計 畫 編 號：NHRI-EX99-9808SI

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

99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： 99 年 1 月 1 日 至 99 年 12 月 31 日

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壹、99年度計畫研究成果摘要

計 畫 名 稱：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 編 號：NHRI-EX99-9808SI

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，形態生成，細胞專一泛素化蛋白體，細胞專一磷酸化蛋白體，原核生物泛素E3連接酵素測定，白色念珠菌CUG密碼子適應之tRNA

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，特別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的瞭解。我們一直對白色念珠菌細胞週期中 G1 時期的調控有興趣。我們以 *CDC4* 及 *CDC7* 和其調節次單元編碼基因 *DBF4* 的白色念珠菌同源基因為主要目標因其為釀酒酵母中分別於 G1 到 S 期 及 DNA 複製所必需且在演化過程中功能高度保留。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*) 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可塑性的關鍵。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形態生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，我們仍朝研究目標獲致些許進展。此計畫可依涉及之特定技術平台分為。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實施特定激酶反應而得其受質。因此，進度報告將據以分項。由於部份進展已於去年報告，本次報告將著重本年進展，謹分述如下。

壹、以二維電泳建構白色念珠細胞形態專一泛素化或磷酸化蛋白體圖譜

一、 細胞形態專一之泛素化蛋白體

我們在第一年已建立將野生型白色念珠菌 SC5314 細胞有效誘導成酵母菌、真菌絲及假菌絲特定細胞形態之方法(**Table 1**)也建立二維電泳膠操作及細胞專一蛋白體圖譜之分析方法。為了就不同白色念珠菌細胞形態分析差異化次蛋白體，我們尋求建立有效之豐富細胞形態專一次蛋白體之方法。為避免我們鑑定之泛素化蛋白質透過蛋白酶體而水解，我們建立了一種化學方法可讓廣汎運用的蛋白酶體抑制劑 MG132 不需像釀酵母般依賴構築特定突變株就能通透入白色念珠菌細胞內。白色念珠菌細胞在萃取蛋白質前以 MG132 處理所得之泛素化蛋白質確實比未處理者多(**Figure 1**)。然而，以目前可取得能豐富泛素化蛋白質之商品化套組，我們仍無法有效取得汎素化蛋白體 (**Table 2, #1 & #2**)，雖然仍有被認為是最有效豐富化泛素化蛋白質的方法迄未嘗試(**Table 2, #4**)。因此，我們決定以二維電泳膠搭配賴氨酸 48 聯結專一多泛素抗體之西方墨點法分析白色念珠菌泛素化蛋白體(**Table 2, #4**)。我們試著分析了酵母菌及菌絲態差異性泛素化蛋白體。雖然細胞專一泛素化蛋白質可被鑑定出(**Figure 2**)，並非所有此類蛋白質於染 instant blue 後可見。然而，有些主要蛋白質只以肉眼可辨識見其持續出現。此類蛋白質將以質譜儀鑑定其身份。

二、細胞形態專一之磷酸化蛋白體

為解析不同白色念珠菌細胞形態差異性磷酸化蛋白質體圖譜，我們採用 Pierce 磷酸化蛋白質豐富化套組。由於磷酸化蛋白質豐富化不需使用蛋白酶體抑制劑 MG132，最佳誘導不同細胞形態之條件有些許不同 (**Table 3**) 而最佳豐富化條件及步驟則列於 **Table 4, #1**。我們首先豐富化磷酸化蛋白體再用二維電泳膠分離磷酸化蛋白體以便分析差異化酵母菌及假菌絲態細胞磷酸化蛋白體。豐富化、濃縮、及 2D clean-up 完成之階段其蛋白質量先行偵測 (**Figure 3A**)，以便進行二維電泳 (**Figure 3B & 3C**)。確實兩種不同細

胞形態磷酸化蛋白體之一致性差異肉眼可辨 (**Figure 3B & 3C**)。然而，我們無法完全肯定差異源於相同蛋白質之不同磷酸化態亦或不同蛋白質，但皆因其等電點之不同而得分離。我們察覺白色念株菌磷酸化蛋白體等電點主要介於 5 至 8 之間，為有效分離其磷酸化蛋白體將於二維分離膠體使用介於 5 至於 8 的酸鹼值。此外，使用磷酸酶 (**Table 4, #3**)去除磷酸化蛋白質之磷酸或能顯現源於多磷酸化態於單一蛋白質造成之差異性磷酸化蛋白體 In addition, the use of phosphatase (**Table 4, #3**)。另外，不同細胞形態之磷酸化蛋白體可於二維電泳膠分離後以 Pro Q Diamond 染色直接偵測 (**Table 4, #2**)，雖然此仰賴能擷取 Pro Q Diamond 染色影像的儀器，因而必須尋得來源。基本上此法以最少步驟完成而有較佳專一性或為一較佳選擇。

貳、於大腸桿菌重建白色念珠菌 SCF 泛素 E3 連接酶及激酶篩選特定受質

一、 構築白色念珠菌 SCF^{Cdc4} 泛素 E3 連接酶及 DDK 激酶

由於以原核生物為基礎之 Skp1-cul1/Cdc53-F-box (SCF) 泛素 E3 連接酶測定需要於大腸桿菌表現包括 SCF 複合體成員及其它相關蛋白質至少一共八種蛋白質(**Figure 4**) [1]，能承接如此多基因之載體系統至為關鍵。我們選用了 pQLinkX 載體系統 [2] 允許選殖多種基因之一種於此載體再將其它基因一一加入此單一之 pQLinkX 載體並配合另外兩種載體 pCDFDuet1 及 pCOLA-3-DEST [3] (**Figure 5**)。由於複製子及抗生素篩選基因之相容性，總共有五個質體可共存，其中之一可表現七種大腸桿菌罕見 tRNA 另外四種質體可同時被誘導表現之白色念珠菌 CUG 密碼子適應編碼絲氨酸之突變 $tRNA_{CAG}$ (**詳見 98NHRI mid-term report**) 及至少八種蛋白質於此系統。我們專注於研究 SCF 泛素化 E3 連接酶蛋白質由載體 pQLinkX and pCDFDuet1 表現的有效性 (**Figure 6**)。載體 pCDFDuet1 可承載兩基因，編碼 E1 的 *UBA1* 及編碼 E2 的 *CDC34*，個別都能以標記蛋白質形式表現

(**Figure 7**)。載體 pQLinkX 可於大腸桿菌產生無標記、7×His 標記、GST 融合及 HA 標記 (我們製備增加的新標記) 蛋白質 (**Figure 8**)。由於能將兩質體以一為載體另一為插入者接續連結，最終多個基因可構築於單一一個 pQLinkX (**Figure 9**)。個別表達單一 GST 融合蛋白質及共表達二至四個蛋白質已被確認 (**Figure 10**)。個別表達單一 7×His 標記蛋白質及共表達二個蛋白質也已被確認 (**Figure 11**)，即便 Cdc53 似乎無法產生。泛素蛋白質用來測試 pQLinkX 載體表現無標記及 HA 標記蛋白質 (**Figure 12**)。由於 Cdc53 想必因對大腸桿菌具毒性而無法以 7×His 標記蛋白質型式產生，我們將構築一 pQLinkX 載體可於大腸桿菌表現 7×His 標記 Skp1、Cdc4、Hrt1，無標記泛素以及 GST 融合 Cdc53。一旦上述蛋白質在大腸桿菌表現能證實有效，由載體 pCDFDuet1 表現之 E1 及 E2 將會被包括整合。如此，建立一有功能的 SCF 泛素 E3 連接酶所需成分可以全在大腸桿菌表現。為了確認 SCF 泛素 E3 連接酶的功能性，我們以由 pCOLA-3-DEST(白色念珠菌 cDNA 庫構築於此)載體表現 Sol1 為受質測試 SCF^{CaCdc4} 泛素 E3 連接酶可否將其泛素化。由於 Sol1 並未正是被證明是 SCF^{CaCdc4} 泛素 E3 連接酶的受質，我們將尋求重建 SCF^{CaGrr1} 泛素 E3 連接酶並以其已知受質 Ccn1、Hof1、或 Cln3 來確認此系統的有效性 (**Figure 13**)。

為了測試白色念珠菌 Dbf4 依賴激酶 (DDK)是否在大腸桿菌有激酶活性，我們尋求將 *CDC7* 及 *DBF4* 選殖入 pCDFDuet-1 載體並確認 DDK 是否能在大腸桿菌形成為一有功能的激酶而磷酸化潛在之受質。此策略異於原先利用由白色念珠菌純化 Cdc7 及 Dbf4 並於體外進行激酶測定。目前單一蛋白質的表現已經確認 (**Figure 14**)。

二、以 Gateway 選殖系統將白色念珠菌 cDNA 庫有效穿梭至最終表現載體

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，我們尋求能夠構築 cDNA 庫的最好方法。我們選用了 Invitrogen CloneMiner cDNA 構築的方法。為了純化高品質的 RNA 及 mRNA，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 且連接 *att*B 配接子(adapter) 。利用 Gateway 系統之捐獻(donor)載體(pDONR)中一個含有 *att*P 位的 pDONR222 並以一含 *att*B 位的 cDNA 庫為受質，可在體外以 BP Clonase 進行重組反應產生含有 *att*L 位帶有 cDNA 庫的入口(entry)載體 pENTR 及含 *att*R 位的副產物 (**Figure 15A**)。我們已得到一個入口 cDNA 庫內含 2.6×10^7 獨立選殖株的，其中 87%具 cDNA 插入片段 (**詳見 98NHRI mid-term report**)，具有足夠代表白色念珠菌 mRNA 數目。因此，帶 *att*L 位含有 cDNA 庫的入口載體 pENTR 可與帶 *att*R 位的目地(destination)載體 (pDEST)以 LR Clonase II 進行重組產生帶有 *att*B 位的表現庫(expression library)(pEXPR)。然而，起初設定以 pCOLA-3-DEST 載體為目地載體以便獲得帶有 cDNA 庫的最終表現載體，其與 pENTR 有相同 kanamycin^R 基因因而不相容。一種兩個輪迴構築過程的替代方案是需要的。在第一輪構築中，Gateway 載體 pET-53-DEST (ampicillin^R) 將用為一種目的載體而產生帶 *att*B 位含白色念珠菌 cDNA 的表現載體 pEXPR (**Figure 15A**)，可於第二輪構築時與帶 *att*P 位捐獻載體 pDONR223 (Spectinomycin^R) 或 pDONR207 (gentamycin^R)利用 BP clonase 反應產生帶 *att*L 位的 pENTR (Spectinomycin^R or gentamycin^R) (**Figure 15B**)。此 pENTR 與 pCOLA-3-DEST 經 LR Clonase II 反應催化可產生最終含白色念珠菌 cDNA 的 pEXPR。雖兩個輪迴構築過

程是必須，高效率重組反應可確保最終表現載體的獨立 cDNA 數量能維持而用於 SCF 泛素 E3 連接酶或激酶測定。

叁、其它相關研究：與此 NHRI 計畫有關研究成果之發表文章及手稿

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。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，結果發表一研究文章[4](**MS1**)，題為「Affinity purification of *Candida albicans Ca*Cdc4-associated proteins reveals presence of novel proteins for morphogenesis」(以 NHRI 計劃 NHRI-EX98-9808SI 為致謝對象)，附於**本報告第拾壹項**中。為使泛素編碼序列引進白色念珠菌 *ADH1* 基因座同時利用四環素類誘導產生一個 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。雖然用於構築能誘導產生一個 6×HisFLAG 標記泛素品系所使用之營養缺陷品系 BWP17 之後確認無法誘導成假菌絲態，因而以 BWP17 為主的品系建構及形態誘導中止。然而，四環素類誘導(Tet-on)基因表現可用於基因功能研究 。 因此，一種 Tet-on 表現系統被建立而得一以被接受之研究文章 [5](**MS2**) 題為「Construction of *Candida albicans* Tet-on tagging vectors with a Ura-blaster cassette」(以 NHRI 計劃 NHRI-EX99-9808SI 為致謝對象) ， 附於**本報告第拾壹項**中。為研究白色念珠菌因失去 *CaCDC4* 而造成細胞聚集的現象，我們分析了白色念珠菌 *Ca*Cdc4 不同結構域的功能而有一篇手稿已稿成，題為「Dissecting *Candida albicans* Cdc4 protein reveals its essential domains involved in morphogenesis and cell flocculation」將投稿，如附於**本報告第拾壹項**中之 **MS3**。

（2）英文摘要

We aim to provide a genome-wide view on genes responsible for morphogenesis mediated by the mechanisms of post-tranlational modifications (PTMs), particularly ubiqitination and phosphorylation, in the opportunistic human fungal pathogen *Candida albicans*. We have been interested in studying the control of G1-phase of the cell cycle in *C. albicans*. *C. albicans CDC4* and *CDC7* along with its regulatory subunit-encoded gene *DBF4* have been our prime research targets as their *S. cerevisiae* counterparts are required for G1-to-S transition and DNA replication, respectively, and their functions are highly conserved throughput evolution. However, contrary to the *CDC4* and *CDC7* of *S. cerevisiae* being essential, *C. albicans* equivalents appear to be nonessential and suppresses yeast-to-filament transition. Since *C. albicans CDC4* (*CaCDC4*) encodes an F-box protein of the Skp-Cul1/Cdc53-F-box (SCF) ubiquitin E3 ligase complex involved in ubiquitinating specific target proteins for degradation, *C. albicans CDC7* (*CaCDC7*) encodes a protein kinase involved in the alteration of activity by phosphorylation of targets, and prior phosphorylation is common as pre-requisite for ubiquitination led us to consider that temporally and spatially controlled phospho-ubiquitin-proteosome dependent proteolysis of proteins may play a pivotal role in *C. albicans* morphological plasticity. Hence, we hypothesized that ubiquitin-dependent proteolysis by SCF and phosphorylation is central to our understanding the morphogenesis of *C. albicans* and initiated a five-year NHRI proposal to validate this hypothesis, despite of final approval being three years. However, some progresses towards the aims are made. The aims can be categorized into two according to the specific platform technologies into which this progress report will be summarized. Firstly, cell-type specific profiling of *C. albicans* ubiquitome and phosphoproteome by 2-D gel electrophoresis. Secondly, establishment of a prokaryote-based assay for targets of specific *C. albicans* SCF ubiquitin E3 ligases or for substrates of specific

kinases. As some of the progresses have been reported last year, we will focus only the progress of this year as follows.

I. Cell-type specific profiling of *C. albicans* ubiquitome and phosphoproteome by 2-D gel electrophoresis

Ia. Cell-type specific ubiquitome

We have been able to induce *C. albicans* cells of the wild-type strain SC5314 into three cell types of yeast, hyphae, and pseudohypae (**Table 1**) and establishment of 2-D gel electrophoresis and differential proteome profiling in the first year of study. To allow analyze the differential subproteome profile among different cell types of *C. albicans*, we have sought to establish the efficient methods to enrich the cell-type specific subproteomes. To avoid ubiquitinated proteins that we identity undergoing proteolysis via proteasome, we have established a chemical methods to allow the commonly used proteasome inhibitor MG132 being permeable to the *C. albicans* cells, devoid of relying on the construction of a specific mutant as that used in *S. cerevisiae*. Indeed, *C. albicans* cells treated with MG132 prior to protein extraction could obtain more ubiquitinated proteins as compared with those without treatment (**Figure 1**). However, we have been unable to sufficiently obtain the ubiqutome by several commercial available ubiquitinated proteins enrichment kits (**Table 2, #1 & #2**), although we have not yet tested the acclaimed most efficient enrichment method (**Table 2, #4**). As a result, we have decided to analyze the *C. albicans* ubiquitome by 2-D gel alongside western blot with the lysine 48-linkage specific polyubiquitin antibody, the 2-D western blot (2D WB)(**Table 2, #4**). We have analyzed the differential ubiquitome between yeast and hyphal cells. Indeed, although cell-type specific ubiquitinated proteins could be identified (**Figure 2**), not all the proteins were visible by the instant blue staining. However, some of the major proteins are revealed constantly even with naked eyes. The identity of the proteins will be sought to determine by mass

spectrometry.

Ib. Cell-type specific phosphoproteome

To permit dissecting the differential phosphoproteome profile among different cell types of *C. albicans*, we have adopted the Pierce Phosphoprotein Enrichment Kit to specifically analyze cell-type specific phosphoproteome. Since the phosphoproteome enrichment required no treatment of proteasome inhibitor MG132, the optimal conditions for induction of specific cell types are listed in **Table 3** and the optimal enrichment conditions and steps are shown in **Table 4, #1**. We have first analyzed the differential phosphoproteome between yeast and pseudohyphal cells by using phosphoproteome enrichment prior to 2-D gel electrophoresis. The amount of proteins was monitored after each step of enrichment, concentration, and 2D clean-up (**Figure 3A**) and the protein samples were subjected to the 2-D gel electrophoresis (**Figure 3B & 3C**). Indeed, the difference in phosphoproteome of the two cell types could be consistently revealed with naked eyes (**Figure 3B & 3C**). However, we were not entirely sure if the differences were due to the same proteins having diverse phosphorylated states or different proteins, but all of which were separable according to differences in their isoelectric point (pI) value. We noted that the pI values of *C. albicans* phosphoproteome fall into the pH range between 5 and 8. To enable proteins separated more efficiently, we would seek to separate the proteins in the second dimension with the pH range between 5 and 8. In addition, the use of phosphatase (**Table 4, #3**) to remove the phosphate from the phosphorylated proteins might be able to reveal the differential phosphorylated proteins among different cell types when the multiple phosphorylations are occurred on a single protein. Moreover, an alternative method to directly detect phosphoproteome by staining 2-D gel with Pro Q Diamond among different cell types was sought (**Table 4, #2**), although an equipment that allows obtaining the image of Pro Q Diamond staining needs to be sought out. Basically, the latter

method with lesser steps but better specificity might be the best choice.

II. Reconstitution of *C. albicans* SCF ubiquitin E3 ligase and kinase in *E. coli* to screen specific substrates

IIa. Reconstitution of *C. albicans* SCF^{Cdc4} ubiquitin E3 ligase and DDK kinase

Since the prokaryote-based Skp1-cul1/Cdc53-F-box (SCF) ubiquitin E3 ligase assay requires expressing a total of at least eight proteins including members of SCF complex and related proteins (**Figure 4**) [1] in *E. coli*, the vector systems that allow accommodating genes for those components are critical. We have adopted the pQLinkX vector system [2] to allow cloning each of multiple genes into the vector, followed by a step-wise joining of those genes into a single pQLinkX vector in combination with two other vectors pCDFDuet1 and pCOLA-3-DEST[3] (**Figure 5**). 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 (**See the 98NHRI mid-term report**) and at least eight proteins were used in this system. We have focused our work on validation of the expression of proteins of SCF ubiquitin E3 ligase involved in vectors pQLinkX and pCDFDuet1 (**Figure 6**). The vector pCDFDuet1 allows accommodating two genes, *UBA1* encoding E1 and *CDC34* encoding E2, each of which can be expressed as a tagged protein (**Figure 7**). The vector pQLinkX can generate non-tagged, 7×His-tagged, GST-fusion, and HA-tagged (additional version made by us) proteins in *E. coli* (**Figure 8**). Because of the ability to consecutively join two pQLinkX together by using one as vector and the other as insert, multiple genes were constructed into a single pQLinkX eventually (**Figure 9**). The expression of a single GST-fusion protein and co-expression of GST-fusion proteins from two up to four were verified (**Figure 10**). The expression of a single 7×His-tagged protein and co-expression of two

7×His-tagged proteins were verified (**Figure 11**), despite that fact that Cdc53 was unable to produced. The ubiquitin was used to test the expression of non-tagged and HA-tagged version of protein from the pQLinkX vector (**Figure 12**). Since the Cdc53 was unable to produce as a 7×His-tagged protein presumably due to toxic in *E. coli*, we will construct a pQLinkX vector capable of expressing 7×His-tagged Skp1, Cdc4, Hrt1, and non-tagged ubiquitin as well as GST-fusion of Cdc53 in *E. coli*. Once the expression of the above mentioned proteins is validated in *E. coli*, the expression of E1 and E2 from the vector pCDFDuet1 will be incorporated such that the components required for constituting a functional SCF ubiquitin E3 ligase can all be expressed in *E. coli*. To verify the functionality of SCF ubiquitin E3 ligase, we will use *C. albicans* Sol1 as a substrate expressed from pCOLA-3-DEST vector, on which the *C. albicans* cDNA library is based, for the ubiquitination of SCF^{CaCdc4} ubiquitin E3 ligase. As the Sol1 has not formally been proved to be the substrate of SCF^{CaCdc4} ubiquitin E3 ligase, we have sought to reconstitute SCF^{CaGrr1} ubiquitin E3 ligase in combination with the know Grr1 substrate Ccn1, Hof1, or Cln3 to validate functionality of the system (**Figure 13**).

