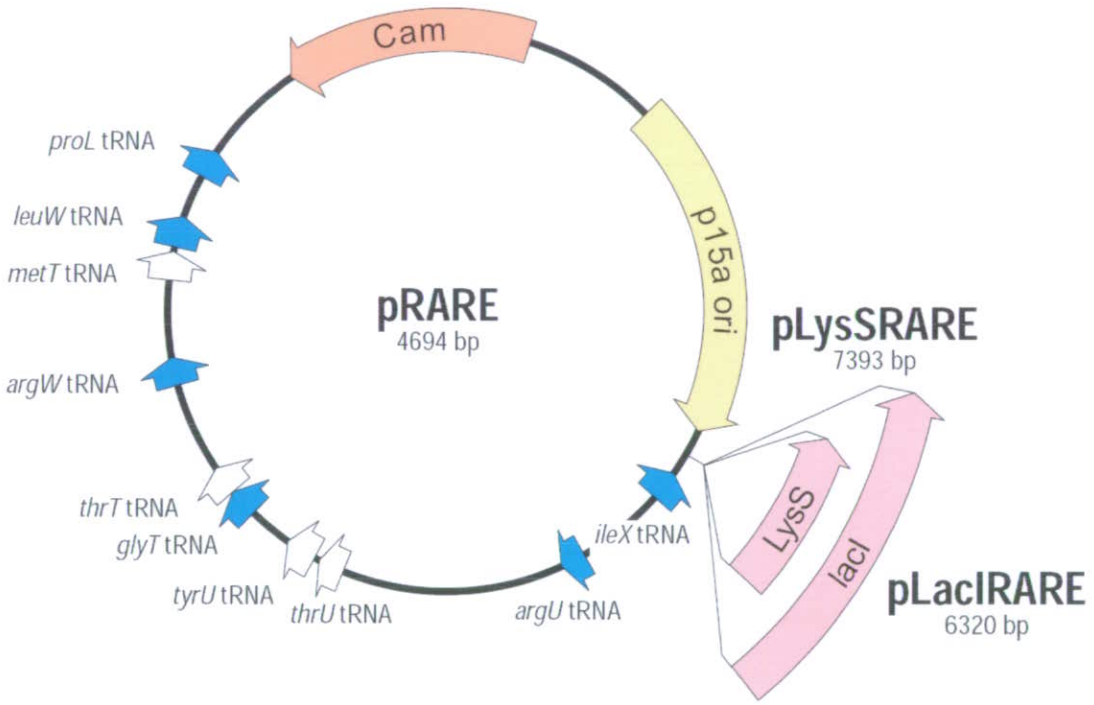

Figure 8. Map of pLysS plasmid. The plasmid is one of pRARE plasmid family. Seven tRNA genes for tRNAs *ileX* (AUA), *leuW* (CUA), *proL* (CCC), *glyT* (GGA), *argU* (AGG/AGA), and *argW* (AGA), shown in blue, which correspond to rare codons in *E. coli*, are under their native promoters control. The tRNA gene for tRNA (CGA/CGG) is not shown on the map.

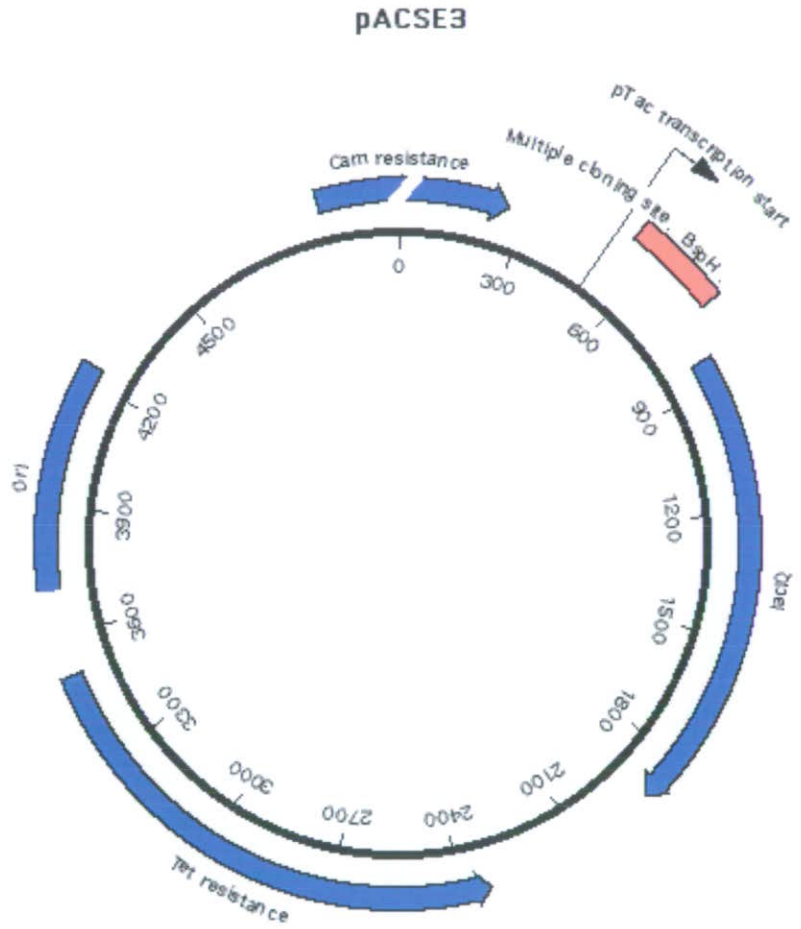

Figure 9. Map of pACSE3 vector. The map is adopted from reference [2].

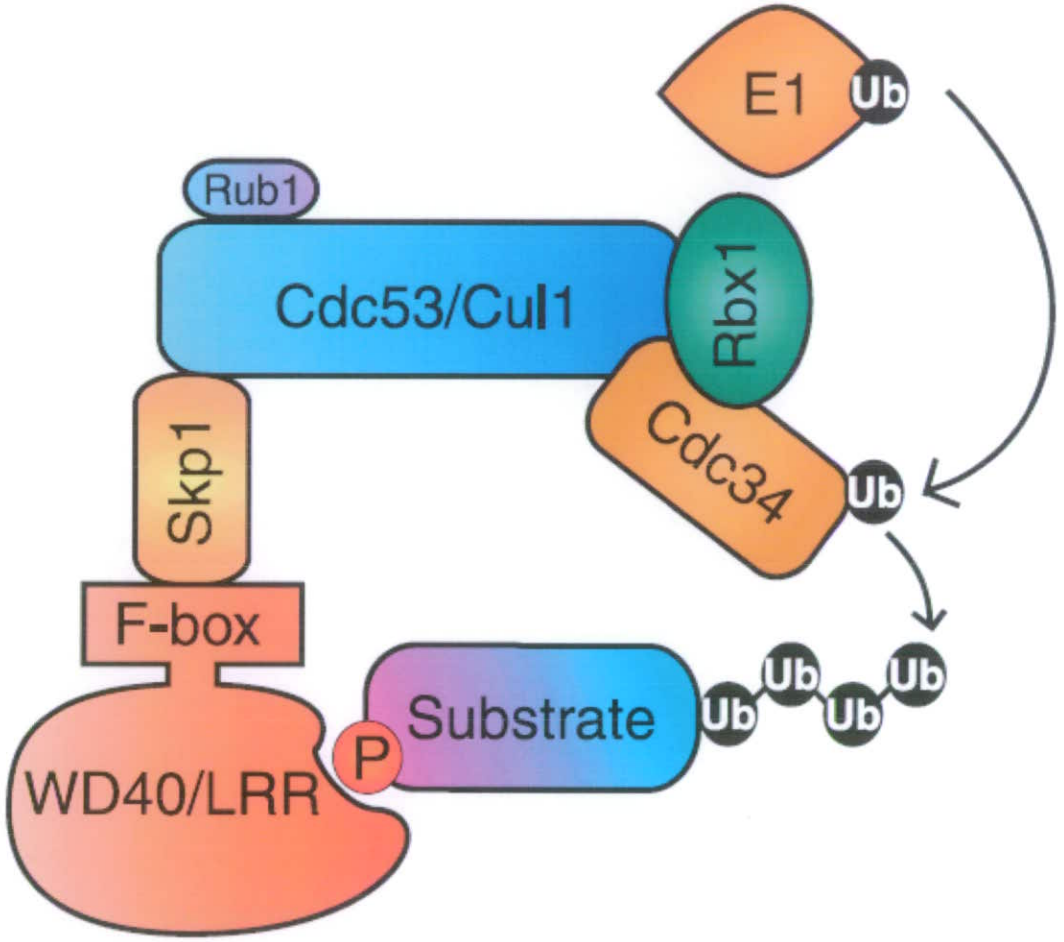

Figure 10. A cartoon exhibiting the essential components for the activity of SCF ubiquitin E3 ligase for ubiqutination of its substrate. The diagram is adopted from reference [4]. Note that the Rub1 regulates Cdc53/Cul1 through neddylation, thus is excluded as an essential component of SCF ubiquitin E3 ligase in our prokaryote-based SCF ubiquitin E3 ligase assay.

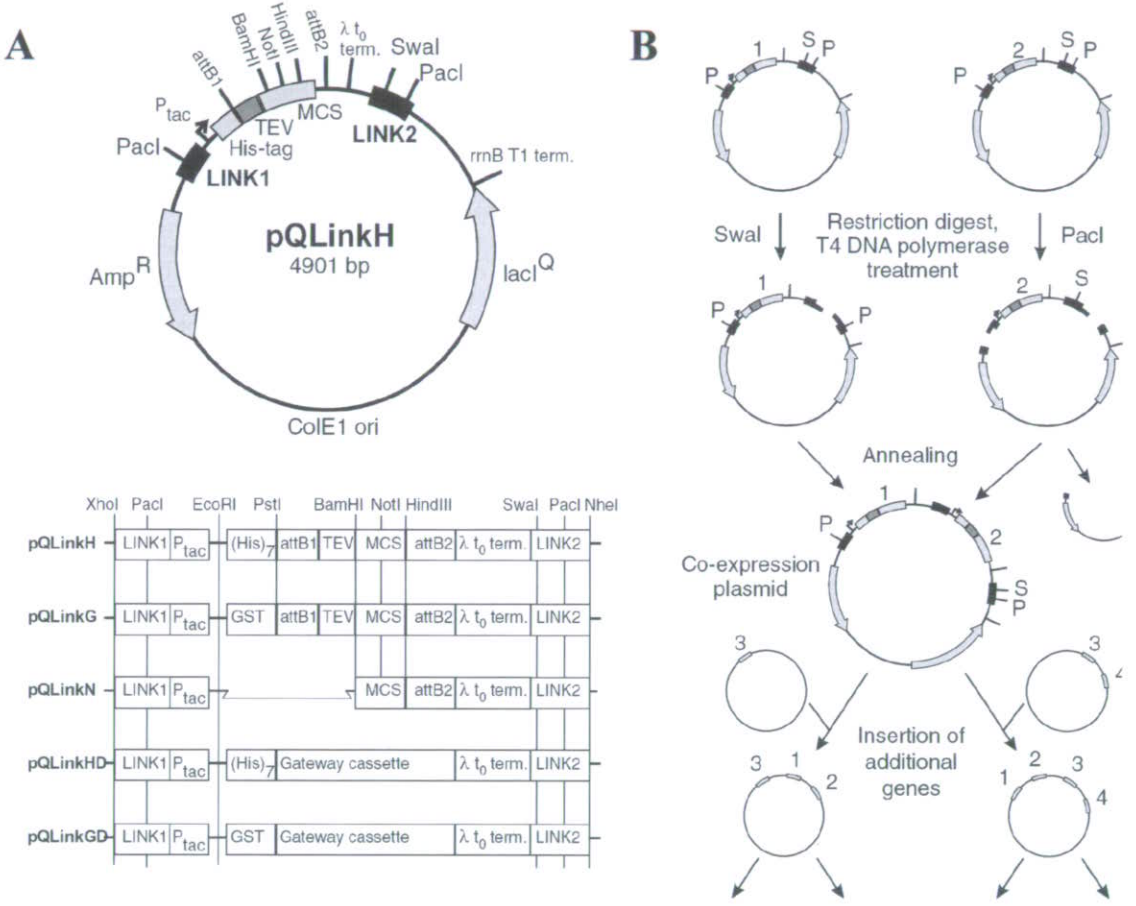

Figure 11. Map of pQLink vector seires and construction of co-expression. (**A**) Map of vector pQLinkH and genetic elements of all pQLink vectors. (**B**) Construction of a co-expression plasmid from two pQLink plasmids with two different cDNA inserts, labeled 1 and 2. The resulting plasmid can accept additional inserts, labeled 3 and 4.

References:

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拾壹、本年度之著作抽印本或手稿

Dissecting domains of *Candida albicans* Cdc4 reveals its instability nature and involvement of cell flocculation

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Running title: *Candida albicans* Cdc4 instability flocculation

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Abstract

Candida albicans CDC4 (*CaCDC4*), encoding an F-box protein of ubiquitin E3 ligase, negatively regulates filamentous development. To further elucidate *CaCDC4* function, a *C. albicans* strain with one *CaCDC4* allele deleted and the other under the repressible control of *C. albicans MET3* (*CaMET3*) promoter was constructed before introducing each of plasmids capable of doxycycline (Dox)-induced expressing various *C. albicans* Cdc4 (*Ca*Cdc4) domains. By expressing each of assorted *Ca*Cdc4 domains under Dox-induced but *CaMET3*-repressed condition, the instability nature of *Ca*Cdc4 was observed as full-length *Ca*Cdc4 was hardly detected and *Ca*Cdc4 was found to be cleaved at N-terminus. The analysis of phenotypic consequences revealed that domains of F-box and WD40- repeat were critical for *Ca*Cdc4 function because strains with or without expression of each of these domains in the absence of full length *Ca*Cdc4 exhibited non-discriminatory phenotypes of filamentation and flocculation. Interestingly, the Dox-induced expression of *Ca*Cdc4 decreased flocculation rather than completely suppress filamentation in the *CaMET3-CaCDC4* repressed strain, which are likely the characteristics of regulation by the Tet-on and *CaMET3* repressible systems and the nature of *Ca*Cdc4 protein. Consequently, we suggest a novel function of *CaCDC4* for negatively regulating flocculation that is independent of filamentation.

Keywords:*Candida albicans*; *CDC4*; domain; Tet-on; flocculation

Introduction

Candida albicans, a natural diploid without complete sexual cycle, exists as yeast, pseudohyphal, and hyphal cells [1]. It is capable of morphological switch by environmental stimuli [2], essentially via cAMP-mediated and MAPK signalling pathways [3]. Importantly, its ability to alter among cell types is associated with virulence to human [4]. Cyclins are also known to control morphogenesis in *C. albicans* [5]. Whereas G1 cyclin of Hgc1 expresses in hyphae [6], that of Ccn1 is essential for hyphal development [7]. By contrast, Cln3 negatively regulates filamentation as depleting Cln3 causes germ tube extension [8, 9].