To test if the *C. albicans* Dbf4 dependent kinase (DDK) have the kinase activity in *E. coli*, we have sought to clone *CDC7* and *DBF4* into the vector pCDFDuet-1 and to verify if the DDK can be formed as a functional kinase to phosphorylate the potential substrates in *E. coli* as opposed to our original planned *in vitro* kinase assay using purified Cdc7 and Dbf4 from *C. albicans* cells. The expression of a single component was verified (**Figure 14**).

IIb. The Gateway cloning system is used to efficiently shuffle *C. albicans* cDNA library to the final expression vector

To enable both a prokaryote-based assay for substrates of specific SCF ubiquitin E3 ligases and substrates for specific kinases successful, a good quality

of *C. albicans* cDNA library was made by the use of the CloneMiner cDNA construction kit (Invitrogen), the MasterPure™ yeast RNA purification kit (EPICENTRE Biotechnologies), and the FastTrack MAG micro mRNA isolation kit (Invitrogen) together with reverse transcribing purified *C. albicans* mRNAs into cDNAs and ligating with *att*B adapter. A donor vector (pDONR) of the Gateway system, pDONR222 with *att*P sites, and a substrate (a cDNA library) with *att*B sites was subjected to the BP Clonase such that an *in vitro* recombination reaction occurs to generate an entry vector pENTR based cDNA library with *att*L sites flanking the cDNA and a by-product with *att*R sites (**Figure 15A**). The entry *C. albicans* cDNA library (pENTR) contains 2.6×10^7 independent clones, 87% of which contain cDNA inserts (**See the 98NHRI mid-term report**) that is sufficiently good. Hence, the entry vector pENTR containing cDNA library 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 library (pEXPR) with *att*B sites. However, the pCOLA-3-DEST, initially designed to be a destination vector to obtain the final expression vector (pEXPR) with *C. albicans* cDNA library, and the pENTR have the same kanamycin^R gene, which are incompatible. Instead, a two-round construction process is needed. The Gateway pET-53-DEST (ampicillin^R) will be used as a destination vector in the first round of construction to generate pEXPR carrying *C. albicans* cDNA with *att*B sites (**Figure 15A**), which can be used in the second round construction with pDONR223 (Spectinomycin^R) or pDONR207 (gentamycin^R) with *att*P sites to generate pENTR (Spectinomycin^R or gentamycin^R) with *att*L sites by the reaction of BP clonase (**Figure 15B**). The pENTR and pCOLA-3-DEST catalyzed by LR Clonase II reaction can generate the final pEXPR with *C. albicans* cDNA. Although the two-round process is required, the highly efficient recombination reaction would ensure maintenance of pool of cDNA clones of the library in the final expression vector for the use

in either SCF ubiquitin E3 ligase or kinase assay.

III. Other related studies: published papers and a manuscript 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. As an alternative of prokaryote-based assay for targets of SCF^{CaCdc4} ubiquitin E3 ligase, the study resulted in a paper [4](**MS1**) entitled "Affinity purification of *Candida albicans Ca*Cdc4-associated proteins reveals presence of novel proteins for morphogenesis" (NHRI grant NHRI-EX98-9808SI being acknowledged), which is attached in the **in the item 11**. To allow ubiquitin encoding sequence introducing into *C. albicans ADH1* locus and is expressed as a 6×His-tagged ubiquitin in a doxycycline induced manner in which the induced 6×His-tagged ubiquitin can compete with the endogenous non-tagged ubiquitin due to being overexpressed, a Tet-on vector has been developed. Although the auxotrophic strain BWP17 used to construct strain capable of inducibly expressing 6×His-tagged ubiquitin was later proved to be unable to induce pseudohyphal form, therefore the approach based on BWP17 was discontinued. However, the Tet-on inducible gene expression can be used in functional study of gene; hence, a Tet-on system was established and resulted in a paper [5](**MS2**) entitled "Construction of *Candida albicans* Tet-on tagging vectors with a Ura-blaster cassette" (NHRI grant NHRI-EX99-9808SI being acknowledged), which is attached in the **in the item 11**. To study the observed flocculation of cells in the absence of *CaCDC4*, we have analyze the domain function of *Ca*Cdc4 in which a manuscript entitled "Dissecting *Candida albicans* Cdc4 protein reveals its essential domains involved in morphogenesis and cell flocculation", was drafted to submit for publication as attached (**MS3**) in **the item 11 of this report**.

貳、99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	10. Tseng, T-L., Lai, W-C, Jian, T., Li, C., Sun, H-F. S., Way, T-D., and Shieh, J-C. Affinity purification of Candida albicans CaCdc4-associated proteins reveals the presence of novel proteins involved in morphogenesis. Biochem Biophs Res Commun 2010;351:152-157. Supported by NHRI-EX98-9808SI (SCI) Published	Foreign	2.548	NHRI
2	Lai, W-C., Tseng, T-L., Ting, J., Lee, T-L., Cheng, C-W., and Shieh, J-C. Construction of Candida albicans Tet-on tagging vectors with a Ura-blaster cassette. Yeast 2010;Supported by NHRI-EX99-9808SI (SCI) Accepted	Foreign	1.805	NHRI
3	Chin, C., Lai, W-C., Tseng, T-L., Cheng, C-W., and Shieh, J-C. Dissecting Candida albicans Cdc4 protein reveals its essential domains involved in morphogenesis and cell flocculation. Yeast 2010; (SCI) Submitted	Foreign	1.085	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、99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99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、99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

（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

以蛋白體學篩選重要有關白色念珠菌形態生成調節基因能進而瞭解致病力

（二）計畫研究成果技術移轉情形

☐即將進行洽談或洽談中

☐已技轉

☑無

請概述 (1)技術或產品名稱(2)內容說明(3)產業別(4)應用範圍(5)技術移轉可行性及預期效益

註：1. 如研究成果尚未申請專利，請勿揭露可申請專利之主要內容

2. 如計畫研究成果沒有技術移轉，請寫「無」

1. 以原核生物之大腸桿菌重建真核生物Skp1-Cul1/Cdc53-F-box (SCF)泛素連接酵素，可用於篩選泛素化蛋白質而排除被蛋白酵素體降解。The use of prokaryotic E. coli to reconstitute the Skp1-Cul1/Cdc53-F-box (SCF) ubiquitin E3 ligase for screening substrates devoid of ubiquitinated protein substrates being degraded by propteasome

2. 構築可多基因選殖入單一質體pQLinkX之系統以便共同表現數個基因於i腸桿菌。Construction of a vector system pQLinkX for accommodating multiple genes to be co-expressed in E. coli.

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。

本計畫為發展技術平台以確認後轉譯修飾中之泛素化及磷酸化在白色念珠菌形態生成扮演關鍵角色。共發展兩個技術平台，其一為鑑定差異性泛素化或磷酸化蛋白體圖譜存在不同形態之白色念珠菌細胞，從而尋求形態專一蛋白質以解析其角色。另一為於原核生物之大腸桿菌系統鑑定激酶受質或測定真核生物特有之 SCF 泛素 E3 連接酶受質，可避免於真核系統中標靶蛋白質因 SCF 泛素 E3 連接酶泛素化後被蛋白酶體降解而難以鑑定。

釀酒酵母菌能充分利用遺傳方法分析的特性在白色念珠菌因其二倍體且無完全有性生殖而難實施，故任何非遺傳法且具基因組層次或如本計劃第一項以次蛋白質體分析形態生成相關蛋白質功能皆具重要意義。固然建構蛋白體圖譜無技術新穎性。但本計劃之標的為調控形態生成之蛋白質，因形態生成能力與白色念珠菌致病力有關，任何與調控形態生成之蛋白質及相關基因皆有潛力成為新穎白色念珠菌藥物標靶以治療白色念珠菌感染。第二部分之以原核生物之大腸桿菌測定真核生物激酶或特有之 SCF 泛素 E3 連接酶之受質由其以後者具技術新穎性。除需引進原核生物罕用之 tRNA 基因但真合生物常用，且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(已於前一年報告討論)，加以牽涉成份極多，所需載體數目亦屬極限，要考慮篩選記號基因及複製子的相容性，也要考慮眾多蛋白質同時表現有無差異性的現象，具高度挑戰性。

目前，第一項技術平台設法豐富化泛素化或磷酸化蛋白體或替代性建構白色念珠菌形態專一次蛋白體圖譜並致完善化階段，而第二項技術平台則是已確認SCF泛素E3連接酶相關成份能以單一基因或多基因共表現於大腸桿菌中；而 cDNA 之構築則已訂出策略解決相容性之問題。

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，但它能發展抗藥性且藥物常具副作用，顯示對其更深入了解是必要的。由於它的致病力與其形態生成能力相關，我們致力瞭解其如何控制形態生成並以兩項方法尋找相關蛋白質及其對應基因。第一種是廣泛尋求在白色念珠菌之蛋白質以分子修飾改變特性及功能而導致酵母菌形、真菌絲形及假菌絲形態的生成。另外一種則是用已知特定形態生成的蛋白質在新建立測定系統中鑑定更多被其調節的蛋白質，而此類蛋白質也一定能控制形態生成。因此，透過尋找更多牽涉形態生成的基因及其蛋白質產物可以更瞭解其如何控制形態生成及致病力，從而尋求更佳治療方法。

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

一為透過二維電泳及西方墨點法建立細胞形態專一白色念珠菌泛素化蛋白體圖譜；其二為利用豐富化及二維電泳建立細胞形態專一白色念珠菌磷酸化蛋白體圖譜；其三為以可相容之不同載體承接多個能於大腸桿菌共表現並構成有活性 SCF 泛素 E3 連接酶的系統以篩選特定 SCF 泛素 E3 連接酶受質，發展大腸桿菌共表現 DDK 激酶的兩個次單元共表現以篩選 DDK 受質；其四為以 Gateway 選殖策略利用 clonase 對特定重組位進行高效能重組而將白色念珠菌 cDNA 庫有效穿梭選殖入最終表現載體可作為 SCF 泛素 E3 連接酶或 DDK 激酶受質篩選來源。

本研究所使用之載體 pQLink 系列因此可同時容納多個基因對需共

表現多個基因於大腸桿菌是必要的，我們同時也將取得之 pQLink 改造使之具多種複製子、篩選記號基因、抗原決定部位標記，如此單一系列 pQLink 載體可相容且足以表現多基因於大腸桿菌，擴大其利用價值於極至具技轉潛力；利用 Gateway 方法構築白色念珠菌 cDNA 庫中之入口 cDNA 庫其優勢為經輸入載體後可選擇不同目的載體而得執行不同用途的終點載體，例如除本研究用於大腸桿菌表現外亦可構築酵母菌雙雜核 cDNA 庫，此技術乃 Gateway 系統其專利權由 Invitrogen 公司掌握；由三及四項完成後可以原核生物篩選真核生物特有之 SCF 泛素 E3 連接酶或特有激酶受質，建立後具廣泛應用性有技轉潛力。

四、 成效評估（技術面、經濟面、社會面、整合綜效）

就技術面而言，白色念珠菌泛素化或磷酸化次蛋白體圖譜建構及分析的能力是為了奠定基礎於往後可研究其他轉譯修飾次蛋白體圖譜，例如組蛋白質甲基化，可往表觀遺傳方向研究；而以原核生物測定真核生物特有之 SCF 泛素 E3 連接酶則是建立無蛋白酶體的蛋白質泛素化反應於大腸桿菌有多項問題需解決，完備後除本身為一廣汎有價值之篩選系統亦可衍生出如其他真核生物複合體酵素可否為避免真核生物可能的干擾而引入原核生物測定的思維。就經濟面而言，一個技術平台待解決多重問題或面向，例如衍生出 pQLink 載體系列的建構，再如 Gateway 系統可於非本計劃之大腸桿菌表現系統實極具成本效益；再者，本計劃技術層面非涉高成本硬體儀器之運作及維護，亟需高度思考力，故其實施具高度經濟上的成本效益。就社會面而言，透過本計劃之實施，相關人員訓練後，除不可取代的專業性亦有多面向發展可能性，極具未來就業競爭力；若運作得宜則計劃得延續以吸引更多人員參與訓練成就更多具不可取代具專業性的研究人員，可供其他研究團隊運用，意味更多的就業人員是社會穩定之源。

本計劃整合綜效而言，技術上具專業性及延展性，經濟上有商品化潛能，社會上提供良好之就業條件而實現社會正義，若有更長時間的計畫支持，除計劃能完成，前述成效更能展現。

五、 下年度工作構想及重點之妥適性

下年度目標首先將以二維電泳及西方墨點法配合泛素化蛋白質專一抗體構建白色念珠菌細胞形態專一泛素化蛋白質體圖譜及部份蛋白質身份鑑定；完成豐富化磷酸化蛋白質或磷酸化專一之蛋白質染色及二維電泳構建白色念珠菌細胞形態專一磷酸化蛋白質體圖譜及部份蛋白質身份鑑定。此兩項分別就泛素化及磷酸化蛋白體取得及鑑別性之差異而進行。

另外，預計完成 SCF 泛素 E3 連接酶及 DDK 激酶於大腸桿菌表現並以其受質進行活性確認；白色念珠菌 cDNA 庫將以 Gateway 系統完成選殖至最終表現載體可於大腸桿菌表現蛋白質；於大腸桿菌表現白色念珠菌 cDNA 庫並分別搭配表現 SCF 泛素 E3 連接酶或 DDK 激酶以篩選鑑定白色念珠菌特定 SCF 泛素 E3 連接酶之特定標靶蛋白質及 DDK 激酶之受質。

最後，將引進第一年已開始嘗試運用之原核生物罕用之 tRNA 基因而真核生物常見及一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用之 CUG 密碼子於大腸桿菌表達，結合 SCF 泛素 E3 連接酶或 DDK 激酶檢視與未引進 tRNA 之差異性。

六、 檢討與展望

在執行本計劃過程中之第一年，因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已以不依賴特定白色念珠菌品系方向設計，然而本年度以能豐富化泛素化蛋白質之套組進行並未能有效達成，雖以二維電泳配合西方墨點法及泛素化

蛋白質專一抗體進行為替代方案仍值得檢討。然而，磷酸化蛋白體順利被豐富化，即便增加可辨識蛋白質數量需完善化，細胞形態專一之磷酸化蛋白體圖譜已可建立。我們也擬以磷酸化蛋白體專一染劑為替代方案來達成建構細胞形態專一之磷酸化蛋白體圖譜。已開始嘗試對主要差異性之泛素化及磷酸化蛋白質進行身分鑑定。

就執行 SCF 泛素 E3 連接酶於大腸桿菌篩選而言，雖運用可同時容納多個基因於同一載體 pQLink 解決多基因承載問題，仍面臨能否共表現該等蛋白質、表現效率是否足以支撐此測定、有無蛋白質對大腸桿菌毒性等問題，下階段驗證有無功能性 SCF 泛素 E3 連接酶於大腸桿菌形成具高度挑戰性；除此而外，功能性 SCF 泛素 E3 連接酶若需以磷酸化為前提或成員之一之 Cul1 需經 neddylation，將增加建構此系統之複雜度，雖已掌握相關資訊但兩者此系統完備之重要性未完全確定，也值得檢討。然而，以 Gateway 系統能完備選殖白色念珠菌 cDNA 的前提下，無論 SCF 泛素 E3 連接酶或 DDK 激酶仍能有以體外篩選受質方式為替代方案。

本計劃發展兩類技術平台，雖然非常具技術性，對欠缺正向遺傳手段操作的白色念珠菌研究而言，任何非遺傳手段之研究方法的發展別具意義。建構白色念珠菌細胞形態專一之泛素化或磷酸化蛋白體圖譜雖無技術新穎性，但對我們而言具一定門檻，若研究方向及人員能維持延用此技術對我們發展其他轉譯後修飾次蛋白體圖譜或其他蛋白體研究為一基石；以原核生物測定真核生物激酶或特有之 SCF 泛素 E3 連接酶，尤其是後者乃非依賴蛋白酶體之泛素化適合轉移任何真核生物特定 SCF 泛素 E3 連接酶系統與大腸桿菌篩選專一受質，具廣泛的應用性；誘導表現一個突變 $tRNA_{CGA\rightarrow CAG}$ 基因以適應白色念珠菌非通用 CUG 密碼子於大腸桿菌表現可對於大腸桿菌生產白色念珠菌重組蛋白質氨基酸組成可能異常提供最終解決方案。

伍、99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	
			已結訓	1	
	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	
			已結訓	0	
	2.	碩士班研究生	訓練中	1	
			已結訓	1	
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99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	0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		6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99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	3 人
3	學士	3 人
4	專科	0 人
5	博士班研究生	1 人
6	碩士班研究生	2 人
7	其他	0 人
合計		12 人

捌、參與99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
中山醫學大學 生物醫學科學學系	單細胞真核生物模式	謝家慶
中山醫學大學 生物醫學科學學系	蛋白質體	李娟
國立成功大學 分子醫學研究所	疾病基因圖譜	孫孝芳

玖、九十九年度計畫執行情形

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，九十九年預計達成之研究內容

The original aims of the project proposal shown below were designed for five years. The original focus on aims #1 was to recruit and train personnel, establish methodology, and purchase the essential equipment in the first year. Focus was also on essential part of *C. albicans* cDNA library construction for the use in specific aim #4 and #5. The rest of work was designed to perform in parallel in the next four years.

As the proposal being shortened to three years, we have re-scheduled the time-frame with respect to the aims. In the first year, we aimed to establish quantitative proteome profiling among different cell types. In parallel, a *C. albicans* strain expressing 6His-FLAG-ubiquitin in replace of ubiquitin in the original aim#1was set to construct but was discontinued due to non-inducible to peudohyphae of the strain. Also, *C. albicans* cDNA library construction was initiated for the use in the original aim#4 and #5. Moreover, recruiting and training personnel, establishing methodology, and purchasing essential equipment were to complete. **In the second year**, we aimed **to establish efficient methods for enrichment of subproteome** that can be used in aim#2 and #3 **for identifying cell-type specific ubiquitome and phosphoproteome**. **All the constructs** including the most appropriate ***C. albicans* cDNA library**, **the SCF ubiquitin E3 ligase**, and **the kinase** were set to be completed for used in the aim#4 and #5. In the final year, we 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、請詳述九十九年度計畫執行情形，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 accomplished most of the aims that we set out to do with some requiring further optimization for aptness.

Indeed, more obstacles occurred during the establishment of cell-type specific ubiquitome; hence, the 2-D gel and western with specific antibody to polyubiquitinated proteins was currently used as an alternative. However, the cell-type specific phosphoproteome was established by the originally designed protocol of enrichment followed by 2-D gel. Additionally, the use of phosphoprotein-specific staining dye, Pro Q Diamond, to reveal the cell-type specific phosphoproteome was initiated as an alternative. Currently, some major cell-type specific ubiquitinated and phosphorylated proteins were subjected to mass spectrometry for identity.

The co-expression of protein components in *E. coli* used in the prokaryote-based SCF ubiuitin E3 ligase and kinase for substrates was verified, although optimization and some more considerations might be required. Due to imcompatibility of the vector sets in the Gateway system to shuffle *C. albicans* cDNA library to the end expression vector in the original design, we have sought a two-round cloning strategy to tackle the problem. A mutant tRNA adapt for *C. albicans* non-universal CUG codon has been constructed for use in

generating recombinant *C. albicans* proteins in *E. coli*. Moreover, we have sought an alternative screening for kinase substrates in *E. coli* instead of the original *in vitro* kinase assay, designed to do the next year.

	Month	1	2	3	4	5	6	7	8	9	10	11	12	%*	Note
1	Optimization of Proteasome inhibition	×	×	×	×	×	×	×						100	Optimal
2	Enrichment of ubiquitome	×	×	×	×	×	×	×	×	×				80	Alternative methods are required
3	Enrichment of phosphoproteome						×	×	×	×	×	×		100	Further optimal will be performed
4	Cell-type specific ubiquitome by 2-D gel and western blotting with the lysine 48-linkage specific polyubiquitin antibody									×	×	×		50	further optimization is required
5	Cell-type specific phosphoproteome by 2-D gel after #2									×	×	×		90	Further optimization is required
6	Cell-type specific phosphoproteome by Pro Q Diamond staining									×	×	×		50	Not being listed as this year's work[§]
7	Co-expression of SCF ubiqutin E3 ligase in *E. coli*	×	×	×	×	×	×	×	×	×	×	×		80	Sufficient for this year
8	Co-expression of DDK kinase in *E. coli*			×	×	×	×	×	×	×	×	×		80	Sufficient for[§] this year
9	Gateway construction of *C. albicans* cDNA library									×	×	×		80	Sufficient for this year
10	Induced expression of a mutant $tRNA_{CGA \rightarrow CAG}$ gene						×	×	×	×	×			70	Sufficient for this year

* 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 10-1~10-4 and 10-5~10-19, respectively.