Recently, F-box proteins encoded by *C. albicans GRR1* (*CaGRR1*) and *CaCDC4* have been shown to play role in filamentous development [10-12]. Cdc4 and Grr1, originally identified in the budding yeast *Saccharomyces cerevisiae*, encode ubiquitin E3 ligases, each of which belongs to a member of Skp1-cullin/Cdc53-F-box (SCF) complex, which are known to play a role in degradation of regulatory proteins in eukaryotes by the ubiquitin-proteasome system [13]. Specific SCF complex is designated by its associated F-box protein, the variable member with two interacting domains of F-box for Skp1 and WD40-repeat (or LRR) for substrates [14], such that Cdc4 can be named as SCF^{Cdc4}. To progress through G1-S transition in *S. cerevisiae*, SCF^{Cdc4} is required for degrading Sic1 [15, 16] and Far1 [17], the cyclin-dependent kinase inhibitors. Hence, *S. cerevisiae CDC4* (*ScCDC4*) is essential in *S. cerevisiae*. Other SCF^{Cdc4} substrates, such as Cdc6 for initiation of DNA replication [18] and Gcn4 for control of amino acid biosynthesis[19], are also known.

Although *Ca*Cdc4 is a structural homolog of *S. cerevisiae* Cdc4 (*Sc*Cdc4) and capable of rescuing the mitotic defect caused by loss of *ScCDC4* in *S. cerevisiae* [12], the function of *Ca*Cdc4 and ScCdc4 are dissimilar. While depleting *C. albicans* Grr1 (*Ca*Grr1) stabilizes Ccn1p and Cln3 for pseudohyphal growth [11, 20], depleting *Ca*Cdc4 causes accumulation of Sol1 (Sic1 1like) for hyphal development [10], suggesting that the control of degradation on target proteins in *C. albicans* requires *Ca*Grr1 and *Ca*Cdc4. Significantly, depletion of *Ca*Cdc4 initiates no cell cycle arrest but yeast-to-filament transition. Hence, *CaCDC4* is nonessential and suppresses filamentation. While *C. albicans* Sol1 is likely a substrate of SCF^{CaCdc4}, demonstrated by the reduction of Sol1 when *Ca*Cdc4 is overexpressed [10], direct evidence has yet to be established. Additionally, mutants of *Cacdc4* null and *Cacdc4 sol1* double null exhibited comparable filamentous form, refuting that Sol1 is the sole target of *Ca*Cdc4.

To further elucidate *CaCDC4* function in *C. albicans*, we have sought to dissect the *Ca*Cdc4 domains associated with filamentation. In this study, we made a *C. albicans* strain deleted one *CaCDC4* allele and repressed the other via *CaMET3* by methionine and cycteine (Met/Cys). We used this strain to introduce each cassette from plasmids capable of Dox-inducing various *Ca*Cdc4 domains. We observed the instability nature of *Ca*Cdc4 and the essentiality of F-box and WD40-repeat for *Ca*Cdc4 function. We also showed that *C. albicans* cells lacking *Ca*Cdc4 resulted in flocculation, suggesting a novel role of *CaCDC4* for negatively regulating cell flocculation.

Materials and Methods

Strains and growth conditions. E. coli strain DH5α was used for routine manipulation of plasmids. They were grown at 37°C in LB broth medium [21] 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*) [22]. They were grown at 30°C in either yeast extract-peptone-glucose (YEPD) or supplemented minimal synthetic defined (SD) medium with 2% glucose with or without 2% agar [23]. While Ura^+ prototrophs were selected on SD agar plates without uridine, His^+ prototrophs on SD plates without histidine. Selection for lost of *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 expression of *CaCDC4* controlled by *CaMET3* promoter, strains were grown on SD medium or plates with 2.5 mM Met/Cys. To induce gene expression under the Tet-on system, 50 μg/ml Dox (Sigma) was added to YEPD or SD medium.

Plasmid DNA manipulation. Plasmid DNA was extracted routinely from *E. coli* cultures using Gene-Spin™ MiniPrep purification Kit-V^2 (PRO TECH, Taipei, Taiwan) by the instruction of manufacturer. Plasmid DNA was transformed into *E. coli* by the method of $CaCl_2$. The plasmid constructs or PCR-generated products were introduced into *C. albicans* by the lithium acetate method as described [24].

Construction of C. albicans strains. A strain with the expression of *CaCDC4* repressible was made initially. Mini Ura-blaster cassette flanked with 60-bp sequences homologous to *CaCDC4* was PCR-amplified using template of plasmid pDDB57 and

long primers of CaCDC4-URA3-F and CaCDC4-URA3-R (Table 1), transformed for integration into *CaCDC4* locus of BWP17 for Ura$^+$ to generate strain JSCA0018. The plasmid pFA-HIS1-MET3-CaCDC4 with partial *CaCDC4* coding sequence encoding N-terminal *Ca*Cdc4 (1-563) was linearized with *Bsp*EI and transformed into JSCA0018 for His$^+$ to generate JSCA0021 (Fig. 1A; Table 1). Cells of JSCA0021 were plated onto plates with 5-FOA to induce recombination between two copies of *dpl200* flanking mini Ura-blaster for loss of *CaURA3* that generates JSCA0022.

To allow express each of cassettes encoding assorted *Ca*Cdc4 domains in *C. albicans*, a Tet-on plasmid, pTET25M, derived from pTET25 [25] for inducing gene expression with Dox, has been developed (W.-C. Lai, *et al.*, manuscript in preparation). To regulate *CaCDC4* expression by the Tet-on system, coding sequence of *CaCDC4* was PCR-amplified using plasmid CaCDC4-SBTA bearing *CaCDC4* (Lai & Shieh, unpublished data), primers CaCDC4-SalI and CaCDC4-BglII (Table 2), and *Pfu* polymerase (5 U/μl, MD bio), digested with *Sal*I and *Bgl*II, and then cloned into pTET25M in which pTET25M-CaCDC4 was generated. Moreover, *CaCDC4*-6HF, coding for 6×histidine and FLAG (6HF) at C-terminal *Ca*Cdc4, was PCR-amplified with primers CaCDC4-6HF SalI and CaCDC4-6HF BglII (Table 2), followed by digesting with *Sal*I and *Bgl*II and cloning into pTET25M to obtain pTET25M-CaCDC4-6HF.

To define the function of distinct *Ca*Cdc4 domains (Figure 2A), different *CaCDC4* portions were used to replace the full length *CaCDC4* coding sequence on pTET25M-CaCDC4-6HF. By using sets of primers listed in Table 2, the following constructs were made: pTET25M-ΔNCaCDC4-6HF (with primers CaCDC4 ΔN AatII and CaCDC4 ΔN XhoI), encoding N-terminal truncated *Ca*Cdc4, pTET25M-F-6HF (with primers CaCDC4

F-box AatII and CaCDC4 F-box XhoI), encoding F-box domain with flanking regions, pTET25M-WD40-6HF (with primers CaCDC4 WD40 AatII and CaCDC4 ΔN XhoI), encoding eight copies of WD40-repeat, pTET25M-ΔNF-6HF (with primers CaCDC4 ΔN AatII and CaCDC4 F-box XhoI), encoding truncated N-terminal *Ca*Cdc4 and F-box domain. All inserts of the constructs were released with *Aat*II and *Xho*I for replacement of the full-length *CaCDC4* on pTET25M-CaCDC4-6HF. Consequently, plasmids bearing those *CaCDC4* segments flanked with common *CaADH1* sites were digested with *Sac*II and *Kpn*I, each of which was transformed into *C. albicans* for integration at *CaADH1* locus. All strains were verified by colony PCR with specific primers (see Table 2).

Protein extraction and Western blot analysis. Cultured cells were collected and the total protein from each sample was extracted as described previously [26]. The proteins were resolved by SDS-PAGE and transferred electrophoretically to PVDF membranes (PerkinElmer, Boston, USA). Proteins on the membranes were probed with polyclonal antibody to FLAG polyclonal antibody (Sigma), detected by SuperSignal West Pico Chemiluminescent Substrate Kit (PIERCE), recorded by Luminescent Image Analyzer (FUJIFILM LAS-1000), and analyzed by ImageGauge 3.46 and L Process v 1.96 (FUJIFILM).

Microscopic observation. Cells were grown in SD medium with the required supplements and were visualized and recorded with Nikon 50i microscope at 400× magnification.

Cell flocculation assay. Cells were grown in SD medium with the required supplements to saturation in the absence of 2.5 mM Met/Cys. To ensure presence of the same number of cells in the initial culture, the cells were diluted into fresh medium with

similar OD_{600} in the presence of 2.5 mM Met/Cys and 50 μg/ml Dox and grown to another 48 hrs before resuspending to observe the ability of cells to flocculate.

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 *Tet* promoter directly in *C. albicans*, BWP17 with both alleles of *CaCDC4* deleted was sought to construct for use in accommodating 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 [27] to generate a strain JSCA0018 (Fig. 1A; Table 1), which was used to delete the second *CaCDC4* allele to obtain *Cacdc4* null mutant . However, *Cacdc4* null mutants with toughened cell walls were filamentous and cumulative (Lai & Shieh, unpublished data), which obstructed transformation.

To overcome this, a strain JSCA0021 (Figure 1A; Table 1) with *CaCDC4* one allele deleted and the other under *CaMET3* control that is Met/Cys repressible was created. To allow introducing Tet-on cassettes with the same *CaURA3* selectable marker as mini Ura-blaster on JSCA0021, 5-FOA was used as a counter-selection agent for removal of *CaURA3* from JSCA0021 in which JSCA0022 was obtained (Fig. 1A; Table 1). The strains made were PCR-confirmed with primers CaCDC4 locus F and CaCDC4 locus R

(Fig. 1B; Table 2). Since integration of plasmid cassette of pFA-HIS1-MET3-CaCDC4 at *CaCDC4* locus created size enlargement of 6916-bp that was hard to detect the link between *CaMET3* promoter and *CaCDC4* by routine PCR, primers MET-F and CaCDC4 locus R (Table 2) were adopted (Fig. 1A).

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

To compare phenotype regarding to yeast-to-filament transition between strain JSCA0021 and JSCA0022, cells of those strains were assessed under either *CaMET3* repressed or derepressed condition. Cells of both strains on SD plates without Met/Cys grew colonies of circular shape with smooth surface (Fig. 1C). By contrast, cells on plates with Met/Cys formed colonies of irregular shape with filaments (Fig. 1C). Under microscope, these strains exhibited equivalent filamentous forms, suggesting their equal ability to deplete *CaCDC4* for morphological alteration. Subsequently, JSCA0022 (without *CaURA3*) was used as a parental strain for introducing the Tet-on cassettes (with *CaURA3*) encoding assorted *Ca*Cdc4 domains.

Establishment of Tet-on cassettes capable of expressing assorted CaCDC4 domains in C. albicans reveals that CaCdc4 is unstable and domains of F-box and WD40-repeat are required for CaCdc4 function

To ensure Tet-on cassettes, encoding assorted *Ca*Cdc4 domains (Fig. 2A), are functional, each of the *Sac*II and *Kpn*I digested cassettes from pTET25M-CaCDC4, pTET25M-CaCDC4-6HF, pTET25M-ΔNCaCDC4-6HF, pTET25M-F-6HF, pTET25M-WD40-6HF, and pTET25M-ΔNF-6HF was transformed into BWP17 for Ura^+ (Fig. 2B), and the presence of cassette in each of the strains was PCR-confirmed with primers pNIM1 inte F and pNIM1 inte R (Fig. 2C; Table 2). By growing in YEPD with 50 μl/ml Dox at 30°C overnight, the inducibly expressed proteins from those strains were assessed by Western blotting (Fig. 2D). Proteins migrating to approximately 55 kDa and 72 kDa were cross-reacted products to anti-FLAG antibody and used as internal control. The BWP17 and JSCA0033 expressing non-tagged *Ca*Cdc4 were used as negative controls. The F-box and WD40-repeat migrated to the position of around 19 kDa and 43 kDa, respectively. While WD40-repeat and F-box were apparent, the later was somewhat stronger. The full-length *Ca*Cdc4 and the N-terminus truncated *Ca*Cdc4 exhibited weak signals at position corresponding to 86 kDa and 77 kDa, respectively. Two distinctive signals were shown from sample expressing △NF *Ca*Cdc4. Thus, we confirmed that Tet-on system functions in *C. albicans* and *Ca*Cdc4 might be unstable.

The filamentous development of JSCA0022 under *CaMET3-CaCDC4* repressed condition with Met/Cys alongside the Tet-on system allows us to study the function of *Ca*Cdc4 domains. To achieve this, a set of Tet-on cassettes encoding each of assorted domains of *Ca*Cdc4 was transformed into JSCA0022, bearing a *CaMET3* repressible *CaCDC4*. The correctness of the strains was confirmed by yeast colony PCR (Figure 2C). Individual domains of *Ca*Cdc4 inducibly expressed by growing cells in YEPD with Dox were assessed by Western blotting (Fig. 2D). Although levels of expression from

endogenous *CaCDC4* loci of BWP17 and partially repressed *CaMET3-CaCDC4* of JSCA0022 in undefined YEPD medium were undetermined, the expression of those assorted *Ca*Cdc4 domains from JSCA0022 was essentially the same as that from BWP17, confirming that Tet-on system operates sufficiently in strain JSCA0022 (Fig. 2D).

To determine the function of assorted *Ca*Cdc4 domains, JSCA0022-based strains capable of repressing *CaCDC4* and inducibly expressing assorted *Ca*Cdc4 domains were grown in SD medium with or without Met/Cys and in the presence or absence of Dox. Similar to those in YEPD, Dox-induced expressions of assorted CaCdc4 domains were revealed (Fig. 3A). Cells of strains in SD medium without Met/Cys grew as yeast form in the presence or absence of Dox (Fig. 3B). By contrast, cells of those strains in medium with Met/Cys grew with filaments (Fig. 3B). Unexpectedly, growing in medium with Met/Cys and Dox, cells of JSCA0023, JSCA0024, and JSCA0025, particularly the former two that produced the full-length *Ca*Cdc4, still grew as filaments, even though to a lesser extent to the rest of other strains.

C. albicans CDC4 negatively regulating cell flocculation

Significantly, differences among strains in ability to form suspension (to resist flocculation) were observed. Strains JSCA0023, JSCA0024, and JSCA0025 were somewhat easier to maintain as suspension (Fig. 4B). Such an ability to remain suspension was unlikely due to increase in cell number as all cultures were of similar optical density (Fig. 4A). The extent of flocculation among strains was more apparent by resuspending cells in cuvettes (Fig. 4B). Under *CaMET3* repressed condition, strains

JSCA0026, JSCA0027, and JSCA0030 showed a similar degree of flocculation as JSCA0021did regardless of presence or absence of Dox (Fig. 4B). By contrast, strains JSCA0023, JSCA0024, and JSCA0025, even though with some filamentous cells, showed a lesser extent of flocculation as with JSCA0021 under *CaMET3* repressed but Tet-on condition (Fig. 4B).

Discussion

In this study, we aimed to dissect the function of *Ca*Cdc4 domains by introducing Tet-on system with cassettes encoding a variety of *Ca*Cdc4 domains in *C. albicans* mutant of *Cacdc4* null. However, due to inability to use the *Cacdc4* null mutant with filamentous form for introducing Tet-on cassettes, instead we constructed a strain JSCA0022 whose *CaURA3* was popped out and *CaCDC4* expression was repressible. Under repressed condition, the JSCA0022 showed similar filamentous morphology with those of previous reports of cells with *CaCDC4* being repressed [10, 12] and of *cacdc4* null [10]. We therefore confirmed that the JSCA0022 under repressed condition could be used as a strain to replace a strain completely lost of *CaCDC4* function for introducing Tet-on cassettes capable of expressing each of *Ca*Cdc4 domains as a sole source for functional assay.

To verify the ability of the Tet-on cassettes operating in *C. albicans*, each of the Tet-on cassettes encoding various *Ca*Cdc4 domains was transformed into BWP17. Individual *Ca*Cdc4 domains from relevant strains were detectable, suggesting that the Tet-on system functions in *C. albicans*. Interestingly, the full-length *Ca*Cdc4 and the N-terminus truncated *Ca*Cdc4 were present far less than those of either F-box or WD40-repeat, suggesting that they were unstable. Interestingly, two distinctive signals were shown from sample expressing △NF of *Ca*Cdc4, implying occurrence of cleavage on △NF of *Ca*Cdc4, from about 45 KDa to 43 kDa, retaining the FLAG epitope-tag. Hence, we suggested that N-terminal *Ca*Cdc4 plays a role in regulating *Ca*Cdc4 function. Perhaps massively overproducing *Ca*Cdc4 under Tet-on system hyper-activates *Ca*Cdc4 itself to

accelerate its own degradation by auto-ubiquitination. This is consistent with the report that Cdc4 and Grr1, having a short half-life, are degraded by ubiquitin-proteasome system [28]. Consequently, we confirmed that Tet-on system works adequately in *C. albicans* and the stability of *Ca*Cdc4 may be important for its function.

Initially, we presumed that Dox-induced full-length *Ca*Cdc4 would completely suppress filamentation in the strain with its *CaMET3-CaCDC4* being repressed when Met/Cys and Dox were present simultaneously. However, filaments somewhat remained under such a condition (Fig. 3B). The simplest explanation is the difference in timing of repression by *CaMET3* and induction by the Tet-on system. While germ tubes form at 2 hrs and true hypha develop at 8 hrs after *CaCDC4* being repressed in JSCA0022 (Lai & Shieh, unpublished data), the induction of *CaCDC4* by the Tet-on system peaks at 6 hrs (W.-C. Lai *et al.*, manuscript in preparation). Alternatively, the Dox-induced expression of active *Ca*Cdc4 hastened degradation of *Ca*Cdc4 in which insufficient active *Ca*Cdc4 were present to support full suppression of filamentation. It is equally possible that the Tet-on system whose rtTA constitutively driven by *CaADH1* promoter in which metabolic pathway associated with *CaADH1* might affect the operation of *CaADH1* promoter in cells at distinct growth phases as noted by the fact that *CaADH1* mRNA levels are regulated during batch growth on glucose in which *CaADH1* mRNA increases to the highest levels in late exponential phase and declined to low levels in stationary phase [29].

Interestingly, cells of all JSCA0022-based strains exhibited flocculation in medium with Met/Cys, but the strains JSCA0023 (CaCDC4), JSCA0024 (CaCDC4-6HF), and JSCA0025 (ΔNCaCDC4-6HF) showed a lesser extent in flocculation by adding Dox

simultaneously. Importantly, no definitive association between filamentation and adhesion or invasion has been reported. Cells of those three strains grew as filaments but flocculated less than the rest of strains. Hence, we concluded that F-box and WD40-repeat are important in suppressing flocculation (adhesion of cells) and that N-terminal region (1-85) is not critical for such a role. Moreover, a function for *Ca*Cdc4 suppressing flocculation implies a role of *CaCDC4* in biofilm formation.

Until the role of ubiquitin-proteasome-mediated regulation of *Ca*Cdc4 is fully explored, such as identification of substrates, including those for morphogenesis besides Sol1 and those involved in adhesion and biofilm formation, comprehensively understanding the function of *Ca*Cdc4 is impossible.

Acknowledgements

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Figure legends

Fig. 1. Construction of a *C. albicans* strain for repressibly expressing *CaCDC4*. (**A**) Strain construction (detailed in the Materials and 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 the loss of *CaURA3* to obtain JSCA0022 for re-introducing the Tet-on plasmid with *CaURA3* marker. (**B**) Verification of constructed strains by yeast colony PCR. Primers CaCDC4 locus F and CaCDC4 locus R were used. Strains with *CaCDC4*, *Cacdc4*::*CaURA3-dpl200*, and *Cacdc4*::*dpl200* yield respective amplicons of 2689, 2279, and 924 bp. Primers of MET-F and CaCDC4 locus R were used to PCR-generate a 2772-bp amplicon for verification of the link between *CaMET3* and *CaCDC4*. Strains used are as follows. Lane 1: BWP17, Lane 2: JSCA0018, Lane 3: JSCA0019, Lane 4&5: JSCA0021, Lane 6&7: JSCA0022 (see Table 1). Black arrows indicate primer pairs of CaCDC4 locus F and CaCDC4 locus R. Broken arrows in black indicate primer MET-F. (**C**) Morphological analysis of 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). The cells in liquid culture were also recorded with Nikon 50i microscope at 400×magnification (bottom panel). Bars represent 10 μm.

Fig. 2. Construction of *C. albicans* strains capable of Dox-induced expression of assorted *CaCDC4* domains. (**A**) Schematic representation of *Ca*Cdc4 domains expressed from the Tet-on system. (**B**) Generation of Tet-on cassettes capable of expression assorted CaCdc4 domains. Different portions of *CaCDC4* were PCR-generated with sets of primers (Table 2) containing common *Aat*II and *Xho*I sites and used to replace full-length *CaCDC4* on pTET25M-CaCDC4-6HF as described in the Material and Methods. The cassettes were obtained by digestion with *Sac*II and *Kpn*I, each of which was transformed into *C. albicans* of either BWP17 or JSCA0022 for integration into *CaADH1* locus. (**C**) Verification of Tet-on cassettes being integrated into *CaADH1* locus. The correctness of integration of the cassette into *CaADH1* locus was confirmed by yeast colony PCR with primers pNIM1 inte F and pNIM1 inte R, as shown, generating a product of 1079 bp. (**D**) Detection of Dox-inducible expression of assorted *Ca*Cdc4 protein domains in *C. albicans*. The Dox-induced *Ca*Cdc4 domains of either BWP17 or JSCA0022 were assessed by Western blotting from overnight cultures of YEPD with 50 μg/ml Dox. The designations of strains are as in Table 1.

Fig. 3. Morphological analysis of *C. albicans* strains. Cells were grown 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). The Dox-inducible expression of assorted *Ca*Cdc4 protein domains under *CaMET3-CaCDC4* repressed condition was verified by Western blotting (**A**). The images were visualized with Nikon 50i microscope at 400 magnification (**B**). The designations of strains are as in Table 1. Bars represent 10 μm.

Fig. 4. Analysis of cell flocculation. (**A**) Ability for cells to stay in suspension. Cells were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration into SD medium in the absence (–Met/Cys) or presence (+Met/Cys) of 2.5 mM Met/Cys, and each with 50 μg/ml Dox (–Met/Cys+Dox and +Met/Cys+Dox) for another 48 hrs growth. The numbers shown on the bottom of each tube represent values of OD_{600}. (**B**) Ability of cells to flocculate. Cells were grown as above and resuspended into cuvettes to compare the level of cell flocculation. The designations of strains are as in Table 1.

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

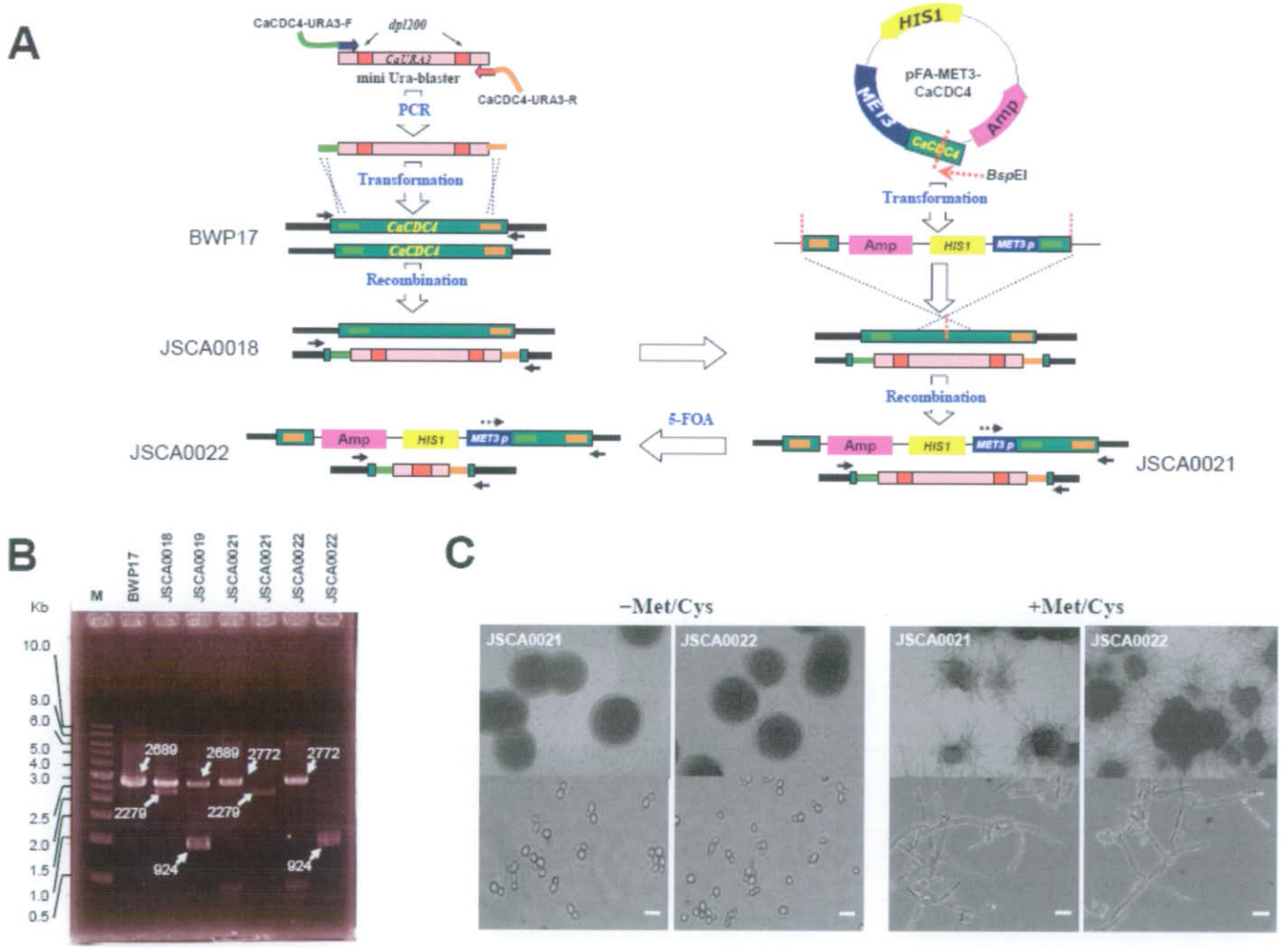
Name of the strain	Parental strain	Genotype
BWP17		*ura3::imm434/ura3:imm434 his1::hisG / his1::hisG arg4::hisG/arg4::hisG*
JSCA0018	BWP17	*CDC4/cdc4::URA3-dpl200*
JSCA0019	JSCA0018	*CDC4/cdc4::dpl200*
JSCA0021	JSCA0018	*cdc4::URA3-dpl200/PMET3-CDC4:HIS1*
JSCA0022	JSCA0021	*cdc4::dpl200/PMET3-CDC4:HIS1*
JSCA0023	JSCA0022	*ADH1/adh1::PTET-CDC4*
JSCA0024	JSCA0022	*ADH1/adh1::PTET-CDC4-6HF*
JSCA0025	JSCA0022	*ADH1/adh1::PTET-CDC4(85-768)-6HF*
JSCA0026	JSCA0022	*ADH1/adh1::PTET-CDC4(241-392)-6HF*
JSCA0027	JSCA0022	*ADH1/adh1::PTET-CDC4(393-768)-6HF*
JSCA0030	JSCA0022	*ADH1/adh1::PTET-CDC4(85-392)-6HF*
JSCA0033	BWP17	*ADH1/adh1::PTET-CDC4*
JSCA0034	BWP17	*ADH1/adh1::PTET-CDC4-6HF*
JSCA0035	BWP17	*ADH1/adh1::PTET-CDC4(85-768)-6HF*
JSCA0036	BWP17	*ADH1/adh1::PTET-CDC4(241-392)-6HF*
JSCA0037	BWP17	*ADH1/adh1::PTET-CDC4(393-768)-6HF*
JSCA0040	BWP17	*ADH1/adh1::PTET-CDC4(85-392)-6HF*

Table 2. Oligonucleotides used in this study

Name	Sequence[a]
CaCDC4 XhoI F	GAACTCGAGATGGATAAGAAATCAAAG
CaCDC4 XhoI R	GAACTCGAGCTGTAAAAGTGGTTGACT
CaCDC4 SalI	TAGCGTCGACATGGATAAGAAATCAAAGC
CaCDC4 BglII	TCGAGATCTTCACTGTAAAAGTGGTTGAC
CaURA3-dpl200 BamHI	AATGGATCCCCAGATATTGAAGGTAAAAGG
CaURA3-dpl200 XhoI	ATTCTCGAGCTAGAAGGACCACCTTTGAT
TET25M KpnI	CAAGGTACCGAACCATCGTGAGTGTAA
TET25M BamHI	GAAGGATCCCGACATTTTATGATGGAA
CaCDC4-6HF SalI	GCGTGTCGACGTCATGGATAAGAAATCAAAGCTA
CaCDC4-6HF BglII[b]	TCGAGATCTTTATTTATCATCATCATCTTTATAATCACCACCGTGGTGGTGGTGGTGGTGCTCGAG CGGCCGCTGTAAAAGTG GTTGACTGAAATC
CaCDC4 ΔN AatII	AATAGACGTCCTTATGCCCTCATGTGACGAC
CaCDC4 ΔN XhoI	ATCCTCGAGCTGTAAAAGTGGTTGACTGA
CaCDC4 F-box AatII	AAGCGACGTCATGAGCAATGAACCTACT
CaCDC4 F-box XhoI	GCCACTCGAGCCACCTATTGACAATTAT
CaCDC4 WD40 AatII	GCTAGACGTCATGGATCCAAAGTTCAAAC
CaCDC4-URA3-F	ATGGATAAGAAATCAAAGCTATTCAAATATCCTTTGAGCGAGGAGACGGCTAAATTTGAGGTTTTCCCAGTCACGACGTT
CaCDC4-URA3-R	TCACTGTAAAAGTGGTTGACTGAAATCTAGAATCTCAATAAACGTTTCACCTTCATCTTCTGTGGAATTGTGAGCGGATA
pNIM1 inte F	CATGTCAAAGGATTCAAC
pNIM1 inte R	GTATGGTGCCTATCTAAC
CaCDC4 locus F	TGCCCATTAGCAATAGTGTA
CaCDC4 locus R	AATCAACCACCAACCAGAAA
MET-F	CCACCTTTGCTAATTGCGTA

[a]Restriction enzyme sites are shaded in grey.
[b]Sequences complementary to those encoding 6×His and FLAG are underlined.