References are on page 10-20.

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

Forms	Yeast	Hyphae	Pseudohypahe
Conditions	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ -- YPD (pH 5.2) -- 2% glucose 30℃, 3hr ↓ -- YPD (pH 5.2) -- 2% glucose -- 0.006% SDS 30℃, 2hr ↓ 1. DMSO 2. 75 μM MG132 [1] 30℃, 4hr	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ -- YPD (pH 7.2) -- 2% glucose -- 10% FBS 37℃, 3hr ↓ -- YPD (pH 5.2) -- 2% glucose -- 10% FBS [2] -- 0.006% SDS 37℃, 2hr ↓ 1. DMSO 2. 75 μM MG132 37℃, 4hr	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ 1st induction -- 2.5 mM KH_2PO_4 (pH 6.5) -- 10.2 mM L-Proline -- 2.6 mM Nacetyl-D-glucosamine -- 3 mM $MgSO_4$. $7H_2O$. -- 2% glucose 37℃, 4hr ↓ 2nd induction -- 300 mM potassium phosphate buffer -- 10.2 mM L-Proline -- 2.6 mM Nacetyl-D-glucosamine -- 3 mM $MgSO_4$ · $7H_2O$. -- 2% glucose 37℃, 4hr ↓
			↓ -- 0.006% SDS 37℃, 2hr ↓ 1. DMSO 2. 75 μM MG132 37℃, 4hr

1 : Z-Leu-Leu-Leu-al is an inhibitor of proteasome activity.

2 : Hyclone Animal Sera, HyClone SH30071.03 Fetal Bovine Serum, Characterized

Table 2. Methods used for ubiquitome enrichment and detection

Forms	Yeast	Hyphae	Pseudohypahe
Methods	1. Ubiquitin Enrichment Kit [1] → Ubiquitin Enrichment Kit, contains sufficient materials for enriching up to 15 lysate samples containing ~0.15 mg total protein per sample → Polyubiquitin Affinity Resin, 300 μl, supplied as a 25% slurry Binding Capacity: ~1 μg per 20 μl of slurry → ✖ 2. S5a UIM, agarose conjugate (500 μl) [2] → Binding Capacity: ? → Elution: SDS and Urea → ! 3. 2D WB → Instant Blue [3] (Total proteins pattern) → 2D WB (Lysine 48-Linkage Specific Polyubiquitin Antibody) [4] → Repeat → ! → Analysis → ! → Artificial analysis		
	4. Agarose-TUBE [5] : Tandem UBAs →Up to 1000-fold higher than single UBA domains. 5. LC/MS/MS→! → pH 3-11 NL, 7cm strip		

1 : Pierce
2 : Enzo Life Sciences
3 : Expedeon
4 : Cell Signaling
5 : Lifesensors

Table 3. Induction of different cell types of *C. albicans* for phosphoproteome

Forms	Yeast	Hyphae	Pseudohypahe
Conditions	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ -- YPD (pH 5.2) -- 2% glucose 30℃, 3hr	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ -- YPD (pH 7.2) -- 2% glucose -- 10% FBS 37℃, 3hr	SC5314 -- YPD (pH 5.2) -- 2% glucose 30℃, O/N ↓ OD_{600}=0.8~1.0 ↓ 1st induction -- 2.5 mM KH_2PO_4 (pH 6.5) -- 10.2 mM L-Proline -- 2.6 mM Nacetyl-D-glucosamine -- 3 mM $MgSO_4$. $7H_2O$. -- 2% glucose 37℃, 4hr ↓ 2nd induction -- 300 mM potassium phosphate buffer -- 10.2 mM L-Proline -- 2.6 mM Nacetyl-D-glucosamine -- 3 mM $MgSO_4 \cdot 7H_2O$. -- 2% glucose 37℃, 4hr

Table 4. Methods used for ubiquitome enrichment and detection

Forms	Yeast	Hyphae	Pseudohypahe
Conditions	1.Pierce® Phosphoprotein Enrichment Kit [1] → Phosphoprotein enrichment (Up to 8 mg of total protein (adjust to 0.5 mg/ml) may be applied to each column for 30 minutes at 4°C.) →Washing →Elution (10ml) →Concentration (iCON column) [1] →2D clean up [2] →2D Quant Kit [2] →Instant blue staining [3] / Silver staining →LC/MS/MS→! → pH 3-11 NL, 7cm strip [2] 2. Pro Q Diamond phosphoprotein gel stain [4] →This proprietary fluorescent stain allows direct, in-gel detection of phosphate groups attached to tyrosine, serine, or threonine residues, without the need for antibodies or radioisotopes. The stain can be used with standard SDS-polyacrylamide gels or with 2-D gels.		
Conditions	3. Dephosphorylation of proteins in cell lysates → Pierce® Phosphoprotein Enrichment Kit [1] → Elution →Concentration →To the cell mixtures was added sequentially deionized water, manganese chloride, and PPase buffer [5]. → The mixture was divided into two aliquots , and λ Protein Phosphatase [5] was added to one of the aliquots. → 2D Clean up [2] →2D electrophoresis *Proteomics* 2002, 2, 1267–1276		

1 : Pierce
2 : GE healthcare
3 : Expedeon
4 : Molecular Probes
5 : NEB

Figures and legends

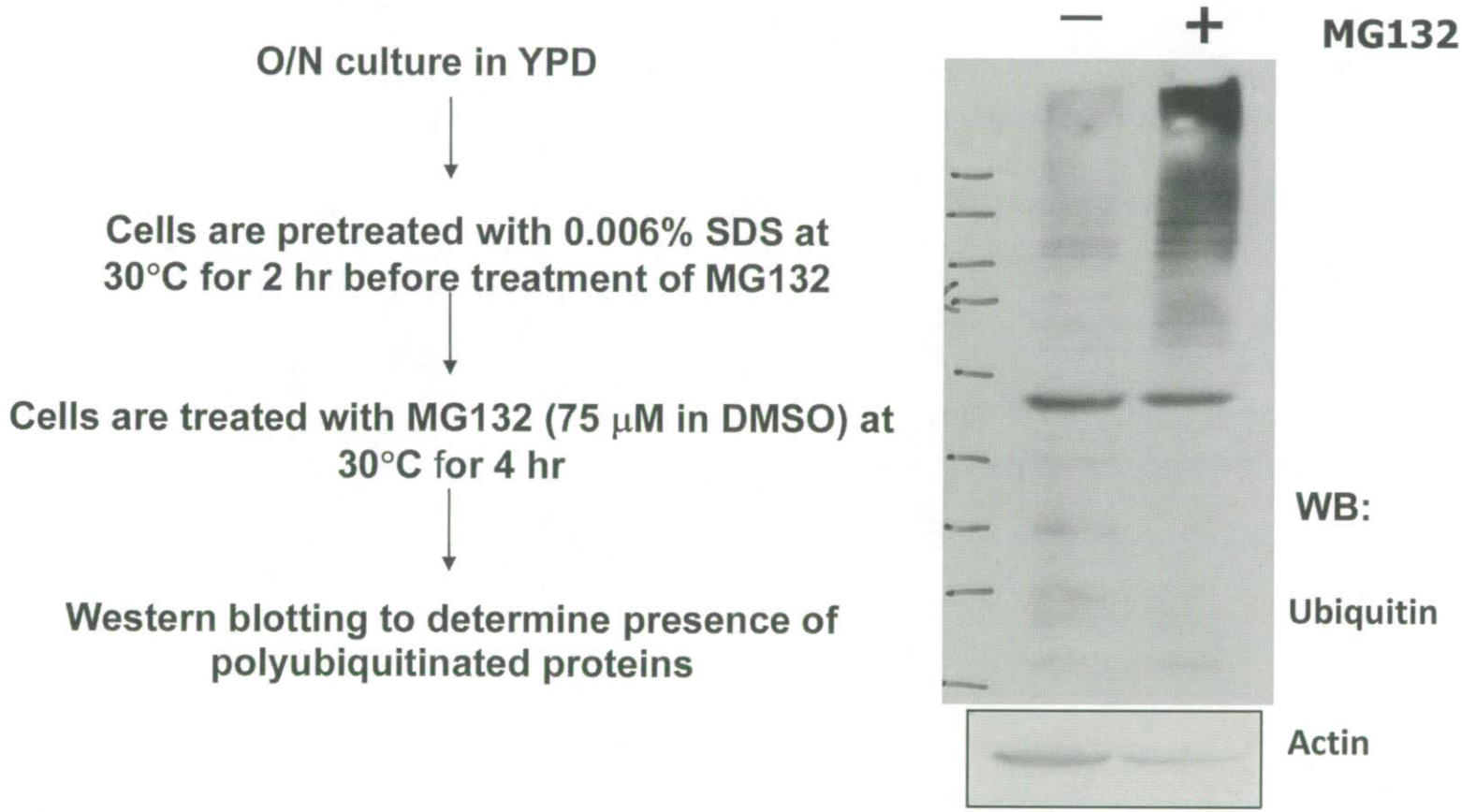

Figure 1. Proteasome inhibition is required to obtain polyubiquitinated proteins from *C. albicans* cells

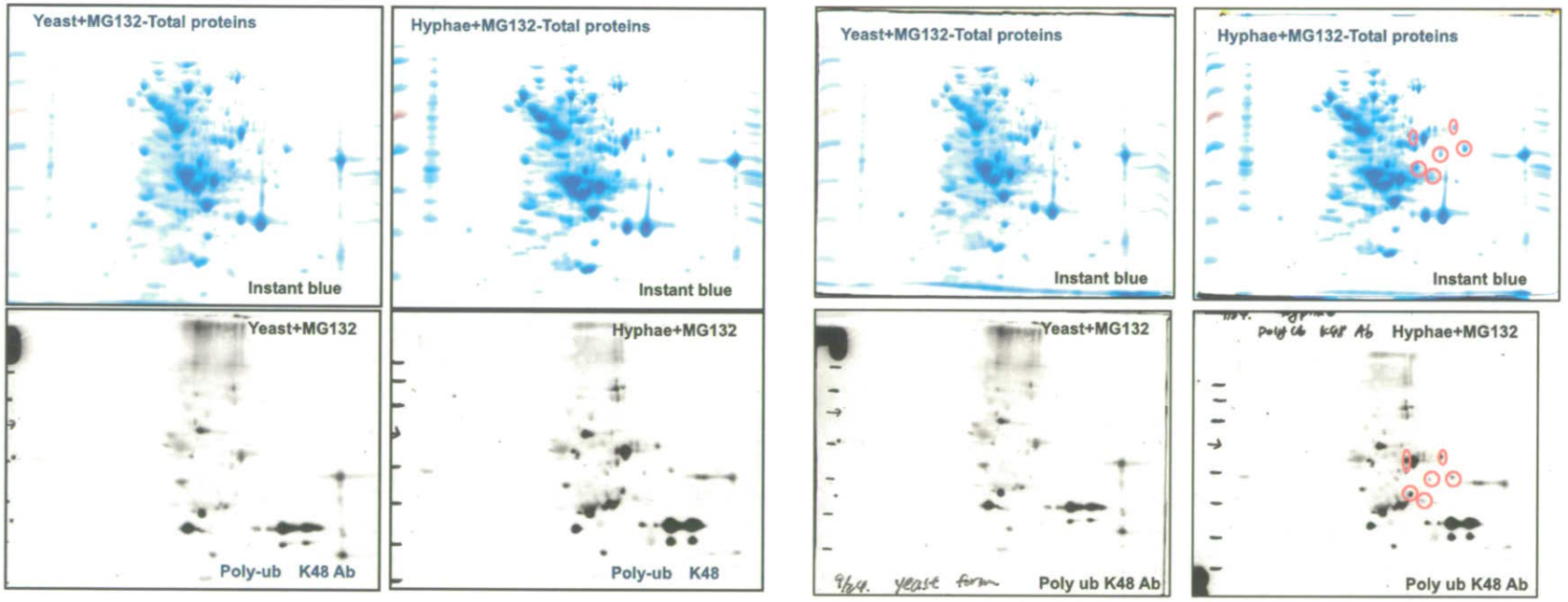

Figure 2. 2-D western blotting to detect the cell-type specific ubiquitome. Two independent 2-D gels of proteins from cells of yeast and hypase are shown. Gels stained with instant blue are on the upper panel, whereas those used for western blotting with the lysine 48-linkage specific polyubiquitin antibody (Poly-ub K48 Ab) are shown on the bottom panel. Representative hyphae-specific ubiquitinated proteins that are also visualized by instant blue staining are circled in red.

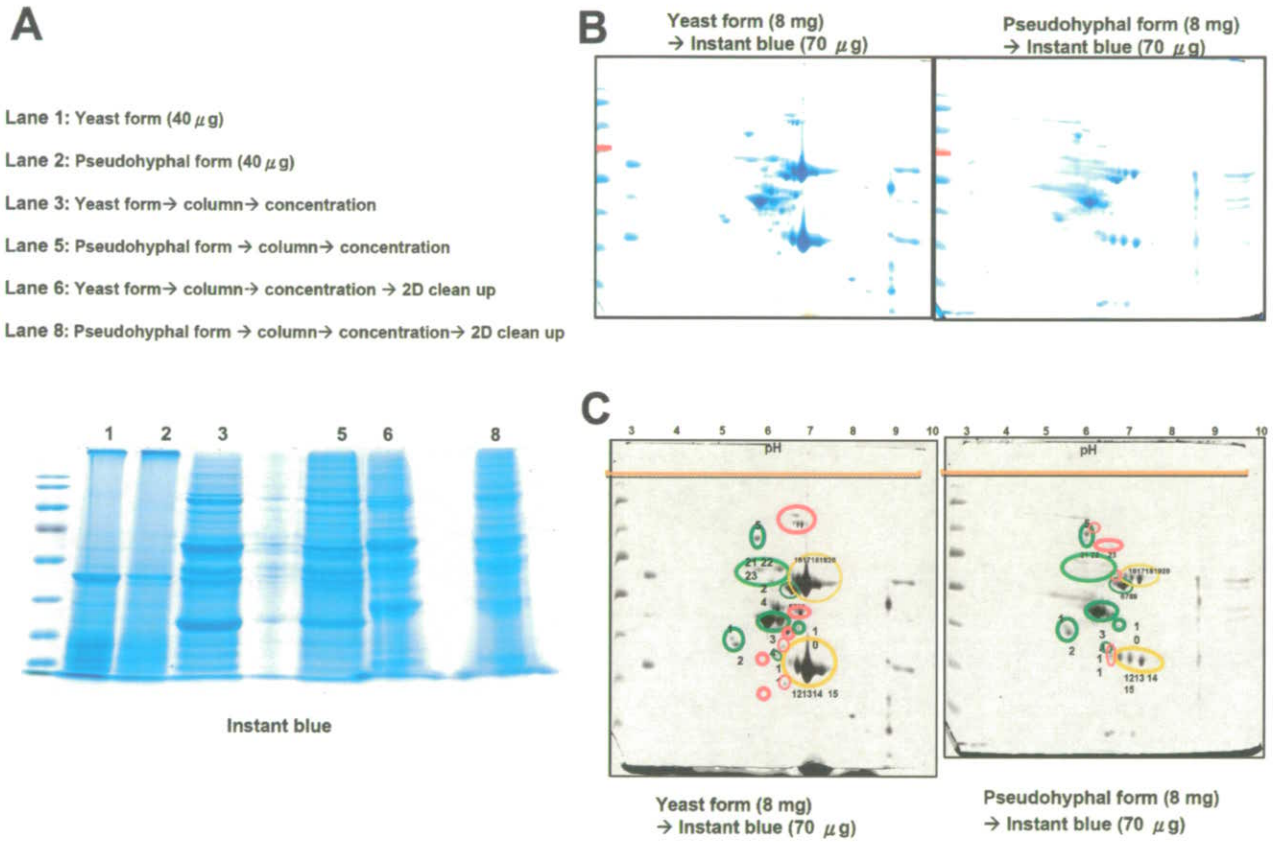

Figure 3. Cell-type specific phosphoproteome profiling. (**A**) Examination of protein contents and patterns of phosphoproteome enrichment, crude extracts (lane 1 & 2), after enrichment and concentration (lane 3 & 5), and 2D clean up prior to electrophoresis (lane 6 & 8). (**B**) and (**C**) are two independent 2-D gels with the phosphoproteome. The common phosphorylated proteins are circle in green. The difference in the status of phosphorylation on proteins are circled in orange. The cell-type specific phosphorylated proteins are circled in red.

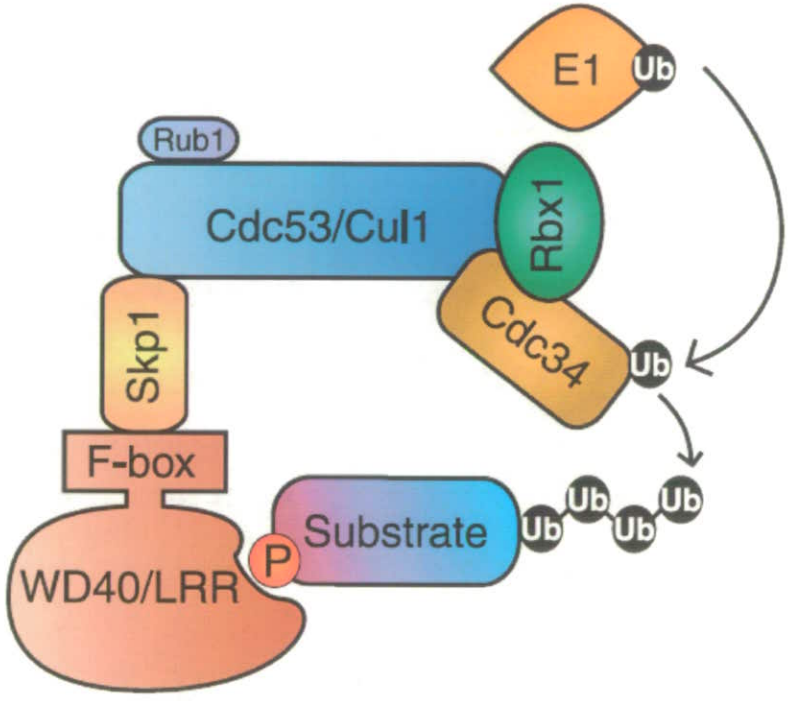

Figure 4. A cartoon exhibiting the essential components for the activity of SCF ubiquitin E3 ligase for ubiqutination of its substrate. The diagram is adopted from reference [1]. Note that the Rub1 regulates Cdc53/Cul1 through neddylation, thus is excluded as an essential component of SCF ubiquitin E3 ligase in our prokaryote-based SCF ubiquitin E3 ligase assay. Rux1 is also called Hrt1. The phsphophorylation of substrates by the cyclin-dependent Cdc28 kinase might be critical but will be included if verification of the system proves to be required.