Fig. 1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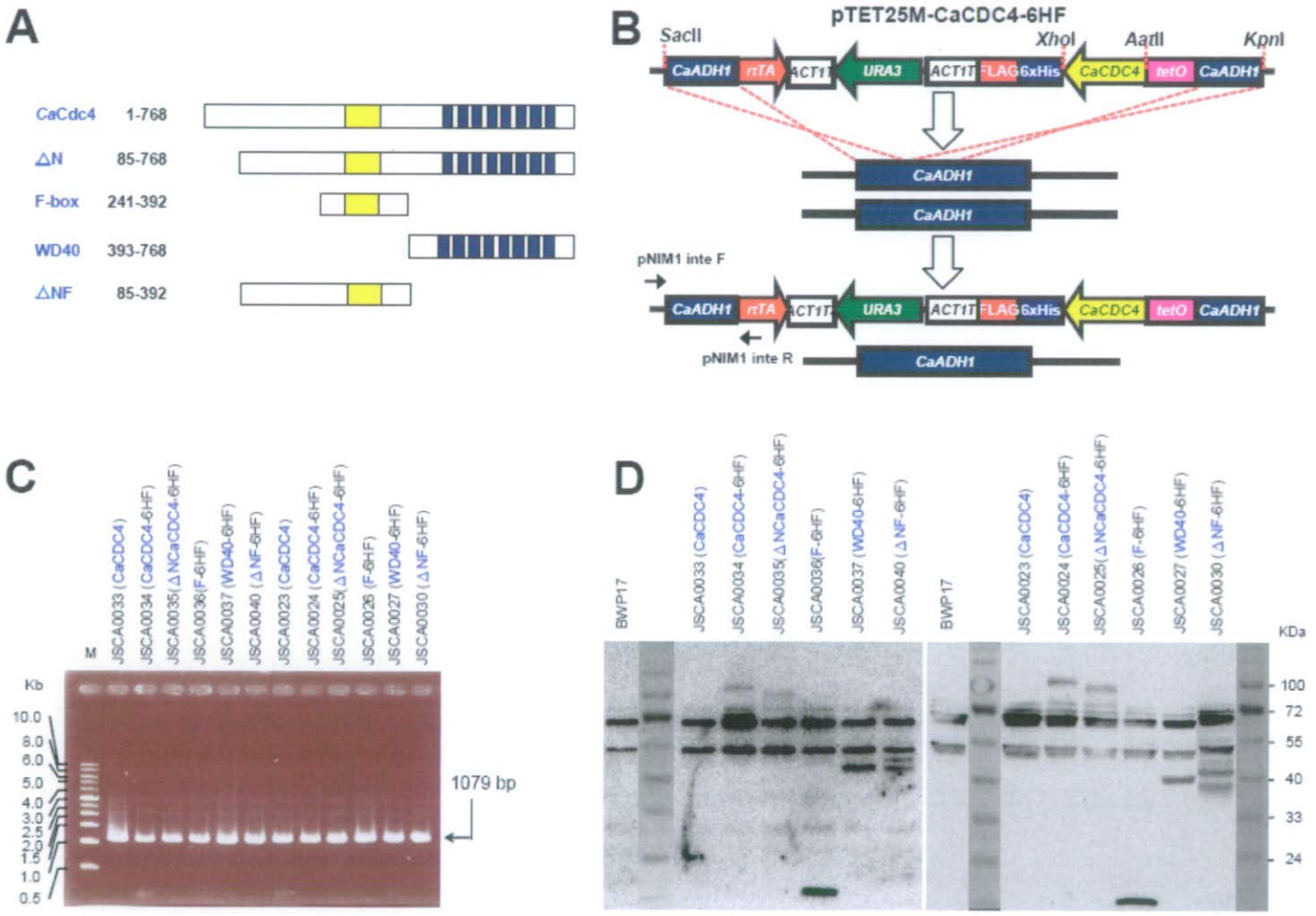
CaURA3
mini Ura-blaster
CaCDC4-URA3-R
PCR
Transformation
BWP17
CaCDC4
CaCDC4
Recombination
JSCA0018
JSCA0022
Amp
HIS1
MET3 p
5-FOA
HIS1
pFA-MET3-CaCDC4
MET3 p
CaCDC4
Amp
BspEI
Transformation
Amp
HIS1
MET3 p
Recombination
Amp
HIS1
MET3 p
JSCA0021
B
M
BWP17
JSCA0018
JSCA0019
JSCA0021
JSCA0021
JSCA0022
JSCA0022
Kb
10.0
8.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5
2689
2689
2772
2772
2279
2279
924
924
C
−Met/Cys
+Met/Cys
JSCA0021
JSCA0022
JSCA0021
JSCA0022

Fig. 2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CaCdc4 1-768
ΔN 85-768
F-box 241-392
WD40 393-768
ΔNF 85-392
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SacII
XhoI
AatII
KpnI
CaADH1
rtTA
ACT1T
URA3
FLAG6xHis
CaCDC4
tetO
pNIM1 inte F
pNIM1 inte R
C
M
JSCA0033 (CaCDC4)
JSCA0034 (CaCDC4-6HF)
JSCA0035(ΔNCaCDC4-6HF)
JSCA0036(F-6HF)
JSCA0037 (WD40-6HF)
JSCA0040 (ΔNF-6HF)
JSCA0023 (CaCDC4)
JSCA0024 (CaCDC4-6HF)
JSCA0025(ΔNCaCDC4-6HF)
JSCA0026 (F-6HF)
JSCA0027 (WD40-6HF)
JSCA0030 (ΔNF-6HF)
Kb
10.0
8.0
6.0
5.0
4.0
3.0
2.5
2.0
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0.5
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BWP17
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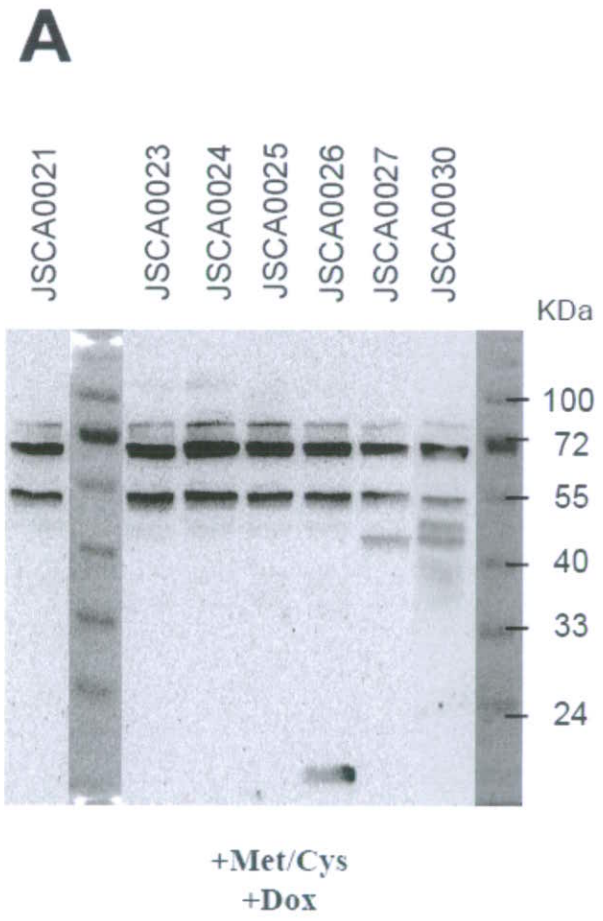
72
55
40
33
24

Fig. 3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JSCA0023
JSCA0024
JSCA0025
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JSCA0027
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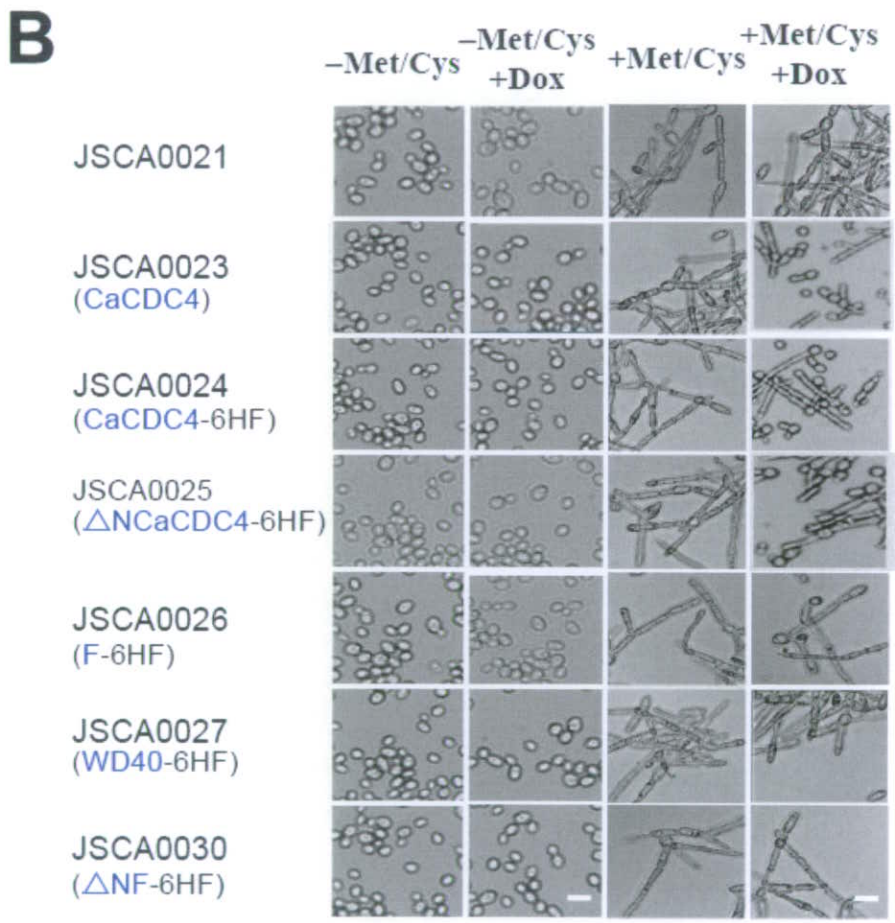
40
33
24
+Met/Cys
+Dox


B
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–Met/Cys +Dox
+Met/Cys
+Met/Cys +Dox
JSCA0021
JSCA0023 (CaCDC4)
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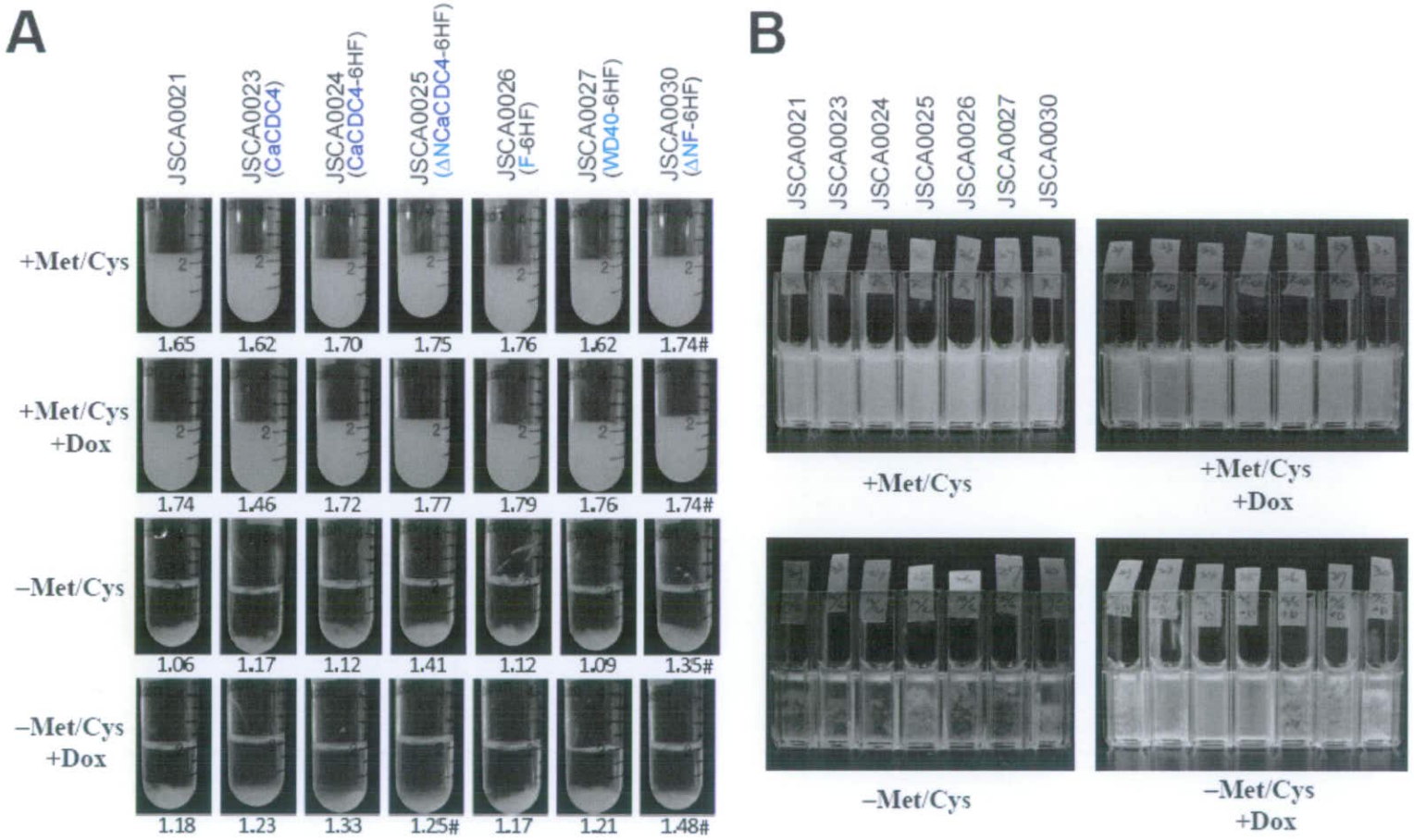
JSCA0030 (△NF-6HF)

Fig. 4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JSCA0026 (F-6HF)
JSCA0027 (WD40-6HF)
JSCA0030 (ΔNF-6HF)
+Met/Cys
1.65 1.62 1.70 1.75 1.76 1.62 1.74#
+Met/Cys +Dox
1.74 1.46 1.72 1.77 1.79 1.76 1.74#
–Met/Cys
1.06 1.17 1.12 1.41 1.12 1.09 1.35#
–Met/Cys +Dox
1.18 1.23 1.33 1.25# 1.17 1.21 1.48#
B
JSCA0021 JSCA0023 JSCA0024 JSCA0025 JSCA0026 JSCA0027 JSCA0030
+Met/Cys
+Met/Cys +Dox
–Met/Cys
–Met/Cys +Dox

Construction of *Candida albicans* Tet-on tagging vectors with an Ura-blaster cassette

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Keywords: *Candida albicans*; Tet-on tagging vector; ura-blaster cassette

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Abstract

Because of its non-standard genetic code and diploid nature without a complete sexual cycle, it has been difficult to develop molecular tool for the fungal pathogen *Candida albicans*. Vector systems, either with regulatable promoters for producing conditional mutants, capable of generating epitope-tagged proteins, or with recyclable selection markers, are useful for functional study of genes. However, most vectors currently available contain only a subset of all desired properties, reducing the value of usefulness. To combine all useful properties in vectors, we have constructed modified version of tetracycline-inducible gene expression system for *C. albicans*. We constructed pTET25M with modified *CaURA3* selection marker of pTET25, capable of producing C-terminal GFP fusion protein induced by doxycycline in *C. albicans*, to *CaURA3* flanked by *dpl*200 repeats that can be used repetitively. We also introduced a multiple cloning site at each end of GFP of pTET25M enabling more choices for cloning. We verified the applicability of the vector by constructing strains of either the C-terminal or the N-terminal GFP fusion of *CaCDC11*. In addition, by replacing the GFP from pTET25M with a 6×His-FLAG epitope tag version of *CaCDC4* coding sequence, we were able to observe expression of *CaCDC4* in a doxycycline-inducible manner after introducing into *C. albicans*. The new system is capable of overexpressing desired *C. albicans* gene product with the advantage of ease of

cloning, recyclable *URA3* marker and ability to be detected as fusion proteins or epitope tagged ones, which will facilitate the functional analysis of gene in *C. albicans* with efficiency.

Introduction

The yeast *Candida albicans* is a member of the normal microflora on mucosal surfaces of gastrointestinal and genitourinary tract in healthy person, however it is also the single most main fungal pathogen in humans (Magee, 1998). Especially, *C. albicans* triggers systemic candidiasis in immunocompromised patients. The molecular analysis of the *C. albicans* is hindered due to its diploid nature without complete sexual cycle, and a non-standard codon usage. However, many approaches have been established to enhance the functional study of *C. albicans*. To date, several gene disruption strategies have been developed for *C. albicans* (Berman and Sudbery, 2002), most common of which is the use of Ura-blaster cassette (Wilson et al., 2000), which allows reusing the auxotrophic *URA3* selection marker. A general usage of of Ura-blaster cassette has also been incorporated into vector systems for sequentially introducing several genes or deleted genes to assess functional interaction among them.

In addition, a number of gene expression vectors have been established, including repressible system using either the *MET3* promoter with methionine and cycteine (Care et al., 1999), the *MAL2* promoter with glucose (Backen et al., 2000), or the tetracycline-repressible system (Nakayama et al., 2000), and inducible systems

such as tetracycline-inducible gene expression (Tet-on) one (Park and Morschhauser, 2005). The expression-regulatable systems are especially useful to assess the consequences of presence or absence of gene expression. Function of gene can also be determined by inducing the expression of specific genes to higher level that disturbs regulatory mechanism for normal function. Function of gene can also be determined by repressing the expression of genes of interest that exhibit functional consequence of depletion of gene product.

Epitope-tagging has also been developed and become a common tool for detecting, purification, and studying function of proteins (Fritze and Anderson, 2000). Several epitope-tags have been established and used widely in a variety of organisms. These include protein tags of glutathione S-transferase (GST)(Smith and Johnson, 1988) and the green fluorescence protein (Cubitt et al., 1995; Heim et al., 1995), single epitope-tags of the haemagglutinin (HA) (Field et al., 1988), the 6×histidine residues (Hagan and Stirling, 1998), the human c-Myc (Evan et al., 1985), FLAG (Brizzard et al., 1994; Overholt et al., 1997; Prickett et al., 1989), as well as double epitope tags such as 6×His-FLAG (Huang et al., 2001; Robeva et al., 1996). In *C. albicans*, the tagging vectors have recently been developed for HA epitope of the Tet-on system (Nakayama et al., 2000), GFP (Cormack et al., 1997), FLAG (Umeyama et al., 2002), and 6×His-FLAG. (Kaneko et al., 2004)

To facilitate detection, purification, and functional studies of *C. albicans* gene products, we constructed tagging expression vectors for use in *C. albicans*. The vectors allow proteins to be expressed ectopically in a doxycycline-dependent manner as GFP fusion proteins C-terminally or N-terminally, or 6×His-FLAG epitope attached to their C-terminus. The vectors also possess an Ura-blaster cassette to allow reintroducing vector with *URA3* marker. The function and applicability of the vector was assessed and the results were presented using the vector encoding *C. albicans CDC4* (*CaCDC4*), an F-box protein that is a member of the *C. albicans* ubiquitin E3 ligase, whose role is a negative regulator of hyphal growth in *C. abicans* (Atir-Lande et al., 2005; Shieh et al., 2005)

Materials and methods

Strains, growth conditions, and DNA methods

Escherichia coli DH5α (F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) was used as a host for the routine maintenance and amplification of plasmids. Bacterial cultures were grown in L-broth, supplemented with 50μg/ml ampicillin as required (Miller, 1972). Plasmid DNA was purified by Gene-Spin™-V^2 Miniprep Purification kit (Taipei,

Taiwan). The oligonucleotide primers used to constructed vectors are listed in Table 1.

The auxotrophic *C. albicans* strain BWP17 (*ura3Δ::λimm434/ura3::λimm434; arg4::hisG/arg4::hisG; his1::hisG /his1::hisG*) (Wilson et al., 1999) was used in strain construction. A complete strain list is shown in Table 2. *C. albicans* strains were grown in either complete medium of yeast extract-peptone-glucose (YEPD) or synthetic minimal medium with or without uridine (Sherman et al., 1986). The oligonucleotide primers in PCR used to constructed vectors and diagnostic analysis for strains are listed in Table 1. Yeast colony PCR was used to determine integrants of *C. albicans* strains (Wang et al., 1996).

Yeast colony PCR (xxxxxx) with diagnostic primers pNIM-inte detect F and pNIM-inte detect R was used to determine integrants of *C. albicans* (Table 1).

Generation of the Ura-blaster cassette

To introduce an Ura-blaster cassette, a *C. albicans URA3* (*CaURA3*) marker fragment was first digested with *Pst*I and *Sal*I from a tetracycline-inducible vector pTET25 and cloned into the vector pUC19 to generate pUC19-CaURA3. The *dpl*200 fragment of *CaURA3* was amplified by PCR from pUC19-CaURA3 with primers CaURA3-dpl200-XhoI and CaURA3-dpl200-BamHI (Table 1), digested with *Xho*I

and *Bam*HI, and cloned into pUC19-CaURA3 to produce pUC19-CaURA3-dpl200 (Figure 1A). To introduce *Bgl*II cloning site downstream of *C. albicans GFP* (*CaGFP*) from pTET25, a DNA fragment containing partial *TetO*, *CaGFP* with SalI and *Bgl*II sites, and *ACT1* transcription termination sequence (T_{ACT1}) from pNIM1 was PCR amplified with primers TET25M-*Kpn*I and TET25M-*Bam*HI (Table 1), digested with *Kpn*I and *Bam*HI, and cloned into pUC19-CaURA3-dpl200 to generate pUC19-TET25M (Figure 1A). A DNA fragment containing *CaGFP*, T_{ACT1}, and partial *CaURA3* was digested with *Sal*I and *Eco*RI from pUC19-TET25 and cloned into pTET25 to become pTET25M (Figure 1A).

Introduction of the multiple cloning sites

To make most use of the pTET25M that is able to express protein as *CaGFP* fusion, restriction cloning sites were introduced into either 5' or 3' end of the *CaGFP*. A DNA fragment was PCR amplified from pTET25M with a forward primer *Ca*GFP-N-MCS containing *Sal*I, *Xho*I, *Eco*RV, *Nhe*I, and *Not*I, and a reverse primer *Ca*GFP-C-MCS containing *BspE*I, *Aat*II, *Afl*II, *Bgl*II, *Stu*I, *Bcl*I along with a stop codon. The PCR amplicon containing *CaGFP* was sequentially digested with *Sal*I and *Bcl*I and cloned into pTET25M that had been released its *CaGFP* by digestion with *Sal*I and *Bgl*II to generate pTET25M-NC with multiple cloning sites at both ends of

the *CaGFP* (Figure 1B).

Creation of the 6×His-FLAG epitope

The coding sequences of *CaCDC4* gene was PCR amplified with primers *CaCDC4-Sal*I and *CaCDC4-Bgl*II (Table 1), digested with *Sal*I and *Bgl*II, and cloned into pTET25M to generate pTET25M-*CaCDC4*. To incorporate the epitope of six histidine and FLAG (6HF) C-terminally, a new reverse primer *CaCDC4*-6HF-*Bgl*II capable of encoding 6HF with adapted codon usage of *C. albican* was introduced. In addition, the reverse primer was incorporated restriction sites of *Xho*I and *Not*I, whereas a new forward primer *CaCDC4*-6HF-*Sal*I was incoporated that of *Aat*II. A DNA fragment was PCR amplified from pTET25M-*CaCDC4* with the new pair of primers. Subsequently, *CaCDC4*-6HF PCR amplicon was digested with *Sal*I and *Bgl*II and cloned into pTET25M to become pTET25M-*CaCDC4*-6HF. *CaCDC4* from the pTET25M-*CaCDC4*-6HF can be replaced by coding sequence of other genes with several restriction sites of choice (Figure 1C).

***Candida albicans* transformation and selection**

Transformation of *C. albicans* and selection of transformants were performed essentially as described (Gerami-Nejad et al., 2001). Briefly, the DNA fragment of

Tet-on part with gene of interest were released from plasmids by *Sac*II and *Kpn*I, purified by G-M™ Gel Extraction System (Taipei, Taiwan), and then transformed into *C. albicans* by the LiAc-PEG-ssDNA method (Gietz and Woods, 2006) with modification (Walther and Wendland, 2003). The DNA was targeted integration to the *ADH1* locus of *C. albicans* genome for stable integrants, the Ura+ prototrophs, on minimal medium plates lacking uridine (Shieh et al., 2005). To recycle the *C. albicans* *URA3* cassette, Ura^+ prototrophs were grown on plate with 5-FOA (1mg/ml) and uridine (80μg/ml) and the Ura^- auxotrophs were selected. Correct insertion of the marker *URA3* gene and the removal of the *URA3* gene after 5-FOA selection were verified by PCR with specific primers pTET25M 5FOA detect F and pTET25M 5FOA detect R (see Table 1)

Western blot analysis

To detect expression of the tagged *Ca*Cdc4 or the CaCdc4 fusion proteins, cells of constructed *C. albicans* strains were grown at 30°C in YEPD overnight, then diluted into fresh YEPD medium with 50 μg/ml (SIGMA) doxycycline, and grown exponentially. Total protein was extracted from the cultured cells by the methods essentially as described previously (Shieh et al., 2007). The proteins were resolved by SDS-PAGE and transferred electrophoretically to PVDF membranes (PerkimElmer,

Boston, MA). The membranes were probed with polyclonal antibody M2 to FLAG epitope (SIGMA) or monoclonal antibody to GFP (xxxxxx). Detection was performed using a peroxidase-conjugated either anti-mouse IgG (xxxxxxx) or anti-rabbit IgG (Pierce, Rockford, IL). The signal was visualized by SuperSignal West Pico Chemiclluminescence Substrate Kit (Pierce, Rockford, IL) in accordance with the manufacturer's instructions.

Microscopy

C. albicans cells were grown to logarithmic phase in liquid of either complete or synthetic minimal media and washed with sterile PBS prior observation. The phase contrast microscopy of either Nikon ECLIPSE 50i or ZEISS Axioskop2 was used for routine assessment and the digital images were acquired using a YOVICA MD130 eyepiece (KOKUSIO BIOMEDICAL PRODUCTS, INC., Taipei, Taiwan) and processed using Webcam Companion and PhotoImpression 5 software. Fluorescence images were obtained using ZEISS LSM 510 inverted confocal microscope with LSM 510 META imaging software (Carl Zeiss Microimaging, Thornwood, NY). The micrographs were digitized and processed by Adobe Photoshop.