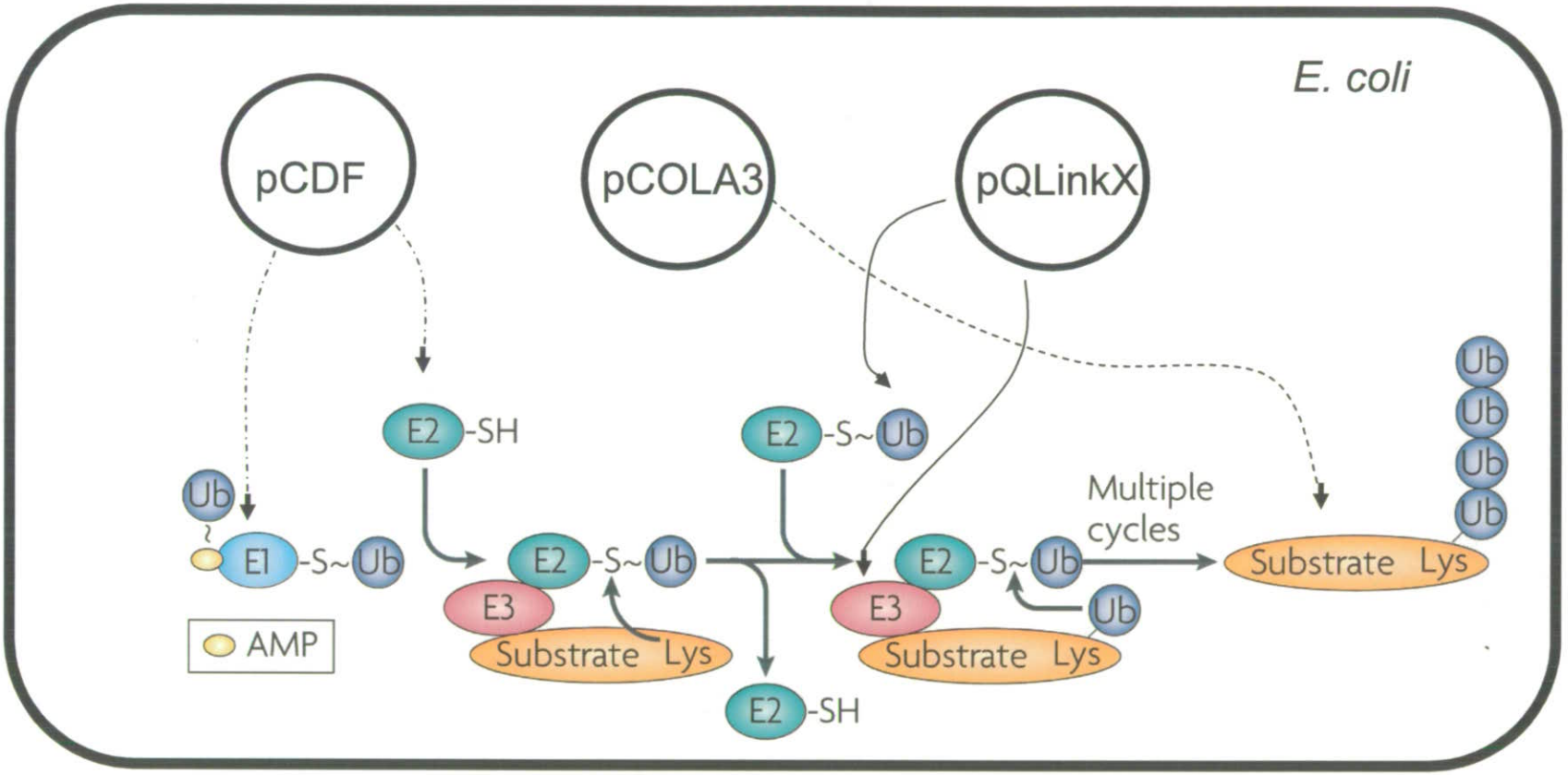

Figure 5. Vectors designed to accommodate genes simultaneously expressing components for a functional SCF ubiquitin E3 ligase in *E. coli*. The pCDF is an abbreviation of pCDFDuet1 and the pCOLA3 is an abbreviation of pCOLA-3-DEST.

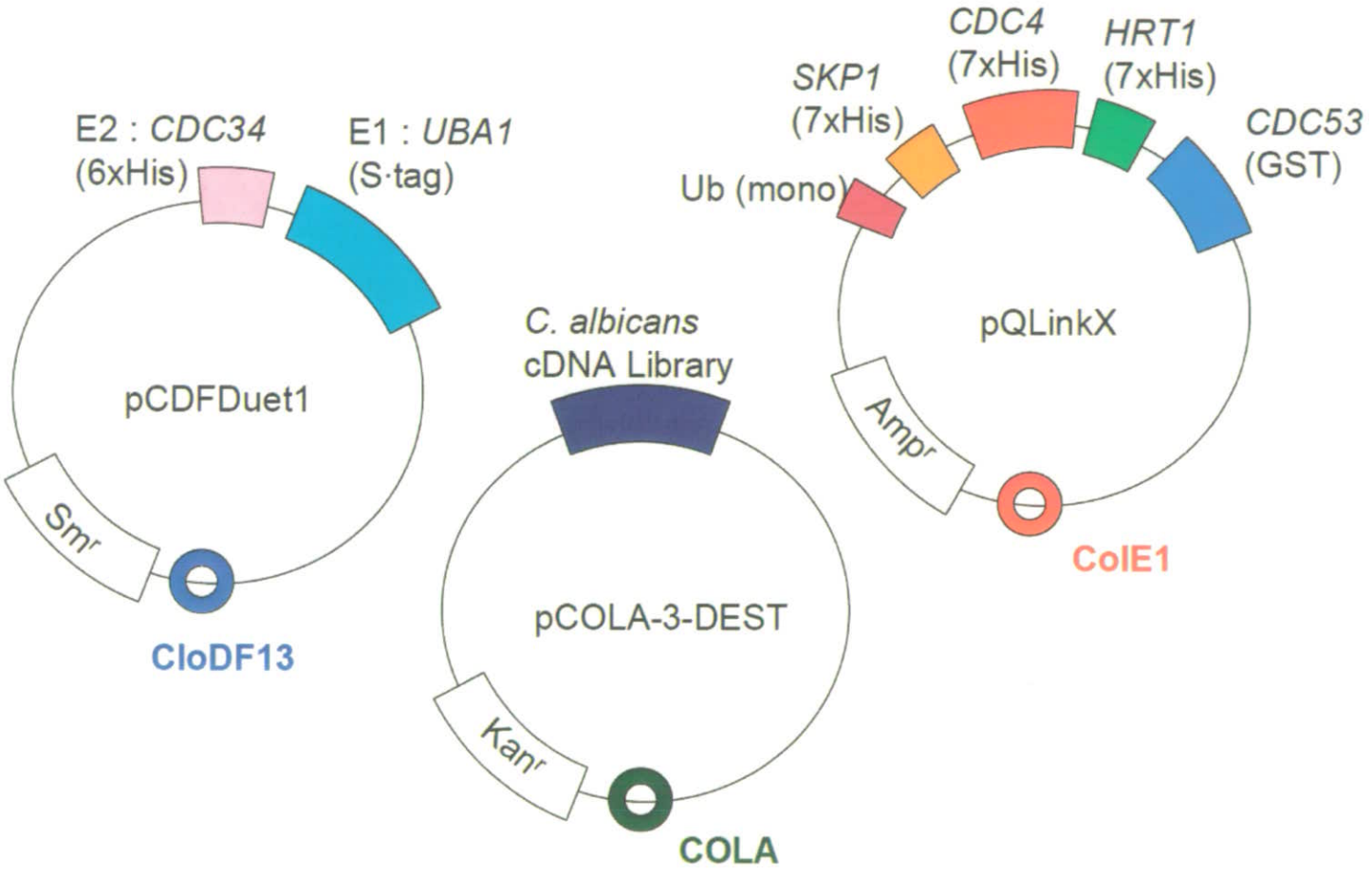

Figure 6. Maps of vectors illustrating their relevant features that are compatible to clone genes to be simultaneously expressed in *E. coli*.

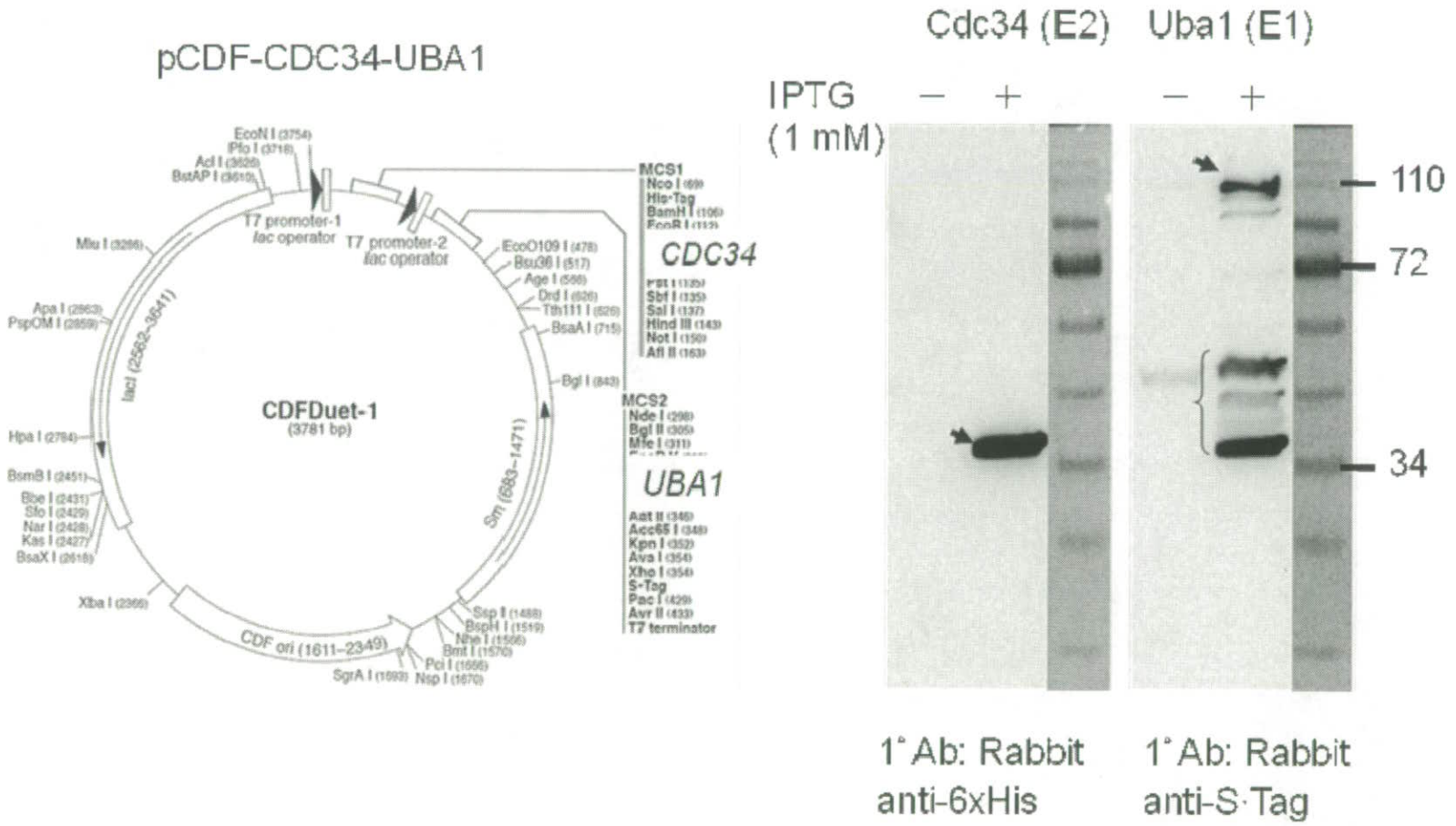

Figure 7. Western blotting to verify the co-expression of Uba1(E1) and Cdc34 (E2) tagged with S·Tag and 6×His, respectively from vector pCDFDuet1 in *E. coli* BL21(DE3). The positions to which the proteins are migrated are indicated by arrows. Breakdown products of Uba1 are indicated.

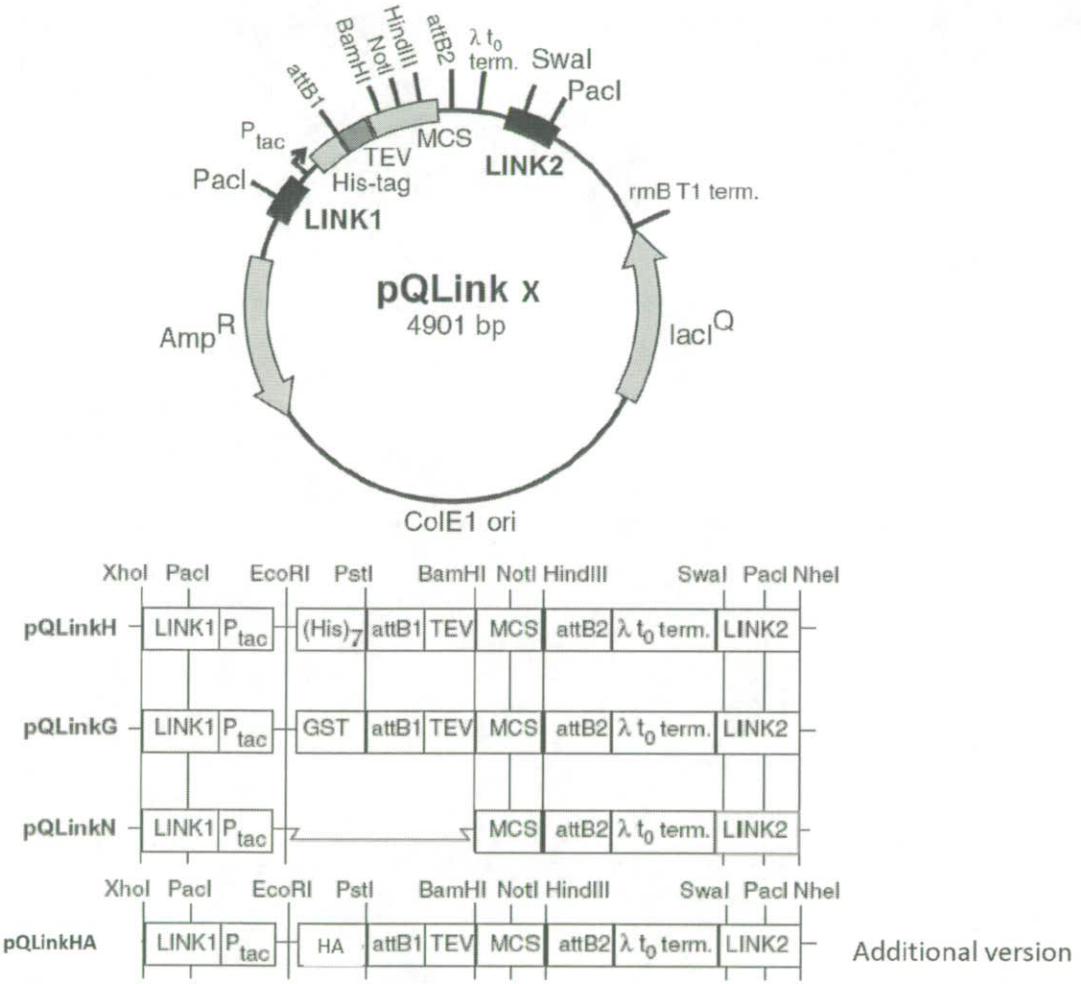

Figure 8. Map of vector pQLinkX with its basic features. The genes cloned into the vector allow IPTG inducibly expressing in *E. coli* BL21(DE3) the non-tagged protein, GST-fusion protein, 7×His-tagged protein, and HA-tagged proteins made in the current study.

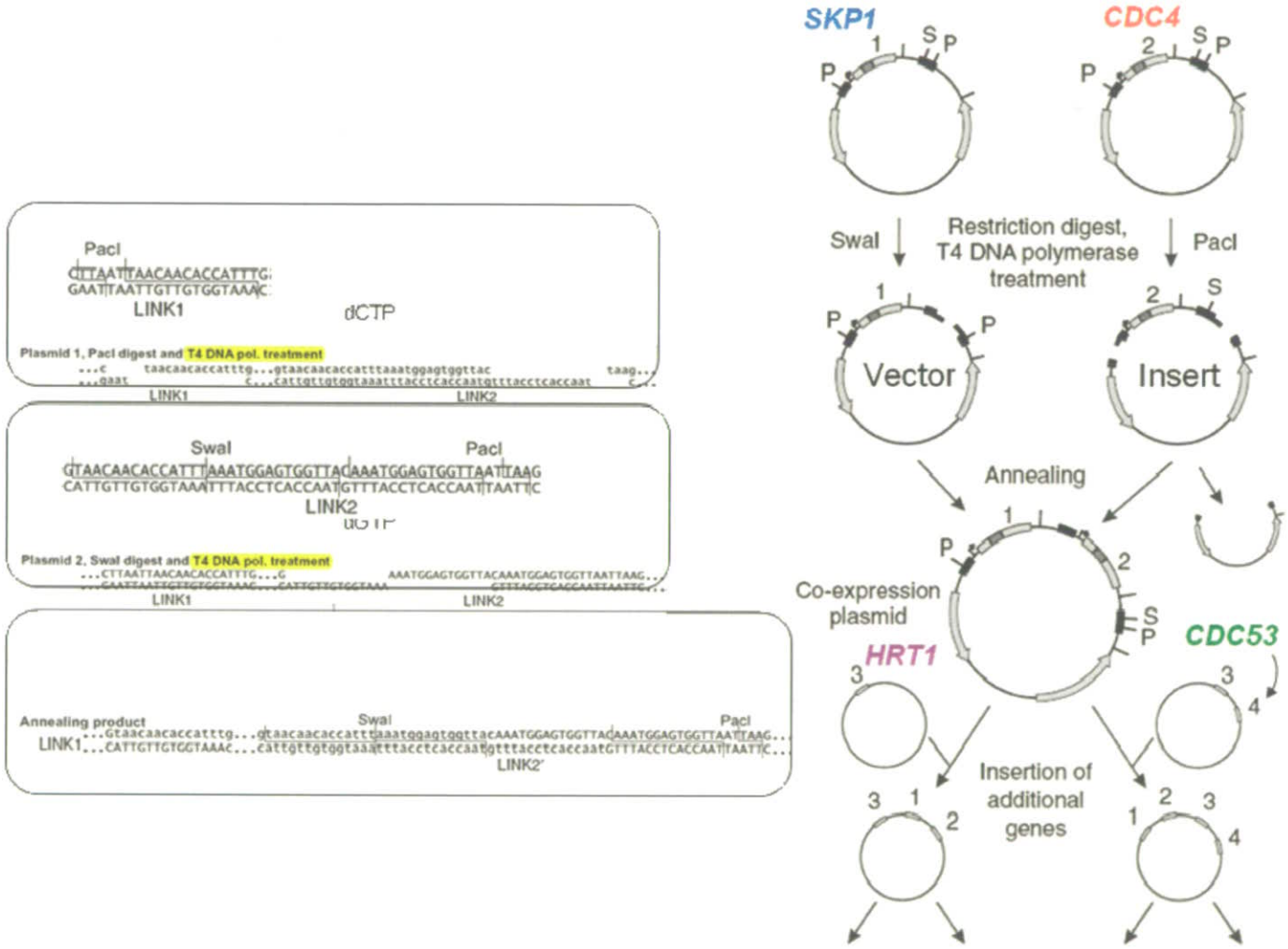

Figure 9. Ligation independent cloning to accommodate multiple genes into a single pQLinkX vector.

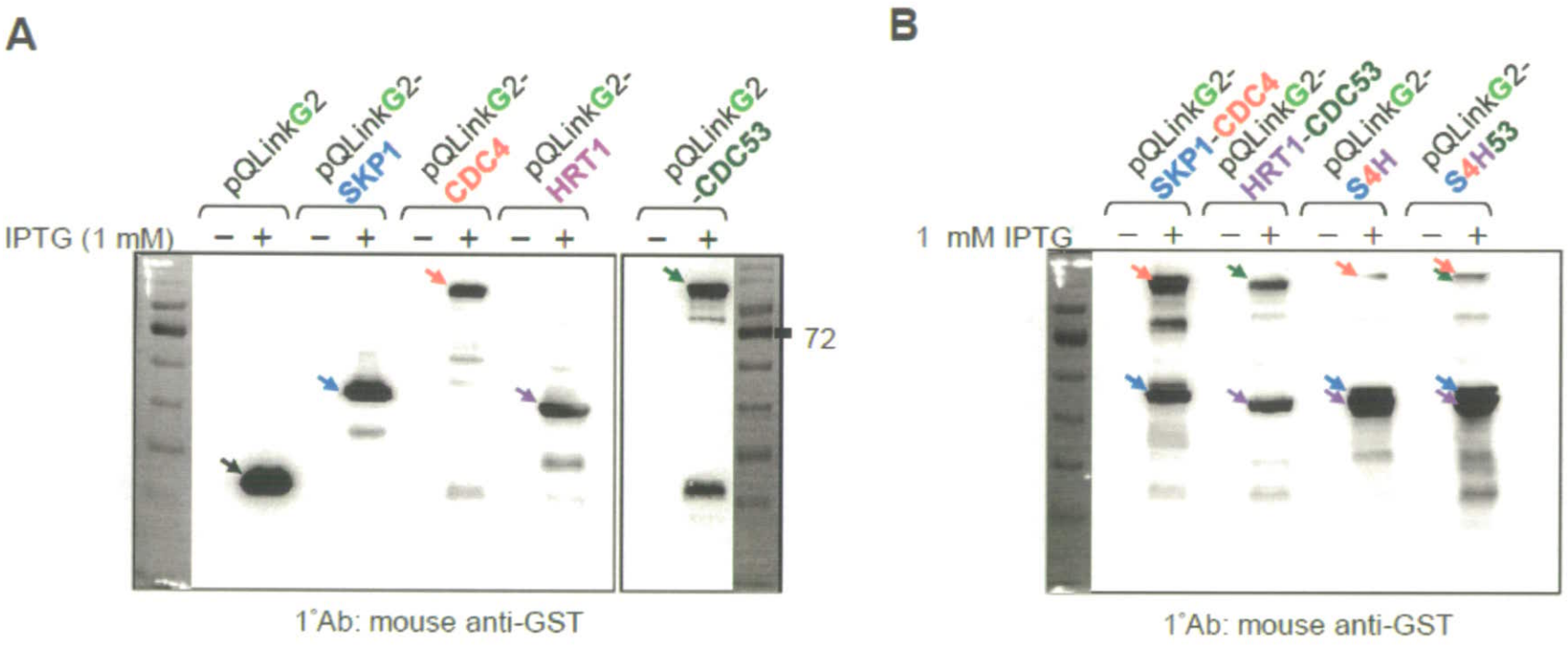

Figure 10. Western blotting to verify the co-expression of GST-fusion proteins from the pQLinkG2 in *E. coli*. (**A**) Expression of GST-fusion protein encoded by gene of *SKP1*, *CDC4*, *HRT1*, and *CDC53*. (**B**) Co-expression of GST-fusion proteins encoded by gene of *SKP1* (S), *CDC4* (4), *HRT1* (H), and *CDC53* (53).

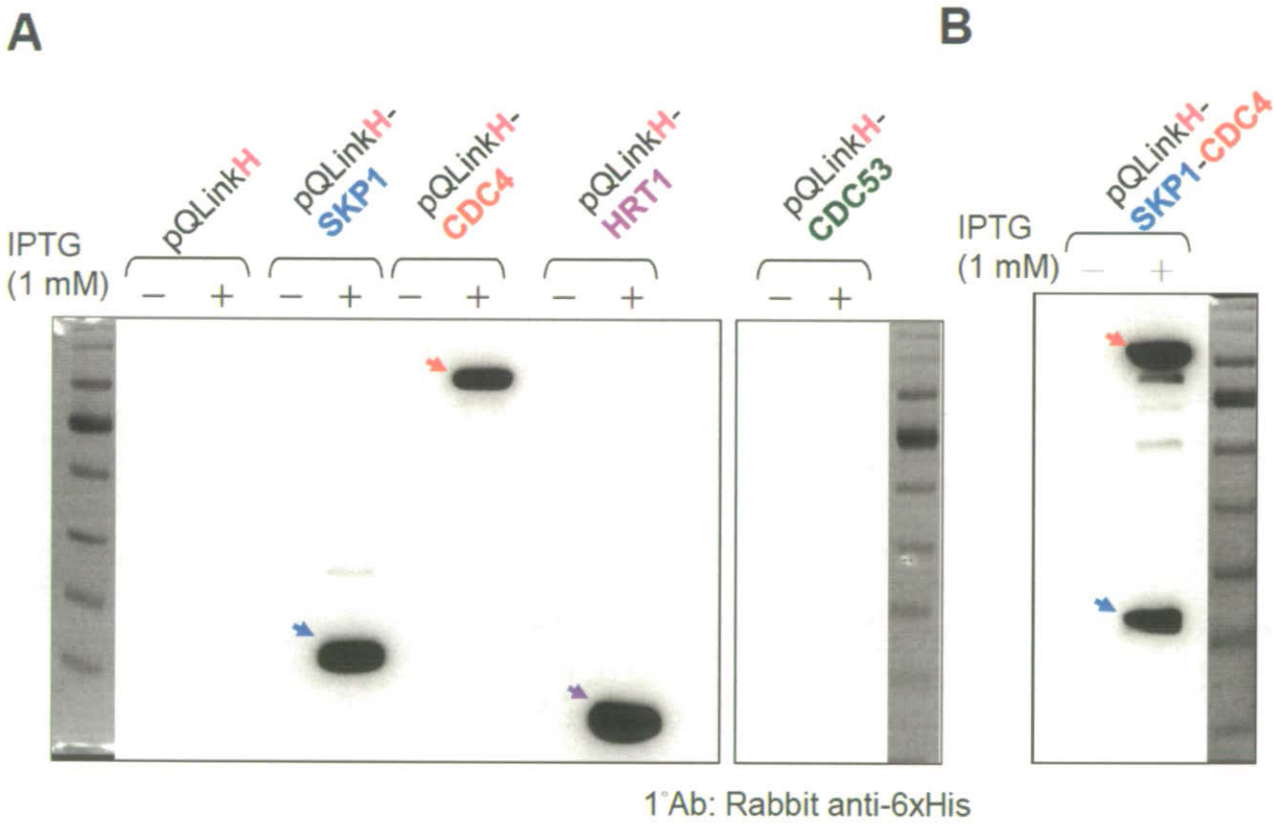

Figure 11. Western blotting to verify the co-expression of 7×His-tagged proteins from the pQLinkH in *E. coli*. (**A**) Expression 7×His-tagged protein encoded by each of gene of *SKP1*, *CDC4*, *HRT1*, and *CDC53*. (**B**) Co-expression of 7×His-tagged proteins encoded by gene of *SKP1* (S), *CDC4* (4), *HRT1* (H), and *CDC53* (53).

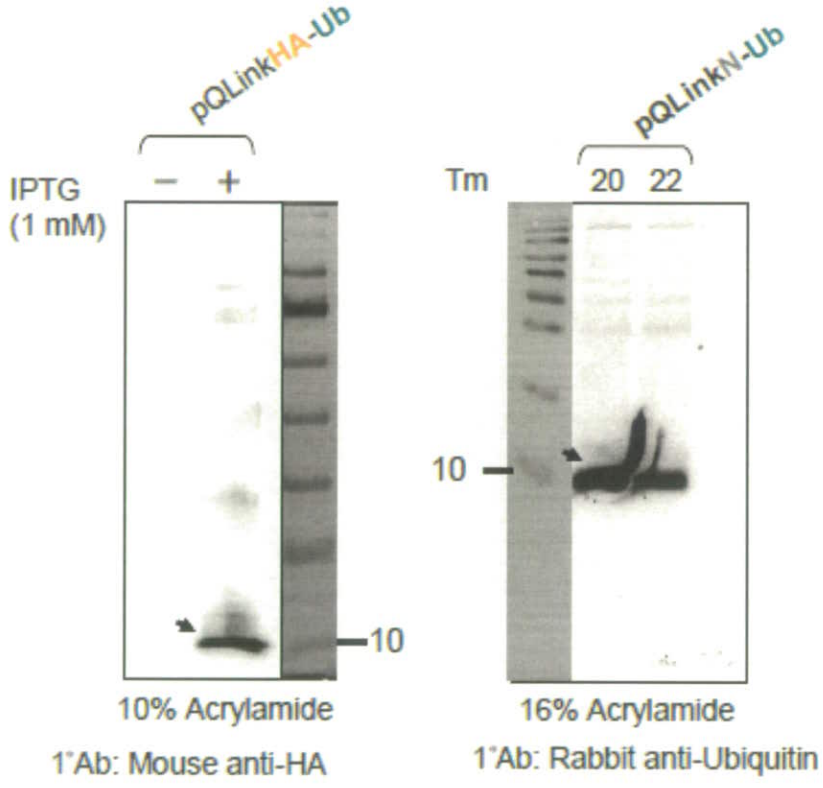

Figure 12. Western blotting to verify the expression of HA-tagged or non-tagged ubiquitin from the pQLinkX in *E. coli* BL21 (DE3). The ubiquitin was detected either by antibody against HA (the left panel) or ubiquitin (the right panel). The Tm represents temerature modification, whereas the numbers donate 20°C and 22°C, respectively. The plasmid pQLinkX carrying gene encoding ubiquitin will be incorporated into pQLinkX carrying genes encoding four other members of SCF ubiquitin E3 ligase to allow simultaneously expressing five proteins in *E. coli*.

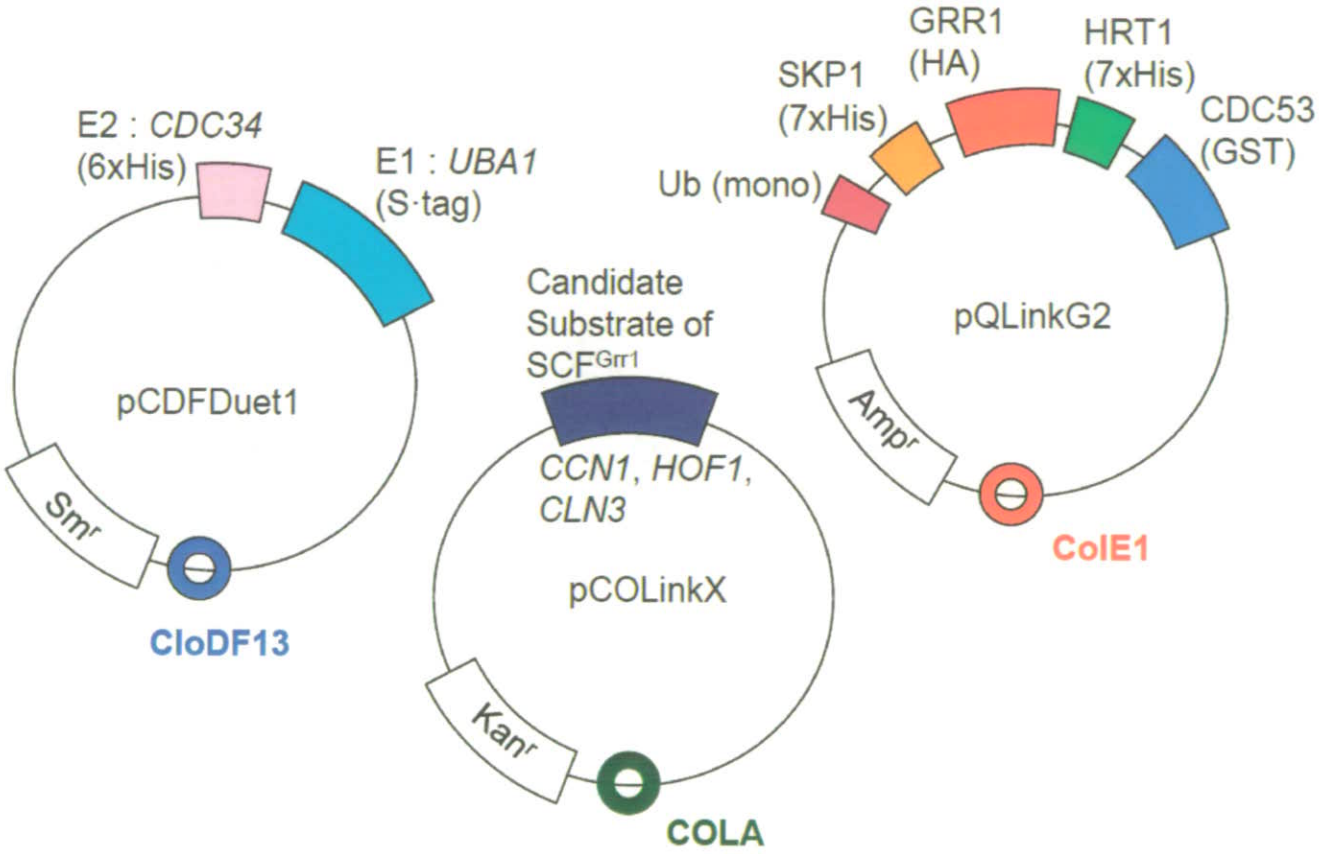

Figure 13. Maps of vectors illustrating their relevant features that are compatible and the genes cloned into which can be simultaneously expressed in *E. coli* BL21 (DE3). The vectors are used to accommodate genes to assemble functional SCF^{Grr1} and each of *CCN1*, *HOF1*, and *CLN3* cloned into pCOLinkX to be expressed as the specific substrate. The pCOLinkX is a derivative of pCOLA-3-DEST made in the current study.

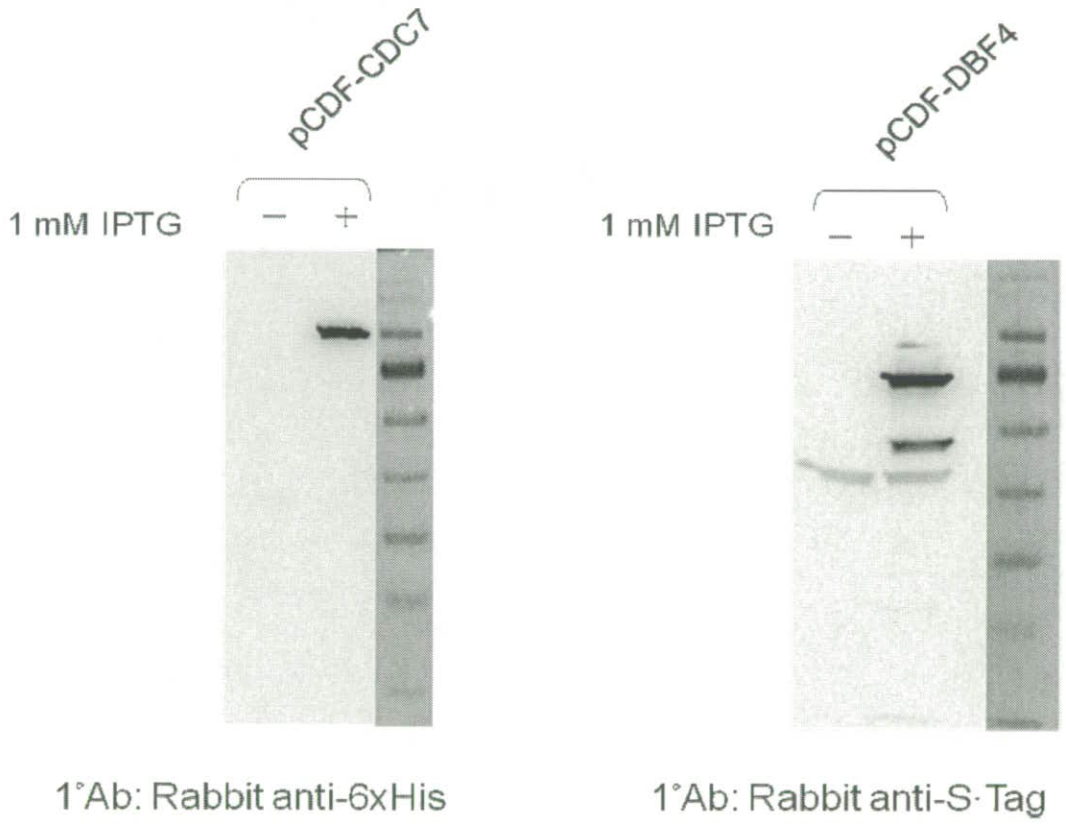

Figure 14. Western blotting to verify the expression of *CDC7* and *DBF4* tagged with 6×His and S·Tag, respectively from vector pCDFDuet1 in *E. coli* BL21 (DE3). The *CDC7* and *DBF4* will be cloned into the same pCDFuet1 for co-expression of *CDC7* and *DBF4* for forming functional DDK that can be used in kinase assay in *E. coli* BL21 (DE3).

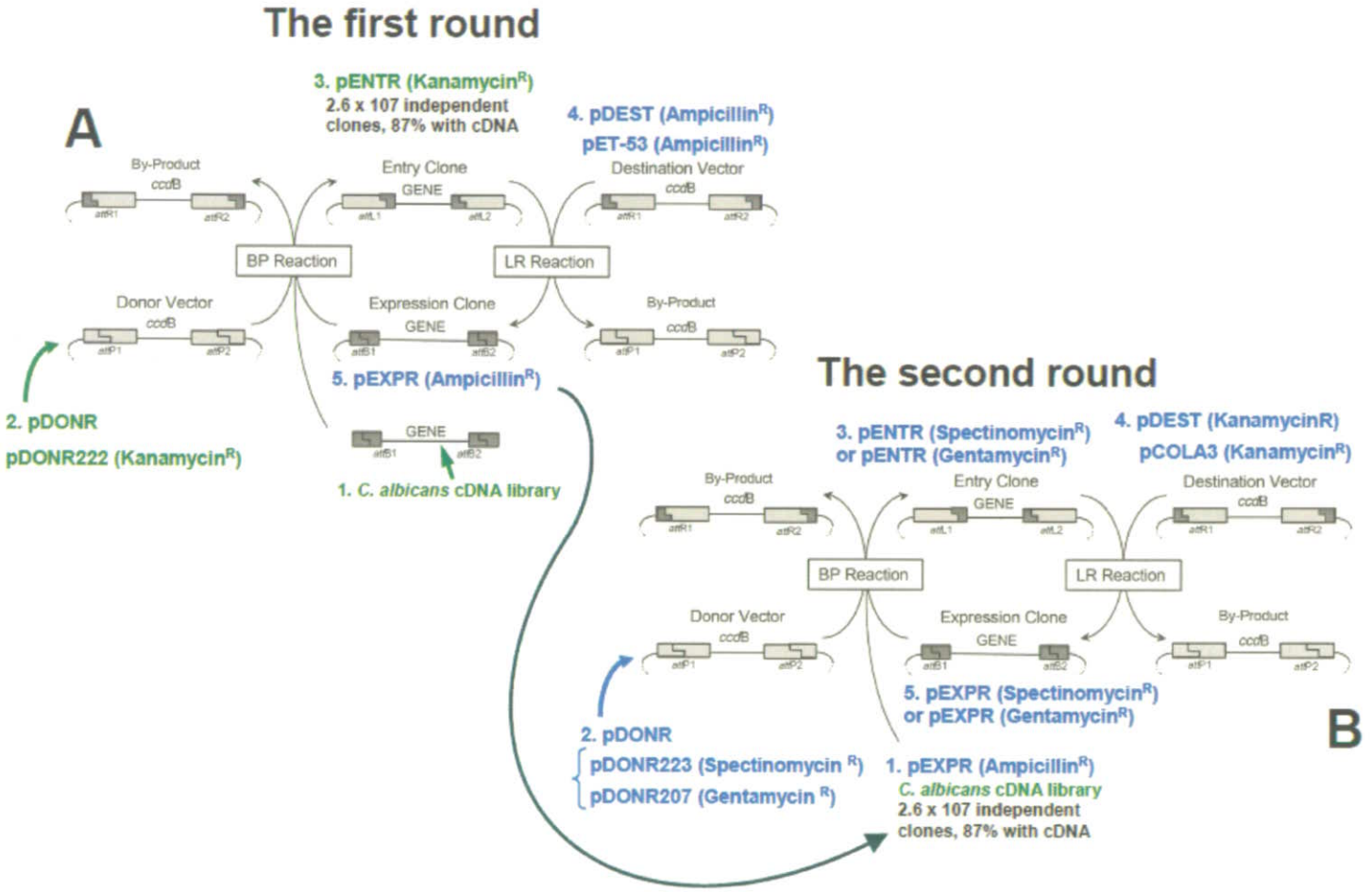

Figure 15. Construction of *C. albicans* cDNA Library by the Gateway cloning approach. Note that the pENTR (#5) obtained in the first round of cloning can be used as the initial clone pEXPR (#1) of the second round of cloning.

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Affinity purification of *Candida albicans* CaCdc4-associated proteins reveals the presence of novel proteins involved in morphogenesis

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ABSTRACT

Candida albicans CDC4 is nonessential and plays a role in suppressing filamentous growth, in contrast to its evolutionary counterparts involved in the G1–S transition of the cell cycle. Genetic epistasis analysis has indicated that proteins besides Sol1 are targets of *C. albicans* Cdc4. Moreover, no formal evidence suggests that *C. albicans* Cdc4 functions through the ubiquitin E3 ligase of the Skp1-Cul1/Cdc53-F-box complex. To elucidate the role of *C. albicans CDC4*, *C. albicans* Cdc4-associated proteins were sought by affinity purification. A 6×His epitope-tagged *C. albicans* Cdc4 expressed from *Escherichia coli* was used in affinity purifications with the cell lysate of *C. albicans cdc4* homozygous null mutant. *Candida albicans* Cdc4 and its associated proteins were resolved by SDS–PAGE and visualized by silver staining. The candidate proteins were recovered and trypsin-digested to generate MALDI-TOF spectra profiles, which were used to search against those of known proteins in the database to reveal their identities. Two out of four proteins encoded by *GPH1* and *THR1* genes were further verified to interact with *C. albicans* Cdc4 using a yeast two-hybrid assay. We conclude that *in vitro* affinity purification using *C. albicans* Cdc4 generated from *E. coli* as the bait and proteins from cell lysate of *C. albicans cdc4* homozygous null mutant as a source of prey permit the identification of novel proteins that physically interact and functionally associate with *C. albicans* Cdc4.