Results and discussion

Modification of a tetracycline-inducible gene expression system

Tetracycline-inducible gene expression system introduced in *C. albicans* in current study was originated from the work developed by Park and Morschhäuser (Park and Morschhauser, 2005), in which reverse tetracycline-controlled transactivator (rtTA) and *GFP* was adapted to *C. albicans* codon usage. Two plasmids, pTET25 and pNIM1, each with *CaURA3* and dominant *CaSAT1* selection markers, were obtained from them. However, inability to re-use the marker limits the functional analysis among genes. To make *CaURA3* selection marker in pTET25 recyclable, the Ura-blaster cassette (Wilson et al., 2000) was sought to be introduced into pTET25. By using pUC19 as an intermediate vector, an Ura-blaster cassette with 200-bp 3' region of *CaURA3* was created and cloned into the 5' region of *CaURA3* to obtain pUC19-CaURA3-dpl200 (Figure 1A). Such a mini Ura-blaster cassette, termed *CaURA3-dpl*200, contains 200-bp flanking repeats that permit 5-FOA induced intrachromosomal recombination. To facilitate further cloning, the *Bgl*II restriction site was introduced downstream of the *CaGFP* (Figure 1A). This was achieved by PCR amplification of a DNA fragment comprises of partial Tet operon, *CaGFP*, and *CaACT1* transcription termination sequence from plasmid pNIM1 and cloned into pUC19-CaURA3-dpl200 to produce pUC19-TET25 (Figure 1A). After replacing the *Eco*RI and *Sal*I region of pTET25 that contains part of *CaURA3*, *CaACT1*

transcription termination sequence, and *CaGFP*, with *Eco*RI and *Sal*I region of pUC19-TET25, pTET25M was generated, which is the initial step of modified version of pTET25. The pTET25M possesses *Sal*I and *Bgl*II flanked the *CaGFP* allow introducing other restriction sites. To verify the maintenance of functionality of *CaGFP* of the pTET25M, both the pTET25M and pTET25 were linearized by *Sac*I and *Kpn*I and introduced separately into *C. albicans* strain BWP17 for Ura+. Stable integrants (TET25 and TET25M, respectively) that target the *ADH1* locus were verified by colony PCR with specific primers. Importantly, doxycycline-induced expression of *CaGFP* was similar in pattern analyzed by Western blot in both pTET25 and pTET25M integrants (data not shown). The expression of *CaGFP* was also comparably evident in both cells of integrants observed by fluorescence microscopy (data not shown). We therefore concluded that pTET25M carrying Ura-blaster cassette was successfully established.

To enhance the application of the vector that lacks adequate numbers of restriction sites for cloning, multiple restriction sites were sought to introduce into flanking ends of *CaGFP* of pTET25M. This was accomplished by using PCR with a pair of long oligonucleotide primers, each with a number of restriction sites in addition to the homologous region of *CaGFP*, to generate modified *CaGFP* from the pTET25M. The PCR product was then to re-introduce into the pTET25M that had

been released its *CaGFP* by digesting with *Bgl*II and *Sal*I to generate pTET25M-NC (Figure 1B). The pTET25M-NC was useful both in replacing its *CaGFP* with other genes of interest with numerous choices of restriction sites and cloning coding region of gene to be expressed as fusion protein of *CaGFP* at either N- or C-terminus. To extend the usage of pTET25M, epitope-tagging other than *CaGFP* was sought to develop. This was achieved by replacing the *CaGFP* from pTET25M with a *CaCDC4* coding sequence at the *Sal*I and *Bgl*II sites to obtain pTET25M-*CaCDC4*. The *Ca*CDC4 from pTET25M-CaCDC4 was then replaced with a sequence encoding 6×His-FLAG tagged *Ca*Cdc4 at the C-terminus to obtain pTET25M-*CaCDC4*-6HF (Figure 1C). Several restriction sites in addition to the cloning sites of *Sal*I and *Bgl*II were also introduced at either end of the tagged *CaCDC4* for future application with other genes.

Verification of applicability of the modified tetracycline-inducible gene expression system

To validate the preservation of functionality of gene fused with *CaGFP* expressed from pTET25M-NC, the *CaCDC4* was chosen as a test gene and cloned into pTET25M-NC at sites of XX and YY to obtain pTET25M-N-*CaCDC4* and pTET25M-C-*CaCDC4*, respectively. These were then linearized by *Sac*II and *Kpn*I

before transforming into *C. albicans* strain BWP17 for Ura+. Stable integrants (C-*CaCDC4* and N-*CaCDC4*, respectively) that target the *ADH1* locus were confirmed by colony PCR with specific primers (Figure 2A). Significantly, the expression of *CaCDC4-CaGFP* encoded fusion protein at either N- or C-terminus was observed and was in a doxycycline-dependent manner (Figure 2B). The expression of *CaCDC4-CaGFP* encoded fusion proteins from the two integrants was also comparably in terms of intensity of fluorescence to that of *CaGFP* from integrants from pTET25M as observed by fluorescence microscopy (Figure 2C). The localization of the fusion proteins encoded by *CaCDC4-GFP* either N- or C-terminus appeared to be at the cytoplasm. We concluded that pTET25M was successfully established.

To validate the applicability of functionality of C-terminal 6×His-FLAG tagged protein, the pTET25M-*CaCDC4*-6HF was linearized by *Sac*II and *Kpn*I and transformed into *C. albicans* strain BWP17 for Ura+. Stable Ura+ integrants (*CaCDC4*-6HF) with their *ADH1* loci being targeted correctly were confirmed by colony PCR (Figure 3A). To verify the recyclable of *CaURA3* from *CaCDC4*-6HF, the Ura+ integrants were treated with 5-FOA for loss of *CaURA3* to obtain (*CaCDC4*-6HF-*dpl*200). The integration of dpl200 at the *ADH1* locus was confirmed by diagnostic colony PCR (Figure 3B). The doxycycline-induced expression of

tagged *Ca*Cdc4 proteins from both *CaCDC4*-6HF and *CaCDC4*-6HF-*dpl*200 strains was confirmed by Western blotting analysis, which was in a time-dependent manner. We concluded that modified pTET25M capable of expressing C-terminal 6×His-FLAG protein was successfully established and that the Ura-blaster cassette from pTET25M was able to be removed and left only the *dpl*200 without disturbing the expressing the tagged protein.

Potential applications

The pTET25M-NC allows construction of strains expressing protein either N- or C-terminal fluorescence fusion proteins for isolation, detection, and localization studies. The ability to generate both N- and C-terminal *Ca*GFP fusion proteins can be critical as in many cases only protein fusions at one particular terminus will not interfere the normal localization of proteins. In addition, numerous restriction sites at either end of *CaGFP* on pTET25M-NC provide optional choice of restriction sites during gene cloning. Furthermore, inducible expression of *CaGFP* fusion proteins by doxycycline that is highest level of overexpression relative to other regulatory expression system () allows visualization of fusion proteins when levels of proteins expressed from their native promoters are too low for detectable images. One extension of this system is that the *CaGFP* may be replaced with *C. albicans* adapted

CFP and *YFP* (Gola et al., 2003), or even the future developed fluorescence proteins. With different fluorescence fusion proteins, interaction between or among functional related proteins may be analyzed with fluorescence microscope.

To fully explore the usefulness of the newly developed system, pTET25M-*CaCDC4*-6HF was constructed such that the *Ca*Cdc4 is expressed as a C-terminal 6×His-FLAG tagged protein when introduced into *C. albicans* with the induction of doxycycline. The presence of several cloning sites flanking *CaCDC4* of the pTET25M-*CaCDC4*-6HF permits replacement of *CaCDC4* with other genes. Such tagging proteins can also be used for isolation, detection, and localization studies in concert with the *Ca*GFP fusion proteins. The epitope-tagged proteins may reflect the function of non-tagged native proteins more appropriate than those of GFP fusion ones due to the size of GFP that is more likely to interfere with the fused proteins than those with 6×His-FLAG epitope-tagging. Moreover, the system allows titration of doxycycline that can be used to monitor protein expression in conjunction with phenotype of either the physiological behavior of proteins when expressed closely to the native level or the perturbed performance pf proteins when fully overexpressed. Furthermore, the system has introduced the recyclable mini Ura-blaster cassette (Wilson et al., 2000). The existence of Ura-blaster cassette on the plasmid allows being introduced into *C. albicans* consecutively with another

expressing plasmid carrying *URA3* selection marker, facilitating the functional assessment of interaction between proteins.

We envisage that the system left room for further improvement. For example, N-terminal 6×His-FLAG epitope and other epitopes on either N- or C-terminus should be introduced to make the system universally applicable in terms of assessment of protein-protein interaction. In addition, it is apparent that replacement of *CaGFP* with genes encoding other *Candida*-adpated fluorescence proteins facilitates the study of interaction between fusion proteins with different florescence. Moreover, in the current system, the *ADH1* locus is the sole integration site and from which the constitutive expressed rtTA is driven. It is desirable to have an additional gene locus for integration and for driving rtTA constitutively. When combined with two proteins fused with different fluorescence proteins or two different epitope-tags, the interaction between proteins can be revealed. Furthermore, as the Ura-blaster cassette can only be used to recycle the *URA3* marker, it is worth of introducing more common systems such as the *FLP/FRT* (Reuss et al., 2004) or the Cre/*loxP* (Dennison et al., 2005) into current plasmid with different selection markers to permit excision of any selection marker for use in functional analysis of several gene products simultaneously.

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Figure legends

Figure 1. Construction of the pTET25M and modified versions for expression of GFP fusion and 6×histidine-FLAG epitope tagged proteins. (**A**) Construction of pTET25M. The *CaURA3* was released by *P* and *S*I and cloned in to pUC19 to make pUC19-*CaURA3*. The 3'-end of *CaURA3* (the *dpl*200) was PCR generated with *B* and *Xh* sites at each end and cloned into *B* and *S*l sites of pUC19-*CaURA3* to make pUC19-*CaURA3-dpl*200 that contains *CaURA3* flanked *dpl*200 repeats but has the *Xh* and *S*I sites destroyed. The *CaGFP* and up- and down-stream region on the pNIM plasmid vector was PCR produced with *B* and *K* at the ends and cloned into pUC19-*CaURA3-dpl*200 at the *B* and *K* sites to make pUC19-TET25. The *S*I and *E*I

region of the pUC19-TET25 containing *CaGFP* and upstream half of *CaURA3* was released and cloned into pTET25 deleted with the *S*I and *E*I region to make pTET25M. (**B**) Construction of pTET25M-NC and derivatives. The *CaGFP* of the pTET25M was replaced by *CaGFP* flanked with several cloning sites (as indicated) that was generated by PCR amplification using *CaGFP* as a template and primers with several cloning sites as indicated. The sites of *Bg* and *Bc* were destroyed. The coding region of *CaCDC4* was PCR-amplified with *A*II and *Bg* flanking sites and was then cloned into pTET25M-NC to generate pTET25M-C-CaCDC4. The coding region of *CaCDC4* was PCR-amplified with *S*I and *N* flanking sites and was then cloned into pTET25M-NC to generate pTET25M-N-CaCDC4. (**C**) Construction of a modified version of pTET25M, pTET25M-CaCDC4-6HF. The *CaGFP* was first replaced with the coding region of *CaCDC4* at *Bg* and *S*I sites to obtain pTET25M-CaCDC4. The *CaCDC4* of the pTET25M-CaCDC4 was then replaced by a DNA sequence encoding *Ca*Cdc4 tagged with 6×histidine-FLAG at its C-terminus to obtain pTET25M-CaCDC4-6HF. The *CaCDC4* of the pTET25M-CaCDC4-6HF was flanked with sites of *Xh* and *N* at 3'-end and those of *A*II and *S*I at 5'-end, which can be replaced with other gene of interest. Restriction enzyme abbreviations: *Sc*II, *Sac*II; *X, Xba*I; *Xh, Xho*I; *S*I, *Sal*I; *B, Bam*HI; *Bg, Bgl*II; *P, Pst*I; *E*I, *Eco*RI; *Sc*I, *Sac*I; *Nc, Nco*I; *K, Kpn*I; *Bc, Bcl*I; *St, Stu*I; *Afl*II, *Afl*II; *A*II, *Aat*II; *Bs, BspE*I; *N, Not*I; *Nh, Nhe*I; *EV,*

*EcoR*V.

Figure 2. Construction of *C. albicans* strain capable of expressing *CaCDC11* fused with GFP at either N- or C-terminus from P_{tet} that is doxycycline inducible. (**A**) To construct strain capable of expressed the GFP-*Ca*Cdc4 fusion protein inducibly by doxycycline (Dox), plasmid DNA from each of pTET25M-N-*CaCDC11* and pTET25M-C-Ca*CDC11* was linearized by *S*II and *K* and transformed into the auxotrophic strain BWP17 (*ura3 his1 arg4*) at the *ADH1* locus for Ura+ to generate *CaADH1/Caadh1*::pTET25M-C-*CaCDC4:CaURA3dpl*200 (*TET*-C-*CaCDC4*) and *CaADH1/ Caadh1*:: pTET25M-N-*CaCDC4:CaURA3dpl*200 (*TET*-N-*CaCDC4*). (**B**) The transformed integrants with correct structure at the *ADH1* locus from **A** were verified by colony PCR with specific primers that generate products with specific sizes. (**C**) The doxycycline-inducibility of GFP-*Ca*Cdc4 fusion protein (indicated by the black arrow) from the strains *TET*-C-*CaCDC4* and *TET*-N-*CaCDC4* was assessed by Western blotting with anti-FLAG antibody. The expression appeared to be maximized at 6hr of doxycycline induction. Note that anti-FLAG antibody appeared to dominantly cross-react to some endogenous proteins. (**D**) To assess fluorescence of GFP in the context of GFP-*Ca*Cdc4 fusion protein at either N- or C-terminus, the *TET*-C-*CaCDC4* and *TET*-N-*CaCDC4* strains were grown in YPD to exponential and

emission of green fluorescence of GFP-*Ca*Cdc4 was examined microscopically. Restriction enzyme abbreveations: *Sc*II, *Sac*II; *X, Xba*I; *Xh, Xho*I; *Sl, Sal*I; *B, Bam*HI; *Bg, Bgl*II; *P, Pst*I; *El, Eco*RI, *Nl, Not*I; *Al, Aat*II; *Sc*I, SacI; *Nc, Nco*I; *K, Kpn*I.

Figure 3. Construction of *C. albicans* strain capable of inducibly expressing epitope-tagged *Ca*Cdc4 with doxycycline. (**A**) To construct strain capable of expressed the tagged *Ca*Cdc4 inducibly by doxycycline (Dox), pTET25M-*CaCDC4*-6HF generated as in Figure 1 was linearized by *Sac*II and *Kpn*I and transformed into the auxotrophic strain BWP17 (*ura3 his1 arg4*) at the *ADH1* locus for Ura+ to generate *CaADH1*/*Caadh1*::pTET25M-*CaCDC4*-6HF: *CaURA3dpl*200 (*TET-CaCDC4-6HF*). (**B**) Yeast colony PCR was used to verify the correct yeast integrants with marker *CaURA3*-specific or *ADH1* locus specific primer pairs. To pop out the *CaURA3* from *TET-CaCDC4-6HF*, the *TET-CaCDC4-6HF* was grown on YPD plates with 5-FOA for Ura– to obtain *CaADH1*/ *Caadh1*::pTET25M -*CaCDC4-6HF*:*dpl*200 (*TET-CaCDC4-6HF-dpl*). (**C**) The doxycycline-inducibility of tagged *Ca*Cdc4 protein (indicated by the black arrow) from the strain *TET-CaCDC4-6HF* and *TET-CaCDC4-6HF-dpl* was assessed by Western blotting with anti-FLAG antibody. The expression was maximized at 6hr of doxycycline induction. Note that anti-FLAG antibody appeared to dominantly cross-react to some

endogenous proteins. Restriction enzyme abbreveations: *Sc*II, *Sac*II; *X, Xba*I; *Xh*, *Xho*I; *Sl*, *Sal*I; *B*, *Bam*HI; *Bg*, *Bgl*II; *P*, *Pst*I; *El*, *Eco*RI, *Nl*, *Not*I; *Al*I, *Aat*II; *Sc*I, SacI; *Nc*, *Nco*I; *K*, *Kpn*I.