1. Introduction

The opportunistic human fungal pathogen *Candida albicans*, a natural diploid lacking a conventional sexual cycle, causes disease in healthy and immunocompromised hosts. Considerable efforts have been made towards elucidating the molecular mechanism controlling morphogenesis in *C. albicans*, as it is associated with virulence and pathogenesis. Research has already revealed an unanticipated complexity in that at least three positive and five negative pathways control morphological transitions in *C. albicans* [1,2]. To add even more difficulty, Cdks and many cyclins, along with their regulators, have also been shown to play a role in controlling morphological transitions in *C. albicans* [3]. As such, a critical and underlying issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incompletely understood. Significantly, an intriguing question has recently been revealed by us and others in which key cell cycle genes such as *CDC4* and *GRR1*, which are conserved throughout evolution, play no essential role in cell cycle but affect morphogenesis in *C. albicans* [4–7].

Candida albicans CDC4 encodes a structural homologue of the *Saccharomyces cerevisiae* Cdc4, which is an F-box protein of the ubiquitin E3 ligase family and part of the Skp1-Cul1/Cdc53-F-box (SCF) complex, termed SCF^{Cdc4}. We and others have found that, in contrast to *S. cerevisiae CDC4* being essential for the 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 [4,7]. The *C. albicans* homologue of Sic1, termed Sol1, has been isolated as a target of *Ca*Cdc4 [4]. However, the hyperfilamentation phenotype of the double mutant of *Cacdc4−/−* and *sol1−/−* has been shown to be similar to that of the single *Cacdc4−/−* mutant. This result suggests that the stability of Sol1 alone does not explain the constitutive hyphal morphology of *Cacdc4−/−*. We therefore postulated the presence of other *Ca*Cdc4 substrates and conducted affinity purification of *Ca*Cdc4 to identify *Ca*Cdc4-interacting proteins.

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In this report, we describe the identification of *Ca*Cdc4-associated proteins by affinity-purifying a recombinant *Ca*Cdc4 generated from *Escherichia coli* with cell lysate from a *Cacdc4* homozygous null mutant. MALDI-TOF analysis of the purified proteins and confirmation by yeast two-hybrid assays revealed two proteins encoded by the genes *GPH1* and *THR1*, which have not been known to be functionally associated with orthologs of *Ca*Cdc4 from other species. Our study uncovers new components that are functionally associated with *Ca*Cdc4 and will lead to a further understanding of the role of *CaCDC4* in regulating morphogenesis.

2. Materials and methods

2.1. Strains, growth conditions, and DNA methods

Escherichia coli DH5α (F⁻, φ80d*lacZ*ΔM15, Δ (*lacZYA-argF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17* (rk⁻, mk⁺), *phoA*, *supE44*, λ⁻, *thi-1*, *gyrA96*, *relA1*) was used as a host for the routine maintenance and amplification of plasmids. *Escherichia 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 50 μg/ml ampicillin as required [8]. Plasmid DNA was purified using the Gene-Spin™-V² Miniprep Purification kit (PRO TECH, Taipei, Taiwan). The oligonucleotide primers used to construct the plasmids and in the diagnostic analysis of the strains are listed in Table 1.

The *Cacdc4* homozygous null mutant of *C. albicans* (*Cacdc4−/−*), *Cacdc4::dpl200/Cacdc4::dpl200 ura3Δ::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG* (Tseng and Shieh, unpublished data), was constructed from the auxotrophic *C. albicans* BWP17 (*ura3Δ::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG*) [9], which was used for the *in vitro* affinity purification of *Ca*Cdc4-associated proteins. *Candida albicans* strains were grown at 30 °C in either complete media of yeast extract–peptone–glucose (YEPD) or synthetic minimal media with uridine [10].

2.2. Expression and purification of recombinant CaCdc4 in bacteria

To express the recombinant protein in *E. coli*, the coding region of *Ca*Cdc4 was PCR amplified with the primers CaCDC4_XhoI_F and CaCDC4_XhoI_R (Table 1), and cloned into the vector pET-29b (+) at the XhoI site. The construct was transformed into *E. coli* BL21 and selected for kanamycin resistant cells. The transformant was pre-cultured in L-broth at 37 °C with 25 μg/ml kanamycin, and the induction of expression of the C-terminally 6×histidine-tagged *Ca*Cdc4 in a total culture of 500 ml was optimized with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 25 °C for 3 h. The cells were lysed in binding buffer containing 0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9, and 0.1% lysozyme (Sigma) by incubating on ice for 30 min followed by successive sonication for 10 min. The lysate containing histidine-tagged *Ca*Cdc4 proteins was centrifuged and the supernatant was filtered through a 0.45-μm filter before purification with HIS-Select nickel affinity gel (Sigma) essentially as described [11]. Protein concentration was determined by the Protein Assay (Bio-Rad) method according to the manufacturer's instruction.

Table 1
Synthetic oligonucleotide primers used in this study.

Name	Sequence
CaCDC4_XhoI_F	5′-GAACTCGAGATGGATAAGAAATCAAAG-3′
CaCDC4_XhoI_R	5′-GAACTCGAGCTGTAAAAGTGGTTGACT-3′
CaCDC4_NcoI_F	5′-TGCGCCATGGTGGATAAGAAATCAAAGCTA-3′
CaCDC4_NcoI_R	5′-TGCGCCATGGTCACTGTAAAAGTGGTTG-3′
CaGPH1_NcoI_F	5′-CATGCCATGGAGATGCCAATGGATTATCTTACC-3′
CaGPH1_BamHI_R	5′-GCGGGATCCCTAAACATTGGATGGTTCAAC-3′
CaSTI1_BglII_F	5′-GGAAGATCTGTATGACAACAGCTGACGAATA-3′
CaSTI1_BglII_R	5′-GGAAGATCTTTATCTGGTACGAATAACACC-3′
CaHMT1_BglII_F	5′-GGAAGATCTGTATGTCTGAATCAGCTACTGAT-3′
CaHMT1_BglII_R	5′-GGAAGATCTCTAACGTAAAAAGTAAGTGTATC-3′
CaTHR1_NcoI_F	5′-CATGCCATGGAGATGAGCGTTATTTCATTTAAAAT-3′
CaTHR1_BamHI_R	5′-GCGGGATCCTTATCGTAAGACATTTAATTTTTTA-3′
pACT2_F_(1)	5′-CTATTCGATGATGAAGATACC-3′
pACT2_R_(1)	5′-AGATGGTGCACGATGCAC-3′

Note: Sequences underlined donate site of restriction enzyme.

2.3. Affinity purification of CaCdc4-interacting proteins in vitro

To purify *Ca*Cdc4-associated proteins *in vitro*, the total cell lysate from a *C. albicans Cacdc4−/−* culture in 500 ml YEPD was first prepared as described previously [12]. The cell lysate was centrifuged and the supernatant was filtered through a 0.45-μm filter before being applied to the HIS-Select nickel affinity gel column (Sigma) pre-bound with 6×histidine-tagged *Ca*Cdc4 prepared from *E. coli* cell lysate, and the *Ca*Cdc4-associated proteins were eluted with the 6×histidine-tagged *Ca*Cdc4 by HIS-Select nickel affinity chromatography (Sigma) according to the manufacturer's instructions. Briefly, the column containing the HIS-Select nickel affinity gel bound with 6×histidine-tagged *Ca*Cdc4 proteins was subjected to binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9) twice before applying the proteins from the *Cacdc4−/−* cell lysate. The column was then subjected to binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9) twice, wash buffer (0.5 M NaCl, 20 mM Tris–HCl, pH 7.9 with either 20, 40, 60, or 80 mM imidazole) four times, and elution buffer (0.5 M NaCl, 20 mM Tris–HCl, and 1 M imidazole, pH 7.9) five times.

The proteins were resolved by SDS–PAGE and visualized with silver nitrate staining using a modified version of a previously described method [13]. Briefly, after electrophoresis, gels were washed twice with ddH_2O and then fixed with 50% methanol and 25% glacial acetic acid in ddH_2O for 2 h, followed by washing with 30% methanol for 15 min and subsequently washing three times with ddH_2O for 5 min each. The gels were incubated with a sensitizing solution of 0.8 mM sodium thiosulphate for 2 min, washed twice with dH_2O for 30 s each, followed by incubation in a 0.2% silver nitrate solution for 25 min at room temperature in the dark. After washing twice with dH_2O for 30 s each, the gels were developed with a solution containing 0.28 M sodium carbonate, 0.185% formaldehyde, and 0.016 mM sodium thiosulphate for about 10 min or until the appropriate visualization of proteins was achieved. The reaction was terminated by the addition of 0.042 M EDTA.

2.4. In-gel tryptic digestion and mass spectrometry

The proteins were recovered from the gels and in-gel digested with trypsin as described previously with some modifications [14]. Upon completion of in-gel trypsin digestion, a 3-μl solution of 98% acetonitrile (ACN)/2% formic acid (FA) was added to the digested solution and the solution was sonicated for 10 min. After recovering the supernatant, 0.5 μl of the supernatant sample was spotted onto a MTP AnchorChip™ 600/384 TF (Bruker-Daltonik GmbH, Bremen, Germany) and air-dried for 10 min, followed by spotting 0.5 μl of 1 mg/ml α-cyano-4-hydroxycinammic acid (CHCA) and air-drying for 10 min prior to analysis with an UltraFlexIII MALDI-TOF/TOF mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany) in the Proteomics Research Core Laboratory, Office of Research and Development, China Medical University, Taichung, Taiwan.

2.5. Database-searching to identify proteins

The monoisotopic masses (*m/z*) of both parent ions and their corresponding fragment ions, parent ion charge states (*z*), and ion intensities from the acquired mass spectra were automatically extracted using the script in the analyst software and directly submitted for an automated database search against the NCBInr. 2008. 11.25, CANDIDA using MS-Fit of Protein Prospector (University of California San Francisco). Carbamidomethyl cysteine was set as a static modification and one missing cleavage was allowed. The minimum number of peptides required to match was set to four. The Pfactor of the MOWSE score was set to 0.4. The best possible candidate proteins from the search results were evaluated manually for functional relevance and further confirmed by a yeast two-hybrid assay.

2.6. Immunological detection

Escherichia coli cells expressing *Ca*Cdc4 proteins were grown under the optimal inducing condition and the total protein was extracted by the methods described in the previous section. The proteins were resolved by SDS–PAGE and transferred electrophoretically to PVDF membranes (PerkinElmer, Boston, MA). The membranes were probed with a polyclonal antibody against poly-histidine (LTK Biolaboratories, Taoyuan, Taiwan). Detection was performed using a peroxidase-conjugated goat anti-rabbit IgG (H+L) (Pierce, Rockford, IL). The signal was visualized using the SuperSignal West Pico Chemiluminescence Substrate Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.7. Yeast two-hybrid analysis

Yeast two-hybrid interaction assays were performed as described previously [15]. To generate pGBKT7-*CaCDC4*, which expresses a fusion protein of the GAL4 DNA-binding domain, 2307 bp of *CaCDC4* was PCR-amplified with *C. albicans* genomic DNA and the primers CaCDC4_NcoI_F/CaCDC4_NcoI_R, incorporating a NcoI site (Table 1), followed by digesting with NcoI before ligating into the NcoI digested pGBKT7. *Candida albicans* genes, including *GPH1*, *HMT1*, *ST11*, and *THR1*, were initially PCR-amplified and cloned into pCR2.1-TOPO with the primer pairs CaGPH1_NcoI_F/CaGPH1_BamHI_R, CaHMT1_BglII_F/CaHMT1_BglII_R, CaSTI1_BglII_F/CaSTI1_BglII_R, or CaTHR1_NcoI_F/CaTHR1_BamHI_R (Table 1). To generate pACT2-*GPH1*, which expresses a fusion protein of the GAL4 activation domain, the coding region of *GPH1* was PCR-amplified with pCR2.1-TOPO-based *GPH1* and the primer pair CaGPH1_NcoI_F, incorporating an NcoI site, and CaGPH1_BamHI_R, incorporating a BamHI site (Table 1), to produce a 2703-bp fragment, followed by digestion with NcoI/BamHI before ligating into the NcoI/BamHI-digested pACT2 (Clontech; GenBank Accession No. U29899). Similarly, pACT2-*THR1*, -*HMT1*, and *STI1* were obtained using the coding sequences of the respective genes on pCR2.1-TOPO as the template for PCR amplification with the respective 5′ oligonucleotide incorporating an NcoI site (Table 1) and the 3′ oligonucleotide incorporating a BamHI site (Table 1) for respective 822-, 1020-, and 1770-bp fragments. The interaction assay was conducted by mating *S. cerevisiae Mat*a AH109 carrying pGBKT7-*CaCDC4* (our unpublished data) with *S. cerevisiae Mat*α Y187 carrying either pACT2-*GPH1*, -*THR1*, -*HMT1*, or -*ST11*. The ability of diploid *S. cerevisiae* cells to grow on plates of selective media lacking histidine was used as an indicator of interaction due to the *HIS3* reporter gene being activated. The possibility of cell growth being attributed to a basal level of *HIS3* transcription was excluded by growing the cells on plates with selective media lacking histidine and titrating with 3-aminotriazole (3-AT), an inhibitor of *HIS3* gene product.

3. Results and discussion

3.1. Optimal expression of recombinant CaCdc4 in E. coli

We have previously identified *Ca*Cdc4 as a hyphal suppressor in *C. albicans*. To determine if *Ca*Cdc4 plays this role as an ubiquitin E3 ligase of the SCF complex and to identify substrates or potential regulators of *Ca*Cdc4, we adopted an *in vitro* affinity purification approach using a recombinant *Ca*Cdc4 protein purified from *E. coli* as the bait. The approach was based on the fact that the ubiquitination activity of the ubiquitin E3 ligase is functional when all of its components are co-expressed in *E. coli* cells [16]. We anticipated that the *E. coli*-expressed and purified recombinant *Ca*Cdc4 has this function, and hence has an affinity for its associated proteins. In addition, using recombinant *Ca*Cdc4 purified from *E. coli* as a bait to probe for *Ca*Cdc4-associated proteins from *C. albicans* cell lysate eliminates the possibility of the *Ca*Cdc4 substrates being degraded as occurs when *Ca*Cdc4 is expressed in *C. albicans* cells. Under the IPTG-induced condition, *Ca*Cdc4 tagged with 6×histidine at the C-terminus was optimally expressed and purified from *E. coli*, despite a greater abundance of *Ca*Cdc4 in the pellet than in the supernatant (Fig. 1), and used in the affinity purification of its associated proteins *in vitro*.

3.2. In vitro affinity purification of the CaCdc4-associated proteins

To ensure *Ca*Cdc4-associated proteins, particularly the potential targets, would be revealed, we used proteins extracted from *C. albicans Cacdc4* homozygous null mutant (Tseng and Shieh, unpublished data) cell lysate as a source of prey proteins. In fact, we have previously adopted an approach that allows patterns of affinity-purify proteins from *C. albicans* cell lysate of either hyphal or yeast form. The approach is capable of using doxycycline to induce the expression of *Ca*Cdc4 that is C-terminally tagged with 6×histidine and FLAG. It appeared that the doxycycline-inducible system was able to induce the expression of *Ca*Cdc4 in a defined window of time and produce a massive amount of *Ca*Cdc4 for affinity purification. Nevertheless, this over-expressed and presumably hyperactive *Ca*Cdc4 might lead to its own degradation (Lai and Shieh, unpublished data). As a result, such an approach proved to be ineffective for the purification of *Ca*Cdc4 *in vivo*.

The *Ca*Cdc4 purified from bacteria was used in the affinity column purification of proteins from *Cacdc4−/− C. albicans* cell lysates. After being washed and eluted, *Ca*Cdc4 and its associated proteins were resolved by SDS–PAGE. The presence of bait *Ca*Cdc4 protein during the wash–elution process was monitored by Western blotting (Fig. 2). The resolved protein samples were also visualized by silver staining (Fig. 2) and the prey proteins selected from the elution steps were those that were not present or were less prominent, although not entirely excluded, in the wash steps. A total of 10 prey proteins (Fig. 2) that appeared consistently in at least two experiments were subjected to further in-gel tryptic digestion and mass spectrometry.

3.3. Determination of the identity of CaCdc4-associated proteins and the implication of their functional relevance to CaCdc4

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 mass spectrometry analysis. After obtaining the specific mass spectra (Fig. 3) of the purified proteins from the bands migrating at positions around 110, 100, 72, 45, 40, 35, 30, 28, 25, or 20 kDa (see Fig. 2), the data were used to search against the NCBInr. 2008. 11.25, CANDIDA using the MS-Fit of Protein Prospector. The 10 examined bands were categorized into 10 groups

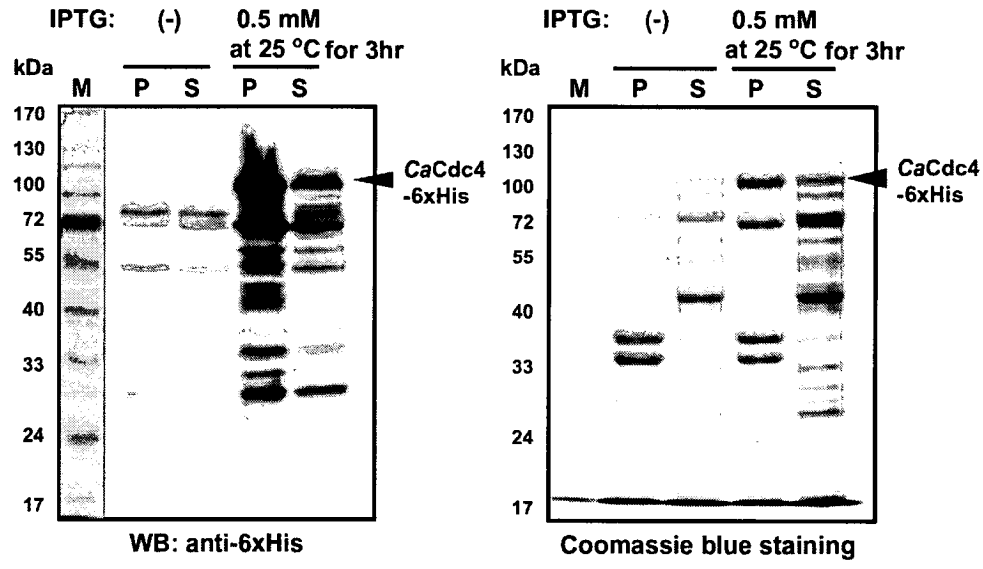

Fig. 1. Optimal expression and purification of *Ca*Cdc4 from *E. coli*. The C-terminal 6xHis-tagged *Ca*Cdc4 was produced in *E. coli BL21* cells containing the plasmid *pET-29b(+)-CaCDC4* grown at 25 °C for 3 h under 0.5 mM IPTG induction. Induction of the *Ca*Cdc4 protein (indicated by arrows) was revealed both by Western blotting and Coomassie blue staining. The generated *Ca*Cdc4 was present both in the pellet (P) and in the supernatant (S) of the *E. coli* cell lysates.

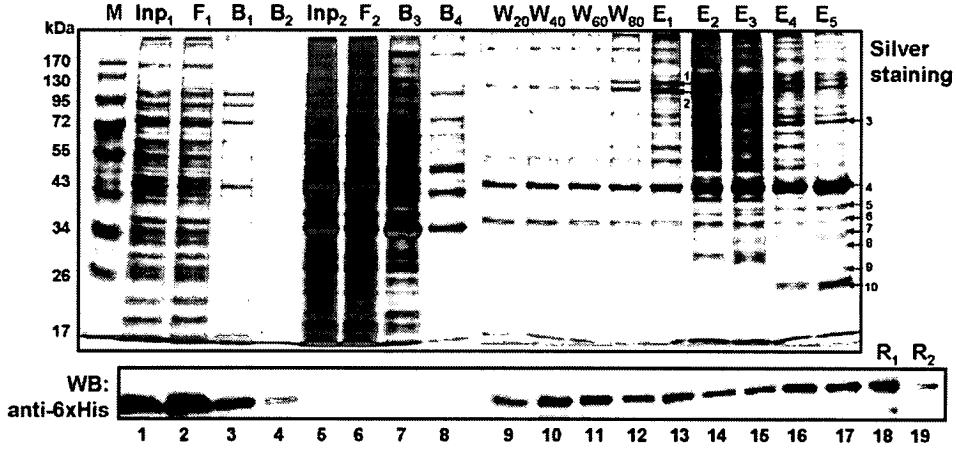

Fig. 2. *In vitro* purification of *Ca*Cdc4-associated proteins. Proteins from *C. albicans Cacdc4−/−* cell lysate were applied to a HIS-Select nickel affinity gel column bound with purified 6×His-tagged *Ca*Cdc4. *Ca*Cdc4-associated proteins purified by *Ca*Cdc4 affinity-chromatography were resolved by SDS-PAGE and the presence of *Ca*Cdc4 was verified by Western blotting (bottom panel). Potential *Ca*Cdc4-associated proteins were eluted and visualized by silver staining (top panel), 10 of which (as indicated by arrows) were subjected to MALDI-TOF analysis. M: size marker; Inp_1: cell lysate from *E. coli* with 6×histidine-tagged *Ca*Cdc4; F_1: flow through after Inp_1; B_1 and B_2: binding buffer (0.5 M NaCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9); Inp_2: cell lysate from the *C. albicans Cacdc4−/−* mutant; F_2: flow through after Inp_2; B_3 and B_4: the same as B_1; W_{20}–W_{80}: wash buffer composed of 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9 with imidazole at a concentration of 20 mM (W_{20}), 40 mM (W_{40}), 60 mM (W_{60}), or 80 mM (W_{80}), respectively; E_1–E_5: 0.5 M NaCl, 20 mM Tris–HCl, and 1 M imidazole, pH 7.9; R_1: 10 μl of resin was recovered after B_2; R_2: 10 μl of resin was recovered after B_4.

numbered 1–10 (Fig. 2), some of which contained more than one potential protein (data not shown). Based on both the highest mass spectrum identity score among the proteins within each group and the possible functional relevance to *Ca*Cdc4 matching our interests, 10 prey proteins were initially listed (Table 2). We were particularly interested in the proteins encoded by *GPH1* from group 2, *STI1* from group 5, *HMT1* from group 7, and *THR1* from group 8. *Candida albicans GPH1*, encoding a putative glycogen phosphorylase that is regulated by Tup1, is involved in the hyphal-specific regulation of gene expression [17] and interacts with Ssk1 to provide a regulatory function in cell wall biosynthesis [18]. *Candida albicans* Sti1 is a hyphae-specific protein [19] and is up-regulated during biofilm formation [20]. *Candida albicans HMT1* encodes a major type I protein arginine methyltransferases that is involved in the nuclear export of Npl3, an mRNA binding protein [21], whose *S. cerevisiae* ortholog is required to export mRNA from the nucleus to the cytoplasm and is involved in regulating the budding pattern [22]. *Candida albicans THR1*, encoding a putative homoserine kinase, is not only transcriptionally regulated by Tup1 [17] but also regulated by Gcn4 [23] and is known to co-ordinate morphogenetic and metabolic responses to amino acid starvation [24,25]. These proteins were further verified as *Ca*Cdc4-interacting proteins by yeast two-hybrid assays.

3.4. Validation with yeast two-hybrid reveals two novel proteins encoded by GPH1 and THR1 that directly interact with CaCdc4

To validate that the affinity-purified proteins are indeed *Ca*Cdc4-interacting proteins, yeast two-hybrid assays were performed. Cells of *S. cerevisiae Mata* AH109 transformed with plasmid

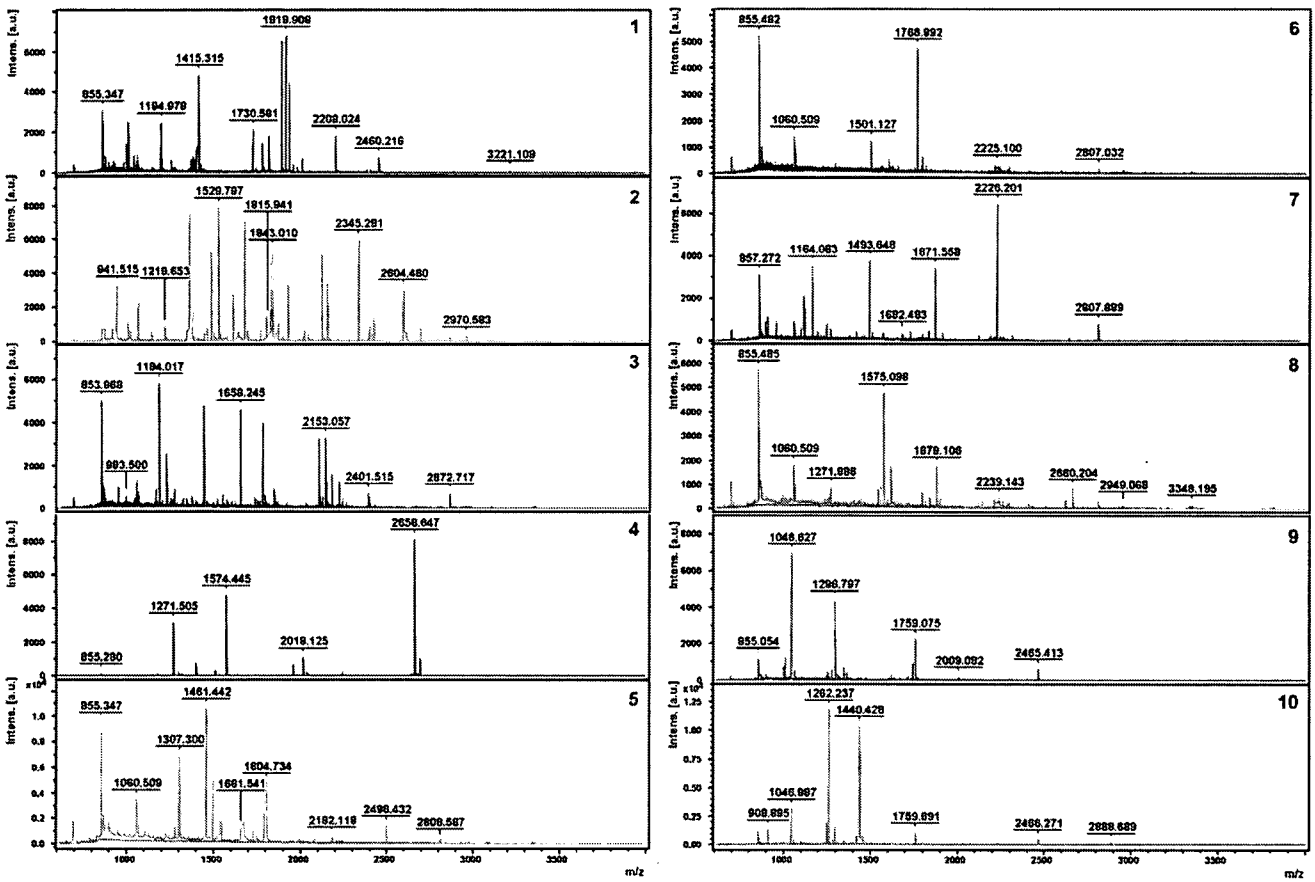

Fig. 3. MALDI-TOF spectra of 10 prey proteins. The *Ca*Cdc4-associated proteins were separated by 10% SDS–PAGE and visualized by silver staining. The bands of interest were excised and in-gel digested with trypsin to generate a spectra profile by mass spectrometry as described in Section 2. The recovered bands were designated as 1–10.

Table 2
Candida albicans Cdc4 associated proteins identified by MALDI-TOF MS.

Band No.[a]	Protein name	CGD systematic name[b]	Score[c]	Seq Cov[d] (%)
1	Potential jumonji-like transcription factor	orf19.5651 (*JHD2*)	64.8	15.4
2	Hypothetical protein CaO19_7021	orf19.7021 (*GPH1*)	1.26E+16	39.2
3	Hypothetical protein CaO19.604	orf19.604 (*PHH1*)	655	8.6
4	Hypothetical protein CaO19.13973	orf19.6652 (*DPB8*)	111	18
5	Hypothetical protein CaO19.3191	orf19.3192 (*STI1*)	4.06	9
6	Likely mitochondrial ribosomal protein MRPL40p	orf19.484 (*MRPL40*)	7.03	13
7	Hypothetical protein CaO19.10801	orf19.3291 (*HMT1*)	486	17.1
8	Likely homoserine kinase	orf19.923 (*THR1*)	47.5	13.2
9	Hypothetical protein CaO19.3689	orf19.3689	62.3	24.4
10	Hypothetical protein CaO19.8055	orf19.425	40.7	20.8

[a] Numbering of the protein bands detected in Fig. 2.
[b] Genes shown in the brackets are those with known homologues of *S. cerevisiae*.
[c] MS-Fit search score of identified proteins.
[d] Sequence coverage (Seq Cov) of the matched peptides in protein.

pGBKT7-*CaCDC4* and capable of expressing a fusion protein of the GAL4 DNA-binding domain were mated with cells of *S. cerevisiae Mat*α Y187 transformed with one of the following plasmids: pACT2-*GPH1*, -*THR1*, -*HMT1*, or -*STI1*. The interaction between *Ca*Cdc4 and the potential associated proteins was demonstrated by their ability to grow on agar plates lacking histidine. As shown in Fig. 4A, in the presence of pGBKT7-*CaCDC4*, only the strains of diploid *S. cerevisiae* carrying pACT2-*GPH1* or pACT2-*THR1* were able to form colonies on agar plates lacking histidine, demonstrating that *Ca*Cdc4 interacts with either Gph1 or Thr1 but not with Hmt1 or Sti1. However, we cannot entirely exclude the possibility that Sti1 and Hmt1 are indirectly associated with *Ca*Cdc4 via other proteins that were affinity-purified with *Ca*Cdc4 but not examined by yeast two-hybrid. To eliminate the possibility of cells growing due to a basal level of *HIS3* transcription, serially-diluted cells were spotted onto agar plates without histidine but with various concentrations of 3-AT, and the ability to form colonies was assessed. It was apparent that the interaction of either Gph1 or Thr1 with *Ca*Cdc4 was moderately strong, as transcriptionally activating *HIS3* in diploid *S. cerevisiae* cells co-expressing *Ca*Cdc4 and Gph1/Thr1 resulted in colonies on the plate with 1.25 mM 3-AT that were comparable to those on a plate without 3-AT, even though growth-

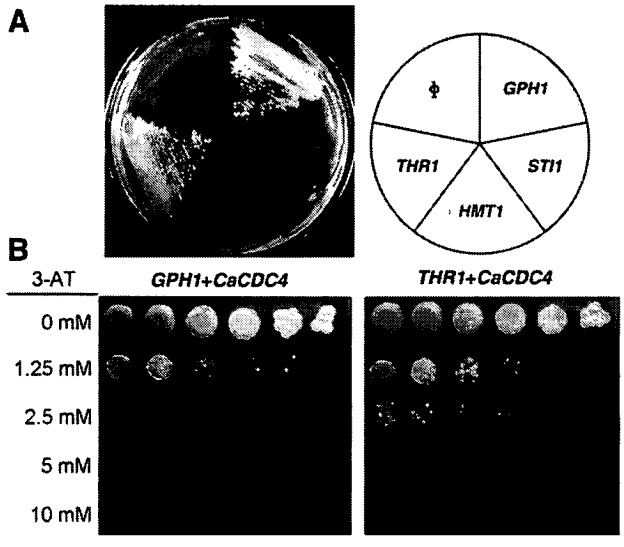

Fig. 4. Interaction of *Ca*Cdc4 with Gph1 or Thr1 using yeast two-hybrid assays with the *HIS3* reporter. (A) Strains of diploid *S. cerevisiae* carrying pGBKT7-*CaCDC4* and the indicated pACT2-based plasmid were streaked onto agar plates with selective media. Diploid *S. cerevisiae* carrying the empty vectors pGKT7 and pACT2 is shown as ϕ. (B) Five-fold serially-diluted cells with a starting concentration of 10^5 cells from (A) were spotted onto agar plate with selected medium and indicated concentration of 3-AT.

inhibition was observed on plates with 3-AT concentrations equal to or greater than 2.5 mM (Fig. 4B). This result indicates that the interaction between Gph1/Thr1 and *Ca*Cdc4 that activates the *HIS3* reporter is indeed genuine and is unlikely to be due to a basal level of *HIS3* transcription.

4. Conclusions

We conclude that two novel proteins encoded by *GPH1* and *THR1* physically interact and functionally associate with *Ca*Cdc4, an F-box protein of the E3 ubiquitin ligase family, in *C. albicans* by using *in vitro* purification in conjunction with mass spectrometry and yeast two-hybrid assays. Establishing the mechanism of how Gph1 and Thr1 regulate *Ca*Cdc4 or how Gph1 and Thr1 are regulated by *Ca*Cdc4 will be critical for further understanding the role *CaCDC4* plays in morphogenesis.

Acknowledgments

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

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Running title: *Candida albicans* Tet-on tagging vectors with a Ura-blaster cassette

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Abstract

It has been difficult to develop molecular tools for studying the fungal pathogen *Candida albicans* because it uses a non-standard genetic code and is diploid without a complete sexual cycle. Vector systems with regulatable promoters to produce conditional mutants, epitope tags for protein detection, and recyclable selection markers are useful for functional study of genes. However, most currently available vectors contain only a subset of desired properties, which limits their application. To combine several useful properties in one vector, the vector pTET25 was initially modified into pTET25M so that the *URA3* gene flanked by *dpl*200 could be used repetitively. To enable more choices for cloning, a multiple cloning site was introduced at both ends of *GFP* in pTET25M. GFP expression was induced by doxycycline in a dose- and time-dependent manner when the plasmid was introduced into *C. albicans* with or without *URA3*. The applicability of the vectors was verified by constructing strains capable of expressing either the N-terminal GFP fusion of Cdc10 or the C-terminal GFP fusion of Cdc11. Additionally, by replacing the *GFP* gene of pTET25M with DNA sequence encoding Cdc10 with an epitope tag of six histidine residues at the C-terminus, doxycycline-induced expression of *CDC10* was achieved when the expression vector was introduced into *C. albicans*. This new system allows for inducible expression of a desired *C. albicans* gene with the

advantage of convenience of cloning. It also allows the presence of a recyclable *URA3* marker and the detectable expression of fusion or epitope-tagged protein.

Keywords: *Candida albicans*; Tet-on tagging vector; Ura-blaster cassette; GFP fusion protein

Introduction

The yeast *Candida albicans* is a member of the normal microflora on the mucosal surfaces of the gastrointestinal and genitourinary tracts in healthy humans. However, it is also the primary fungal pathogen in humans (Magee, 1998). Particularly, *C. albicans* triggers systemic candidiasis in immunocompromised patients. Molecular analysis of *C. albicans* has been hindered because it is diploid in nature, does not have a complete sexual cycle, and uses non-standard codons. However, many approaches have been undertaken to facilitate the functional study of *C. albicans*. To date, several gene disruption strategies have been developed for *C. albicans* (Berman and Sudbery, 2002), the most common of which is the use of a Ura-blaster cassette (Wilson, et al., 2000) that allows the reuse of the auxotrophic *C. albicans URA3* selection marker. The Ura-blaster cassette has also been incorporated into vector systems to sequentially introduce or delete genes to assess their functional interactions.

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

Morschhauser, 2005). Regulatable expression systems are especially useful for assessing the consequences of the presence or absence of gene expression. Gene function can be determined by either inducing the overexpression of a specific gene that disturbs the regulatory mechanism of its normal function, or repressing the expression of a gene of interest that reveals the functional consequence of depletion of the gene product.

Epitope tagging has become a common tool for detecting, purifying, and studying the function of proteins (Fritze and Anderson, 2000). Several single epitope tags such as glutathione S-transferase (Smith and Johnson, 1988), green fluorescent protein (GFP) (Cubitt, et al., 1995; Heim, et al., 1995), haemagglutinin (HA) (Field, et al., 1988), six histidine residues (6×His) (Hagan and Stirling, 1998), human c-Myc (Evan, et al., 1985), and FLAG tags (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), are widely used in a variety of organisms. In *C. albicans*, tagging vectors have recently been developed for the HA epitope of Tet-off (Nakayama, et al., 2000), GFP (Cormack, et al., 1997), FLAG (Umeyama, et al., 2002), and 6×His-FLAG systems. (Kaneko, et al., 2004)

To facilitate the detection, purification, and functional studies of *C. albicans* gene products, we constructed expression tag vectors that can be used in *C. albicans*.

The vectors allow proteins to be ectopically expressed in a doxycycline-dependent manner with either a C-terminal or N-terminal GFP tag or a C-terminal 6×His epitope. The vectors also possess a Ura-blaster cassette to allow one to reintroduce a *URA3* marker. The function and applicability of the vectors were assessed using Cdc10 and Cdc11 proteins, members of the septin family that are components of the septum complex at the bud neck between mother and daughter cells (Sudbery, 2001).

Materials and methods

Strains, growth conditions and DNA manipulation

The auxotrophic *C. albicans* strain BWP17 (Wilson, et al., 1999), shown in Table 1, was used in strain construction. The *C. albicans* strains used and generated in this study are also shown in Table 1. All strains were grown in either a complete rich medium of yeast extract-peptone-glucose (YEPD) or in a synthetic minimal medium with or without 50 μg/ml uridine, as previously described (Tseng, et al., 2010). The strains were stored as frozen stocks in 20% glycerol at –80°C. Transformants were selected on synthetic minimal medium without uridine and screened for integrants by yeast colony PCR (Wang, et al., 1996) using the oligonucleotide primers pNIM-inte detect F and pNIM-inte detect R listed in Table 2.

The *Escherichia coli* strain DH5α was used as a host for plasmid DNA

construction and routine plasmid maintenance and amplification. Bacterial cultures were grown in L-broth or L-broth supplemented with 50 μg/ml ampicillin as required. Plasmid DNA was purified using the Gene-Spin™-V^2 Miniprep Purification Kit (Protech, Taipei, Taiwan). The oligonucleotide primers used for vector construction are listed in Table 2.

Generation of the Ura-blaster cassette

To introduce a Ura-blaster cassette, the DNA fragment of the *C. albicans URA3* marker from the doxycycline-inducible pTET25 vector (Park and Morschhauser, 2005) was removed by digestion with *Pst*I and *Sal*I and cloned into the pUC19 vector to generate pUC19-URA3. The DNA fragment of *dpl*200 in *URA3* was amplified by PCR from pUC19-URA3 using the primers CaURA3-dpl200-XhoI and CaURA3-dpl200-BamHI (Table 2), digested with *Xho*I and *Bam*HI, and cloned into pUC19-URA3 to generate pUC19-URA3- dpl200. To introduce the *Bgl*II cloning site downstream of *C. albicans GFP* to pTET25, a DNA fragment containing partial *TetO*, *GFP* with *Sal*I and *Bgl*II sites, and the *ACT1* transcription termination sequence (*ACT1t*) from pNIM1 was amplified with the primers TET25M-KpnI and TET25M-BamHI (Table 2) by PCR. It was then digested with *Kpn*I and *Bam*HI and cloned into pUC19-URA3-dpl200 to generate pUC19-TET25M. A DNA fragment

containing *GFP*, *ACT1t*, and partial *URA3* was cut out of pUC19-TET25 by digestion with *Sal*I and *Eco*RI and cloned into pTET25 to generate pTET25M (Figure 1), the sequence of which was deposited into GenBank (accession no. HM776584). Importantly, the coding sequence of *GFP* in pTET25M has been modified to allow for CUG-codon adaptation (Morschhauser, et al., 1998)

Introduction of the multiple cloning sites

To enable the expression of a desired protein as a GFP fusion protein, restriction cloning sites were introduced at both ends of the *GFP* coding sequence in the pTET25M vector. A DNA fragment was amplified from pTET25M by PCR with a forward primer, CaGFP-N-MCS (Table 2), containing the restriction sites for *Sal*I, *Xho*I, *Eco*RV, *Nhe*I, and *Not*I and a reverse primer, CaGFP-C-MCS (Table 2), containing the restriction sites for *BspE*I, *Aat*II, *Afl*II, *Bgl*II, *Stu*I, and *Bcl*I. A stop codon was also included. The PCR amplicon containing *GFP* was sequentially digested with *Sal*I and *Bcl*I and cloned into the pTET25M vector that had been digested with *Sal*I and *Bgl*II to cut out its copy of *GFP* to generate pTET25M-NC (Figure 1), which contains multiple cloning sites at both ends of the *GFP* coding sequence. Another DNA fragment was amplified by PCR with the primers CaGFP-N-MCS and CaGFP-MCS-R (Table 2), digested with *Sal*I and *Bgl*II, and

cloned into pTET25M to generate pTET25M-N with multiple cloning sites at one end of the *GFP* coding sequence that allows the generation of a C-terminal GFP fusion protein.

Generation of the 6×His epitope and GFP fusion proteins

The coding sequence of either *CaCDC10* or *CaCDC11* was amplified by PCR with the primer pairs of either CaCDC10-AatII and CaCDC10-BglII or CaCDC11-NheI and CaCDC11-XhoI (Table 2). The two PCR amplicons were digested with either *Aat*II and *Bgl*II or *Nhe*I and *Xho*I. The digested products were subsequently cloned into pTET25M-NC to generate pTET25M-GFP-CDC10, which is capable of encoding an N-terminal GFP fusion of Cdc10, and pTET25M-CDC11-GFP, which is capable of encoding a C-terminal GFP fusion of Cdc11. To incorporate a 6×His epitope at the C-terminus of a protein, a new reverse primer, CaCDC10-6×His-BclI (Table 2), which is capable of encoding 6×His, was introduced. In addition, the reverse primer was incorporated into the *Xma*I and *Bgl*II restriction sites. A new forward primer, CaCDC10-Tag-SalI (Table 2), was incorporated into the *Aat*II and *Not*I sites. A DNA fragment of *CaCDC10*-CH was amplified by PCR from pTET25M-GFP-CDC10 with the new pair of primers. Subsequently, the *CaCDC10*-CH PCR amplicon was digested with *Sal*I and *Bcl*I and

cloned into pTET25M to generate pTET25M-CDC10-CH (Figure 1). The *C. albicans* *CDC10* in the pTET25M-CDC10-CH vector can be replaced by coding sequences of other genes with several restriction sites of choice (Figure 1).