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Figure 1

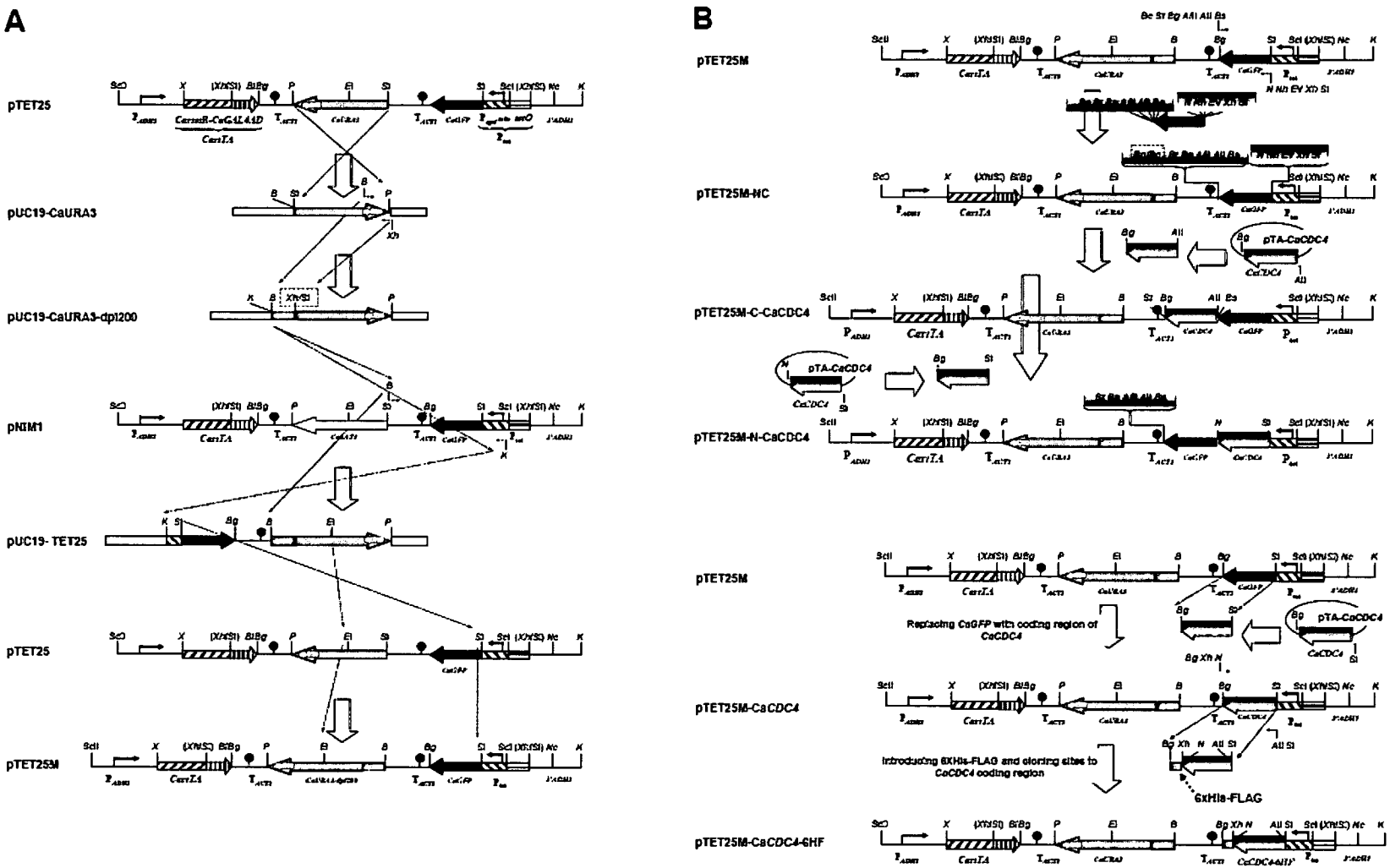
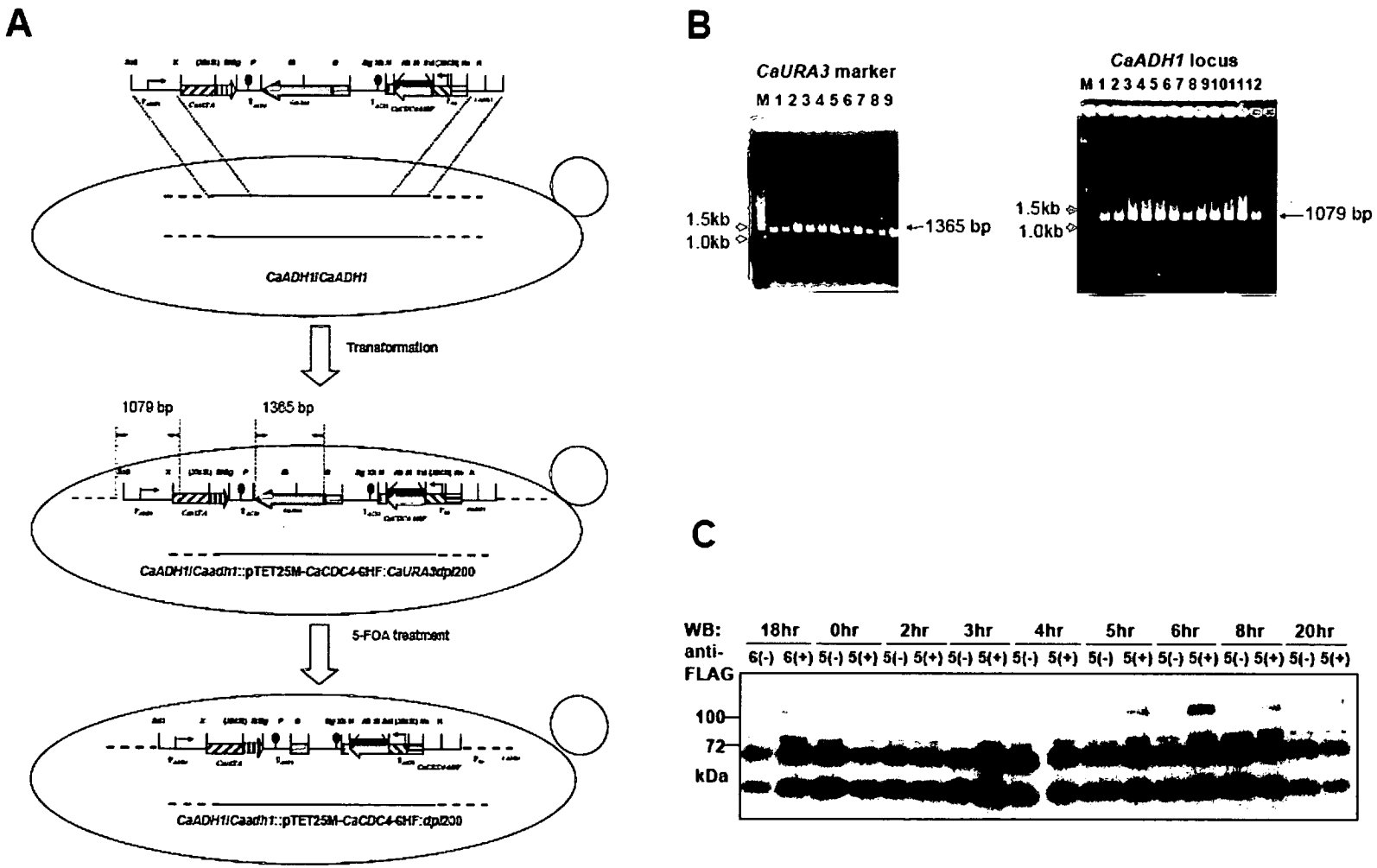

A
pTET25
pUC19-CaURA3
pUC19-CaURA3-dp1200
pNIM1
pUC19- TET25
pTET25
pTET25M
B
pTET25M
pTET25M-NC
pTET25M-C-CaCDC4
pTET25M-N-CaCDC4
pTET25M
Replacing CaGFP with coding region of CaCDC4
pTET25M-CaCDC4
Introducing 6XHis-FLAG and cloning sites to CaCDC4 coding region
6xHis-FLAG
pTET25M-CaCDC4-6HF

Figure 3


A
CaADH1/CaADH1
Transformation
1079 bp
1365 bp
CaADH1/Caadh1::pTET25M-CaCDC4-6HF:CaURA3dpl200
5-FOA treatment
CaADH1/Caadh1::pTET25M-CaCDC4-6HF:dpl200
B
CaURA3 marker
M 1 2 3 4 5 6 7 8 9
1.5kb
1.0kb
1365 bp
CaADH1 locus
M 1 2 3 4 5 6 7 8 9 10 11 12
1.5kb
1.0kb
1079 bp
C
WB:
anti-FLAG
18hr 0hr 2hr 3hr 4hr 5hr 6hr 8hr 20hr
6(-) 6(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+) 5(-) 5(+)
100
72
kDa

Affinity purification of *Candida albicans Ca*Cdc4 associated proteins reveals presence of novel proteins for morphogenesis

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Running title: *Candida albicans* Cdc4 associated proteins

Key words: *Candida albicans*; *CDC4*; affinity-purification; yeast two-hybrid; mass spectrometry

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Abstract

The *Candida albicans CDC4* is nonessential and plays a role in negatively regulating filamentous growth that is opposed to its evolutionary counterparts for G1-S transition of the cell cycle. Genetic epistasis analysis has indicated that proteins in addition to *C. albicans* Sol1 are targets of *C. albicans* Cdc4. Moreover, no formal evidence suggests that the *C. albicans* Cdc4 acts its function through ubiquitin E3 ligase of Skp1-Cul1/Cdc53-F-box complex. To elucidate the function of *C. albicans CDC4*, we have sought to identify *C. albicans* Cdc4 associated proteins by affinity-purification. 6×His epitope-tagged *C. albicans* Cdc4 expressed and purified from *E. coli* was used to incubate with the lysates from cells of *C. albicans CDC4* null homozygous mutant. After several steps of washing and elution, the *C. albicans* Cdc4 together with its potential associated proteins were resolved on SDS-PAGE and followed by silver staining. Ten candidate proteins were extracted and subjected to analysis by MALDI-TOF mass spectrometry. Among ten candidate protein groups whose identities were revealed, two proteins encoded by *GPH1* and *THR1* genes were further verified to be *C. albicans* Cdc4 interactors by using yeast two-hybrid. We conclude that due to the nature of *C. albicans* Cdc4, *in vitro* affinity-purification using *C. albicans* Cdc4 as a bait generated from *E. coli* and proteins from cell lysates of *C. albicans CDC4* null homozygous mutant as a source prey permit identifying novel

proteins physically interacted and functionally associated with *C. albicans* Cdc4.

Introduction

The opportunistic human fungal pathogen *Candida albicans*, a natural diploid without conventional sexual cycle, causes disease in healthy and immunocompromised hosts. Considerable efforts have been made towards elucidating the molecular mechanism for the control of morphogenesis in *C. albicans* as it has been known to be associated with virulence and pathogenesis. Research progress has already revealed an unanticipated complexity in that at least three positive and five negative pathways control morphological transition in *C. albicans* (Berman and Sudbery, 2002; Biswas et al., 2007; Whiteway and Bachewich, 2007). To add even more impediment, Cdks and many cyclins with their regulators have also been shown to play roles in the control of such morphological transition in *C. albicans* (Berman, 2006). As such, a critical and underlying issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incomplete understood. Significantly, an intriguing question has recently been revealed by us and others that some key cell cycle genes such as *CDC4* and *GRR1* conserved throughout evolution play no essential role on cell cycle but morphogenesis in *C. albicans* (Atir-Lande et al., 2005; Bensen et al., 2005; Bensen et al., 2002; Butler et al., 2006; Li et al., 2006; Shieh et al., 2005).

C. albicans CDC4 encodes a structural homologue of the *Saccharomyces*

cerevisiae Cdc4, whcih is an F-box protein of ubiquitin E3 ligase of Skp1-Cul1/Cdc53-F-box (SCF) complex, termed SCF^{Cdc4}. We and others have found that contrasting to *S. cerevisiae CDC4* being essential for progression through G1-S transition in the cell division cycle, *C. albicans CDC4* (*CaCDC4*) appears to be nonessential and is a negative regulator of filamentous growth (Atir-Lande et al., 2005; Shieh et al., 2005). *C. albicans* homologue of Sic1, the Sol1, has been isolated as a target of *C. albicans* Cdc4 (*Ca*Cdc4) (Atir-Lande et al., 2005). However, a hyperfilamentation phenotype of double mutant of *Cacdc4* -/- and *sol1*-/- has been shown to be similar to that of the single *Cacdc4* -/- mutant. The result suggests that stability of Sol1 alone does not explain the constitutive hyphal morphology of *Cacdc4* -/-. We therefore postulated that presence of other substrates for *Ca*Cdc4 and conducted affinity-purification of *Ca*Cdc4 to allow identification of CaCdc4 interacting proteins.

In this report, we describe the identification of the *Ca*Cdc4 associated proteins by purifying the incubation of the recombinant *Ca*Cdc4 generated from *E. coli* with the lysates from *CaCDC4* homozygous null mutant. Analysis by MALDI-TOF of the associated proteins and confirmation by the yeast two-hybrid reveal two proteins encoded by *GPH1* and *THR1* genes, which have not been known to be functionally associated with orthologs of *Ca*Cdc4 among other species. Our study uncovers new

components functionally associated with *Ca*Cdc4, which will lead to further understanding the role of *CaCDC4* regulating morphogenesis.

Materials and Methods

Strains, growth conditions, and DNA methods

Escherichia coli DH5α (F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) was used as a host for the routine maintenance and amplification of plasmids. *E. coli* BL21 (F^- *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]) was used to generate the recombinant *Ca*Cdc4 protein. Bacterial cultures were grown in L-broth, supplemented with 50μg/ml ampicillin as required (Miller, 1972). Plasmid DNA was purified by Gene-Spin™-V^2 Miniprep Purification kit (Taipei, Taiwan). The oligonucleotide primers used to constructed plasmids and diagnostic analysis for strains are listed in Table 1.

The *CaCDC4* homozygous null mutant of *C. albicans* (T.-L. Tseng *et al.*, manuscript in preparation) was constructed based on the parental auxotrophic *C. albicans* BWP17 (*ura3Δ::λimm434/ura3::λimm434; arg4::hisG/arg4::hisG; his1::hisG /his1::hisG*) (Wilson et al., 1999), which was used in the *in vivo* affinity purification of *Ca*Cdc4 associated proteins. *C. albicans* strains were grown at 30°C in either complete medium of yeast extract-peptone-glucose (YEPD) or synthetic

minimal medium with or without uridine (Sherman et al., 1986).

Expression and purification of recombinant *Ca*Cdc4 in bacteria

To express recombinant protein in *E. coli*, the coding region of *Ca*Cdc4 was PCR amplified with primers xxxx (sequence) and yyyy (sequence), and cloned into pET-29b(+) at the sites of xxxx and yyyy. The construct was transformed into *E. coli* BL21 for ampiciline resistant. The transformant was grown in L-broth at 25°C with 50μg/ml ampicillin, and the induction of expression of C-terminal 6×histidine tagged *Ca*Cdc4 was optimized with 5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hrs. The cells were lysed in the lysis buffer containing 1xPBS, 5% Glycerol, 1mM Sodium EDTA, 1mM Sodium EGTA, 1mM Dithiothreitol, 1x Complete EDTA-free protease inhibitor Cocktail (Roche), 1mM PMSF, Triton-X-100. The histidine-tagged proteins were then purified with nickel-nitrilo-triacetic acid (NTA) agarose chromatography (Qiagen) essentially as described (Shieh et al., 1998). Protein concentrations were determined by the method of Protein Assay (Bio-Rad) according to the manufacturer's instruction.

Affinity purification of *Ca*Cdc4 interacting proteins *in vitro*

To purify *Ca*Cdc4 associated proteins *in vitro*, the total cell lysates of *CaCDC4*

homozygous null *Cacdc4* -/- mutant of *C. albicans* were first prepared as described previously (Shieh et al., 2007). The cell lysates with approximately X μg were then incubated with X μg of purified 6×histidine tagged *Ca*Cdc4 from *E. coli* and the CaCdc4 associated proteins were eluted together with the 6×histidine tagged *Ca*Cdc4 by nickel-NTA agarose chromatography (Qiagen) according to the manufacture's instruction. The proteins were resolved by SDS-PAGE and visualized with staining of silver nitrate by the method as described (Shevchenko et al., 1996) with modifications. Briefly, after electrophoresis, gels were washes twice and then fixed with 50% methanol and 25% glacial acetic acid in dH_20 for 2 hrs, followed by washing with 30% methanol for 15 min and subsequently washing three times with dH_20 for 5 min each. The gels were incubated with sensitizing solution of 0.8 mM sodium thiosulphate for 2 min, washed twice with dH_20 for 30 sec each, followed by incubation in a 0.2% silver nitrate solution for 25 min at 4°C in the dark. After washing twice with dH_20 for 30 sec each, the gels were developed with solution containing 0.28M sodium carbonate, 0.185% formaldehyde, and 0.016 mM sodium thiosulphate for about 10 min or until appropriate visualization of proteins. The reaction was terminated by addition of 0.042 M EDTA.

In-gel tryptic digestion and mass spectrometry

The proteins were recovered from the gels and in-gel digested with trypsin as described previously (Shevchenko et al., 2006) with some modifications (Marchetti-Deschmann et al., 2009). Upon completion of in-gel digestion using trypsin, the digested solution was transferred to a clean 0.6-mL tube prior to addition of 50 mL solution of 50% acetonitrile (ACN)/5% formic acid (FA) with sonication for 30 min. After repeating the extraction procedure for three times, a total of about 150 μL of extracts was collected and concentrated to less than 10 μL using a vacuum centrifuge. Subsequently, the samples were reconstituted with trifluoroacetic acid (TFA) to a final concentration of 0.1% prior to cleaning up with C18 ZipTips (Millipore) for MALDI-TOF analysis. The C18 ZipTips were conditioned with 100% ACN, followed by equilibration three times with 0.1% TFA. By alternatingly aspiring and dispensing for at least 15 rounds, and subsequently washing with 0.1% TFA and eluting with 20 μL of elution buffer (75% ACN, 0.1%TFA), the samples were bound to the ZipTip pipet tip. The peptide sample was mixed with an equal volume of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% CAN) and spotted onto a 384-well stainless steel MALDI target plate prior to analysis with an UltraFlexIII MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in Medical Research Core Facilities Center of Research and Development, China Medical University, Taiwan.

Data analysis for database searching and protein identification

The monoiootopic masses (*m/z*) of both parent ions and their corresponding fragment ions, parent ion charge states (z), and ion intensities from the MS/MS spectra acquired were automatically extracted using the script in the Analyst software and directly submitted for automated database searching against the Candida Genome Database (CGD) using Ms-Fit of Protein Prospector (University of California San Francisco). Cysteine Carbamidomethyl at cysteine residue was set as static modifications and one missing cleavage was allowed. Minimum number of peptides required to match was set to four. The MOWSE score, Pfactor, was set to 0.4. The best possible candidate proteins from the search results were evaluated manually with functional relevance and further confirmed by the yeast two-hybrid assay.

Immunological detection

Cells of *E. coli* expressed *Ca*Cdc4 proteins were grown in the optimal induced condition as described in previous section. Total protein was extracted from the *E. coli* cells by the methods as described in (). The proteins were resolved by SDS-PAGE and transferred electrophoretically to PVDF membranes (PerkimElmer, Boston, MA). The membranes were probed with monoclonal antibody to poly histidine. Detection was performed using a peroxidase-conjugated either anti-mouse IgG () or anti-rabbit IgG

(Pierce, Rockford, IL). The signal was visualized by SuperSignal West Pico Chemicllluminescence Substrate Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Yeast two-hybrid analysis

Yeast two-hybrid interaction assays were performed as described previously (Wilkinson et al., 1996). To generate pGBKT7-*CaCDC4* capable of expressing a fusion protein of GAL4 DNA binding domain, the *CaCDC4* of xxxx-bp was PCR-amplified with *C. albicans* genomic DNA and oligonucleotides x and x incorporating respective xxxx and xxxx sites (Table 1) and digested with xxx and xxxx before ligation into the xxxx and xxx digested pGBKT7. Genes of the *C. albicans*, including *GPH1*, *HMT1*, *ST11*, *DPB8*, and two *THR1s*, *THR1*/orf19.923 [*THR1* (1)] and *THR1*/orf19.8539 [*THR1* (2)], were initially PCR-amplified and cloned into pCR2.1-TOPO with oligonucleotides of either x and y, x and x, x and y, x and y or x and y (Table 1). The coding sequence of *GPH1* was amplified by PCR using the oligonucleotide x incorporating an *Nco*I site (Table 1) and the oligonucleotide x incorporating a *Bam*HI site (Table 1) with the pCR2.1-TOPO-based *GPH1* to produce a 2703-bp fragment that was digested with *Nco*I and *Bam*HI and ligated into the *Nco*I and *Bam*HI sites of pACT2 () to form pACT2-*GPH1* capable of

expressing a fusion protein of GAL4 activation domain. Similarly, the pACT2-*THR1*(1), - *THR1*(2), -*HMT1*, -*ST11* and -*DPB8* were obtained by using coding sequences of genes based on pCR2.1-TOPO as templates for PCR amplification with the 5' oligonucleotide x incorporating an *Nco*I site (Table 1) and the 3' oligonucleotide x incorporating a *Bam*HI site (Table 1) for respective 822-bp, xxx-bp, xxx-bp, and xxx-bp fragments. The interaction assay was conducted by mating the *S. cerevisiae Mat***a** AH109 transformed with pGBKT7-*CaCDC4* with *S. cerevisiae Mat*α Y187 transformed with respective pACT2-*GPH1*, -*THR1*(1), -*THR1*(2), -*HMT1*, and -*ST11*. The growth of diploid *S. cerevisiae* cells on semi-solid agar plate with medium lacking histidine was used as an indicator of interaction.

Results and Discussion

Expression of recombinant *Ca*Cdc4 in *E. coli*

We have previously identified *Ca*Cdc4 as a negative regulator of hyphal growth in *C. albicans*. To determine if the *Ca*Cdc4 plays it role through ubiquitin E3 ligase of SCF complex and to identify substrates or potential regulators of *Ca*Cdc4, we adopted *in vitro* affinity purification approach using recombinant *Ca*Cdc4 protein purified from *E. coli* as bait. The approach was based on the fact that ubiquitination of the ubiquitin E3 ligase is functional with all its components co-expressed within *E. coli*

cells (Su et al., 2006). We anticipated that the *E. coli* expressed and purified recombinant *Ca*Cdc4 has the function, hence the affinity to its associated proteins. In addition, the use of recombinant *Ca*Cdc4 purified from *E. coli* as a bait to probe *Ca*Cdc4 associated proteins from *C. albicans* cell lysates is devoid of the possibility of degradation of the *Ca*Cdc4 substrates, in which purification of the *Ca*Cdc4 associated proteins *in vivo* was proven to be ineffective (our unpublished observation), or the *Ca*Cdc4 itself (P.C. Lai *et al.*, manuscript in preparation) when *Ca*Cdc4 is expressed in cells of *C. albicans*. Under IPTG-induced condition, the *Ca*Cdc4 tagged with 6×histidine at the C-terminus was optimally expressed and purified from *E. coli* (Figure 1) and used for affinity purification of its associated proteins *in vitro*.

In vitro affinity-purification of the *Ca*Cdc4 associated proteins

To ensure *Ca*Cdc4 associated proteins, particularly the potential targets, being revealed, we used proteins extracted from cell lysates of *CaCDC4* homozygous null mutant (*Cacdc4* -/-) of *C. albicans* (T-L.Tseng *et al.*, manuscript in preparation) as a source of prey proteins. In fact, we have previously adopted an approach that allow comparison of patterns of affinity-purify proteins from lysates of *C. albicans* cells capable of doxycycline-induced the expression of *Ca*Cdc4 tagged with 6×histidine and FLAG C-terminally either induced for hyphal form or remaining in yeast from. It

appeared that although the doxycycline-inducible system was able to induce the expression of *Ca*Cdc4 in a defined window of time with massive amount of *Ca*Cdc4 for affinity purification. Nevertheless, this over-expressed, and presumably hyperactive *Ca*Cdc4 might lead to its own degradation (P.C. Lai *et al.*, manuscript in preparation). As a result, such an approach of purification of *Ca*Cdc4 *in vivo* proved to be ineffective.

The bacterial purified *Ca*Cdc4 was used to incubate *in vitro* with proteins of *C. albicans* cell lysates from *Cacdc4* -/-. After being washed and eluted, the *Ca*Cdc4 along with the associated proteins were resolved on SDS-PAGE. The presence of bait *Ca*Cdc4 protein during the wash-elution process was monitored by Western blotting (Figure 2A). The resolved protein samples were also visualized by silver staining (Figure 2B) and the prey proteins selected from the elution steps were those not present or less prominent but not entirely excluded in the wash steps. A total of 10 prey proteins (Figure 2B) that appeared consistently in at least three experiments were subjected to further in-gel tryptic digestion and mass spectrometry.

Determination of the Identify of *Ca*Cdc4-associated proteins and implication of their functional relevance to *Ca*Cdc4

To determine the identity of proteins detected by affinity purification of *Ca*Cdc4,

each of the visualized proteins from SDS-PAGE was recovered and in-gel digested with trypsin to generate peptides for the analysis of tandem mass spectrometry. After obtaining the specific MS/MS spectra of the purified proteins (Figure 3) from the bands migrating at the position around 100, 110, 72, 45, 40, 35, 30, 28, 25, or 20 kDa (see Figure 2B), the data were used to search against *C. albicans* database CGD using the Ms-Fit of Protein Prospector. Ten bands categorized into 10 groups of G1 to G10 (Figure 2B), some of which contained more than one potential protein as listed in Table 3. Based on both the highest score of MS/MS spectrum for identity among proteins within each group and the possible functional relevance to *Ca*Cdc4 of our interests, the genes associated with those four proteins were initially selected for further analysis. We were particularly interested in and used for further verification of their being the *Ca*Cdc4-interacting proteins by the yeast two-hybrid assay.

Validation by yeast two-hybrid reveals two novel proteins interacted with *Ca*Cdc4

Discussion

The different pattern of profile in terms of in the amount of protein and presence-absence of protein between samples of hyphae-induced and non-induced conditions were compared. As indicated in Figure 1C, even though higher background

occurred with the sample of *C. albicans* cell lysates as a negative control, some proteins appeared to be presence from both the hyphae-induced and non-induced conditions, which we expected to be members of ubiquitin E3 ligase of SCF complex. Interestingly, at least xxxx proteins presence only from hyphae-induced condition, whereas at least yyyy proteins presence only from hyphae-non-induced condition. Several proteins showed variation in the amount between hyphae-induced and hyphae-non-induced conditions. We anticipated that the latter two categories of proteins are likely to be either substrates or regulators of *Ca*Cdc4.

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Figure legends

Figure 1. *In vitro* purification of *Ca*Cdc4 associated proteins. (**A**) The C-terminal 6xHis-tagged *Ca*Cdc4 was produced in cells of *E. coli BL21* with plasmid *pET-29b*(+)-*CaCDC4* grown at 25°C for 3 hrs under 0.5mM IPTG induction (the arrow). However, the *Ca*Cdc4 generated was confined in the pellet (P) and in the supernatant (S) of the *E. coli* cell lysates. Purification of 6xHis-tagged CaCdc4 from *E. coli* cell extracts. W1 and W2 designate washing with buffer without imidazole. E1 and E2 designate elution with 250mM imidazole. R represents residuals of CaCdc4-6xHis fusion protein on the Ni2+ resin. (**B**) Purified 6xHis-tagged *Ca*Cdc4 bound with Ni2+-beads were incubated with cell lysates of either yeast or hyphal cells of SC5314 grown overnight at 4°C. *Ca*Cdc4 associated proteins purified by *Ca*Cdc4 affinity chromatography were resolved by SDS-PAGE and the presence of *Ca*Cdc4 was first verified by Western blotting. (**C**) Potential *Ca*Cdc4-associated proteins that were differentially purified in yeast and hyphal forms were visualized by silver staining and subjected to analysis with MALDI-TOF or LC/MS/MS (the arrows).

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