Candida albicans transformation and selection

Transformation of *C. albicans* cells and selection of transformants were performed as previously described (Shieh, et al., 2005). Briefly, the DNA fragment of the Tet-on part containing the gene of interest was removed from the plasmid by digestion with *Sac*II and *Kpn*I, purified using the G-M™ Gel Extraction System (Viogene, Taipei, Taiwan), and then introduced into *C. albicans* cells by the LiAc-PEG-ssDNA method (Gietz and Woods, 2006) with some modification (Walther and Wendland, 2003). The DNA was specifically integrated into the *ADH1* locus of the *C. albicans* genome as a stable integrant; the Ura^+ prototrophs on minimal medium plates lacking uridine (Shieh, et al., 2005) and the integrants were determined by colony PCR using the diagnostic primers pNIM1-inte detect F and pNIM1-inte detect R (Table 2). To recycle the *C. albicans URA3* cassette, the Ura^+ prototrophs were grown on a plate with 5-FOA (1 mg/ml) and uridine (50 μg/ml), and the Ura^- auxotrophs were selected. Correct insertion of the *URA3* gene and removal of the *URA3* gene after 5-FOA selection were verified by PCR with the specific primers

CaURA3-dpl200 BamHI and CaURA3-dpl200 XhoI (Table 2).

Western blot analysis

To detect expression of the tagged *Ca*Cdc10 and the *Ca*Cdc10 and *Ca*Cdc11 fusion proteins, *C. albicans* strains were grown at 30°C in YEPD overnight, diluted (1: 10) into fresh YEPD, grown to log phase before addition of 40 μg/ml doxycycline (Sigma), and then grown for 3 h, unless otherwise stated. Total protein was extracted from the cultured cells as previously described (Shieh, et al., 2007). The proteins were resolved by SDS-PAGE and electrophoretically transferred onto PVDF membranes (PerkimElmer Boston, MA). The membranes were probed with an anti-6×His epitope monoclonal antibody (Novagen) or an anti-GFP polyclonal antibody (Abcam). Proteins were detected using either a peroxidase-conjugated goat anti-mouse IgG (Millipore) or a peroxidase-conjugated donkey anti-goat IgG (Santa Cruz). The signal was visualised using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL), according to the manufacturer's instructions.

Microscopy

C. albicans cells were grown at 30°C in YEPD overnight, diluted (1: 10) into fresh YEPD, grown to log phase prior to addition of 40 μg/ml doxycycline (Sigma),

and then grown for 3 h before being analysed by microscopy. The cells were collected and washed with sterile PBS prior to analysis. Both DIC and the green fluorescence images were assessed using a ZEISS Axioskop 2 microscope (Zeiss, Jena, Germany). Digital images were acquired using a MicroFirr digital camera (Olympus, Melville, NY) and processed using the imaging software Optronics PictureFrame 2.1 (Optronics, Goleta, CA). The micrographs were digitised and processed by the software Adobe Photoshop.

Results and discussion

Modification of a tetracycline-inducible gene expression system

The tetracycline-inducible gene expression system introduced in *C. albicans* in the current study originated from the system developed by Park and Morschhauser (Park and Morschhauser, 2005), in which a reverse tetracycline-controlled transactivator (rtTA) (Park and Morschhauser, 2005) and GFP (Morschhauser, et al., 1998) were adapted to the codon usage of *C. albicans*. The pTET25 plasmid with a *URA3* selection marker and the pNIM1 plasmid with a dominant *SAT1* selection marker were obtained from Park and Morschhauser. However, the inability to reuse the markers in the same system limits functional analysis of genes. To make the *URA3* selection marker in pTET25 recyclable, a Ura-blaster cassette (Wilson, et al., 2000)

was introduced into pTET25 that allows 5-FOA-induced intrachromosomal recombination. To facilitate further cloning procedures, subsequent cloning was made to generate pTET25M (Figure 1). This is the initial product of the modified version of pTET25. The pTET25M vector possesses *Sal*I and *Bgl*II restriction sites flanking the *GFP* coding sequence, which allow for the introduction of other restriction sites.

To verify the preservation of doxycycline-inducible GFP expression in pTET25M, both pTET25M and pTET25 were linearised by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura^+ prototrophs. Stable integrants with either pTET25 or pTET25M targeting the *ADH1* locus were verified by colony PCR with specific primers. Importantly, the level of doxycycline-induced GFP expression was comparable between the integrant of pTET25 (JSCA0050) and that of pTET25M (JSCA0051), as shown by the western blot results (Figure 2A). To determine whether doxycycline-inducible GFP expression in cells with *URA3* removed from the Ura-blaster cassette of pTET25M was preserved, the strain JSCA0051F without *URA3* was obtained from the strain JSCA0051 by 5-FOA treatment. It was then verified by yeast colony PCR. The ability of doxycycline-inducible GFP expression in JSCA0051F was verified (Figure 2A). Importantly, the induced GFP expression levels in JSCA0051 and JSCA0051F were comparable (Figure 2A). In addition, GFP expression levels were similar in both cells

of the integrants, as observed by fluorescence microscopy (Figure 4). These results suggest that no significant alteration in the regulation of GFP expression occurs in cells with the pTET25M vector in the absence of *URA3*.

To further analyse GFP expression in cells with pTET25M, two aspects of doxycycline inducibility, namely dose and time, were examined. Cells capable of expressing GFP either with *URA3* (JSCA0051) or without *URA3* (JSCA0051F) were grown in YEPD with 40 μg/ml doxycycline, and GFP expression was assessed at various time points, up to 36 h. GFP expression in both strains gradually increased until 24 h, after which time levels remained by and large the same until 36 h (Figure 2B), suggesting that the doxycycline-induced GFP expression in cells with pTET25M with or without *URA3* is controlled in a time-dependent manner. In addition, cells capable of expressing GFP (JSCA0051) were grown in YEPD with various amounts of doxycycline, and GFP expression was assessed at 3 h. The GFP expression in JSCA0051 cells exhibited a dose-dependent response, with a peak at 40 μg/ml and a dip at 2.5 μg/ml doxycycline (Figure 2C). Therefore, we concluded that the pTET25M vector carrying the Ura-blaster cassette was successfully established.

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

To enhance the application of a vector that lacks an adequate number of restriction sites, multiple restriction sites were introduced flanking the *GFP* coding sequence in the pTET25M vector to generate pTET25M-NC (Figure 1). In addition, by introducing multiple cloning sites at only one end, an accompanying plasmid, pTET25M-N, was also generated. To examine the preservation of doxycycline-inducible GFP expression in pTET25M-N and pTET25M-NC, both plasmids were linearised by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura^+ prototrophs. The stable integrants with either pTET25M-N (JSCA0052) or pTET25M-NC (JSCA0053) targeting the *ADH1* locus were verified by colony PCR with specific primers. The ability of doxycycline-inducible GFP expression was verified (Figure 3A) and was found to be comparable to that of the JSCA0051 strain carrying pTET25M (Figure 2A).

The *C. albicans* genes *CDC10* and *CDC11* were chosen to validate the preservation of functional GFP fusion proteins expressed from pTET25M-NC. The *CDC10* gene was cloned into pTET25M-NC at the *Aat*II and *Bgl*II sites to obtain pTET25M-GFP-CDC10, capable of encoding an N-terminal GFP fusion Cdc10 protein. The *CDC11* gene was cloned into pTET25M-NC at the *Nhe*I and *Xho*I sites to obtain pTET25M-CDC11-GFP, capable of encoding a C-terminal GFP fusion Cdc11 protein. The two plasmids were then linearised by *Sac*II and *Kpn*I and introduced into

the *C. albicans* strain BWP17 for Ura^+ prototrophs. The stable integrants with either pTET25M-GFP-CDC10 (JSCA0063) or pTET25M-CDC11-GFP (JSCA0064) targeting the *ADH1* locus were confirmed by colony PCR with specific primers. Significantly, the expression of both the N- and C-terminal GFP fusion proteins was doxycycline-dependent (Figure 3A).

To assess the preservation of doxycycline-inducible expression of GFP fusion proteins in cells with *URA3* removed from the Ura-blaster cassette of pTET25M-NC, the JSCA0063F and JSCA0064F strains, which had *URA3* removed, were obtained from the JSCA0063 and JSCA 0064 strains by treatment with 5-FOA and were verified by yeast colony PCR. The ability of doxycycline-inducible expression of GFP fusion proteins in the JSCA0063F and JSCA0064F strains was verified and was comparable to that of the parental strains with *URA3* (data not shown). This suggests that the doxycycline-inducible expression of either N-terminal or C-terminal GFP fusion proteins is not affected by the absence of *URA3*.

To extend the application of pTET25M, the vector system expressing an epitope-tagged protein rather than a *GFP* fusion protein was developed. The *GFP* coding sequence on pTET25M was replaced by DNA sequence encoding Cdc10 with a 6×His tag at the C-terminus to obtain pTET25M-CDC10-CH (Figure 1). Several restriction sites, in addition to the cloning sites of *Sal*I and *Bgl*II, were also introduced

at either end of *CDC10* (Figure 1) for future application to other genes. The pTET25M-CDC10-CH plasmid was linearised by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura^+ prototrophs. The stable integrant with pTET25M-CDC10-CH targeting the *ADH1* locus (JSCA0073) was confirmed by colony PCR with specific primers. The ability of doxycycline-inducible expression of Cdc10 with a C-terminal 6×His tag in JSCA0073 cells was confirmed by western blot (Figure 3B). Hence, we concluded that the modified pTET25M was capable of expressing a C-terminal 6×His protein.

To assess the functionality of the GFP fusion proteins, the localisation of the N-terminal GFP fusion of Cdc10 (GFP-Cdc10) in JSCA0063 cells and C-terminal GFP fusion of Cdc11 (Cdc11-GFP) in JSCA0064 cells was examined by fluorescence microscopy. Green fluorescence was observed in the cytoplasm of JSCA0051 cells expressing GFP alone (Figure 4). In contrast, green fluorescence was clearly present at the septum between two cells and in the cytoplasm of JSCA0063 and JSCA0064 cells expressing GFP-Cdc10 and Cdc11-GFP, respectively (Figure 4). In addition, the absence of *URA3* in JSCA0063F and JSCA0064F cells did not significantly alter the distribution and intensity of the green fluorescence (data not shown). Therefore, we concluded that pTET25M-NC enables doxycycline-inducible expression of functional GFP fusion proteins.

Potential applications

The pTET25M-NC vector allows the construction of strains expressing either N- or C-terminal fluorescence fusion proteins for purification, detection, and localisation studies. The ability to express both N- and C-terminal GFP fusion proteins can be critical. In many cases, GFP fusion at one terminus disrupts proper localisation of the protein of interest while a GFP tag at the other terminus maintains proper localisation. In addition, numerous restriction sites at either end of *GFP* on the pTET25M-NC vector provide multiple options for gene cloning. Furthermore, the doxycycline-inducible system can overexpress protein at a higher level compared to other regulatory expression systems (Park and Morschhauser, 2005). Hence, this newly-developed doxycycline-inducible system enables visualisation of the fusion protein when protein expression levels from the native promoter are too low to be detected. One extension of this system is that the *GFP* can be replaced by the *C. albicans* adapted *CFP* and *YFP* (Gola, et al., 2003), mCherry (Keppler-Ross, et al., 2008), DsRFP (Gerami-Nejad, et al., 2009), or other fluorescent protein-encoding genes developed in the future.

To fully explore the application of this newly developed system, pTET25M-CDC10-CH was constructed, in which Cdc10 is expressed as a C-terminal 6×His-tagged protein when the vector is introduced into *C. albicans* with doxycycline

induction. The presence of several cloning sites flanking the *CDC10* coding sequence on pTET25M-CDC10-CH allows *CDC10* to be replaced by other genes. Such tagged proteins can also be used for isolation, detection, and localisation studies in concert with the GFP fusion proteins. The epitope-tagged proteins may reflect the function of the non-tagged native proteins more accurately than GFP fusion proteins because the relatively larger size of GFP compared to the 6×His epitope makes it more likely to interfere with the protein function. Moreover, the system allows titration of doxycycline to monitor protein expression in conjunction with the phenotypes of either the physiological behaviour of proteins when expressed near the native level or the perturbed performance of proteins when significantly overexpressed. Furthermore, the system includes a recyclable mini-Ura-blaster cassette (Wilson, et al., 2000). The presence of the Ura-blaster cassette in the plasmid allows it to be introduced into *C. albicans* consecutively with another plasmid carrying a *URA3* selection marker, facilitating the functional assessment of protein-protein interactions.

We believe that there is room for further improvement in this system. For example, an N-terminal 6×His epitope and other epitope tags on either the N- or C-terminus should be introduced to make the system universally applicable in terms of assessing protein-protein interactions. Moreover, in the current system, the *ADH1* locus is the sole integration site from which constitutive rtTA expression is driven. It

is desirable to have an additional gene locus for integration and for constitutively driving rtTA expression. A system capable of targeting two loci combined with the doxycycline-inducible expression of two proteins that are fused with different fluorescent markers or epitope tags could be used to reveal protein-protein interactions. Furthermore, because the Ura-blaster cassette can only be used to recycle the *URA3* marker, it is worth introducing more common systems, such as *FLP/FRT* (Reuss, et al., 2004) or Cre/*loxP* (Dennison, et al., 2005), into the current plasmid with different selection markers to allow excision of different selection markers for the functional analysis of several gene products simultaneously.

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Figure legends

Figure 1. Maps of the pTET25M vector and its derivatives. Only the *KpnI*/*Sac*II DNA cassettes, which are based on the pBluescript, used to integrate into the *ADH1* locus of *C. albicans* are shown. The restriction sites shown on pTET25M are those that are relevant to its construction. The sequences shown under pTET25M-NC and pTET25M-CDC10-CH are relevant multiple cloning sites, featuring the unique restriction sites, the reading frames, and the flanking sequences. The gene name followed by a small letter "*p*" represents promoter, whereas that followed by a small letter "*t*" donates transcription termination sequence. *URA3-dpl200* contains a functional *C. albicans URA3* gene with an extra downstream sequence upstream of the *URA3* gene. *GFP* and *rTetR* genes use the *C. albicans*-adapted codons. *TetO* contains a fusion of the minimal promoter of *OP4* gene and an operator sequence capable of binding the *rTetR*-encoded protein in the presence of doxycycline to induce gene expression.

Figure 2. Western blotting analysis to assess protein expression of *C. albicans* strains based on the cassette of the pTET25M vector. (**A**) The doxycycline-induced expression of GFP in the strain with (JSCA0051) or without (JSCA0051F) *URA3*. The cells were grown in YEPD to log phase, and the protein was induced by adding 40

μg/ml doxycycline for 3 h before western blot analysis. The strain JSCA0050 carrying the original cassette of the pTET25 vector was used as a positive control. The doxycycline-induced state is designated as "+"; the non-induced state is designated as "–". Two independent isolates (JSCA0051F11 and JSCA0051F22) of the strains without *URA3* were examined. (**B**) Demonstration of the time-dependent manner of doxycycline-induced protein expression. Both the JSCA0051 and JSCA0051F (without *URA3*) strains were examined in parallel. (**C**) Demonstration of the dose-dependent manner of doxycycline-induced protein expression. JSCA0051 cells were grown in YEPD to log phase before adding the indicated concentration of doxycycline to induce protein expression for 3 h. Arrows indicate the predicted positions to which the GFP proteins migrated.

Figure 3. Western blotting analysis to assess protein expression in *C. albicans* strains based on the cassettes of plasmids derived from pTET25M. (**A**) Assessment of ability of the JSCA0063 and JSCA0064 strains to express N-terminal GFP tagged Cdc10 and C-terminal GFP tagged Cdc11, respectively. The strains JSCA0052 and JSCA0053 carrying cassettes from plasmids pTET25M-N and pTET25M-NC, respectively, from which the plasmids for the strains JSCA0063 and JSCA0064 were derived were also assessed. (**B**) Assessment of the JSCA0073 strain in expressing C-terminal 6×His

epitope-tagged Cdc10, along with the wild-type strain BWP17. The cells were grown in YEPD to log phase, and protein expression was induced by adding 40 μg/ml doxycycline 3 h before western blot analysis. Arrows indicate predicted positions to which the relevant proteins migrated.

Figure 4. Assessment of protein expression of the representative *C. albicans* strains by fluorescence microscopy. Cells of the strain expressing N-terminal GFP fusion Cdc10 (JSCA0063) and C-terminal GFP fusion Cdc11 (JSCA0064) with those expressing GFP with or without *URA3* (JSCA0051 or JSCA0051F) were grown in YEPD to exponential phase before adding 40 μg/ml doxycycline to induce protein expression for 3 h. The emission of green fluorescence alongside the DIC images was examined microscopically. The representative cells in which the GFP fusion proteins are localized to the septin complex at the mother-daughter junctions are indicated with white arrows. The bar represents 10 μm.

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

Name of the strain	Parental strain	Genotype	Plasmid	Protein expressed	Reference
BWP17		*ura3::imm434/ura3:imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	None	None	(Wilson, et al., 1999)
JSCA0050	BWP17	*ADH1/adh1::Ptet-GFP:URA3*	pTET25	GFP	This study
JSCA0051	BWP17	*ADH1/adh1::Ptet-GFP:URA3-dpl200*	pTET25M	GFP	This study
JSCA0051F	JSCA0051	*ADH1/adh1::Ptet-GFP:dpl200*	pTET25M	GFP	This study
JSCA0052	BWP17	*ADH1/adh1::Ptet-GFP:URA3-dpl200*	pTET25M-N	GFP	This study
JSCA0053	BWP17	*ADH1/adh1::Ptet-GFP:URA3-dpl200*	pTET25M-NC	GFP	This study
JSCA0063	BWP17	*ADH1/adh1::Ptet-GFP-CDC10:URA3-dpl200*	pTET25M-GFP-CDC10	N-terminal GFP fusion Cdc10	This study
JSCA0063F	JSCA0063	*ADH1/adh1::Ptet-GFP-CDC10:dpl200*	pTET25M-GFP-CDC10	N-terminal GFP fusion Cdc10	This study
JSCA0064	BWP17	*ADH1/adh1::Ptet-CDC11-GFP:URA3-dpl200*	pTET25M-CDC11-GFP	C-terminal GFP fusion Cdc11	This study
JSCA0064F	JSCA0064	*ADH1/adh1::Ptet-CDC11-GFP:dpl200*	pTET25M-CDC11-GFP	C-terminal GFP fusion Cdc11	This study
JSCA0073	BWP17	*ADH1/adh1::Ptet-CDC10:URA3-dpl200*	pTET25M-CDC10-CH	C-terminal 6×His tag Cdc10	This study

Table 2. Table 2. Synthetic oligonucleotide primers used in this study

Name	Primer Sequence
CaURA3-dpl200 *Bam*HI	AAT***GGATCC***CCAGATATTGAAGGTAAAAGG
CaURA3-dpl200 *Xho*I	ATT***CTCGAG***CTAGAAGGACCACCTTTGAT
TET25M *Kpn*I	CAA***GGTACC***GAACCATCGTGAGTGTAA
TET25M *Bam*HI	GAA***GGATCC***CGACATTTTATGATGGAA
CaGFP-N-MCS	AATT*GTCGACTCGAGATATCCAGCTAGCGGCCGC*GTGAGCATGAGTAAGGGAGAAGAAC
CaGFP-C-MCS	CCG*TGATCA*TTATGC*AGGCCTAGATCTTAAG*CTG*ACGTCCGGA*CCTTTGTATAGTTCATCCATGCC
CaGFP-MCS-R	CTCTAGTTTTGACGCTCG
pNIM1-inte detect F	CATGTCAAAGGATTCAAC
pNIM1-inte detect R	GTATGGTGCCTATCTAAC
CaCDC10 AatII	ATCA***GACGTC***ATGTCCATCGAAGAACCTAGT
CaCDC10 BglII	ACGC***AGATCT***TTATCTAGCAGCAGCAGTACC
CaCDC11 NheI	CTACTA***GCTAGC***GATTACTTTTACTTCATTTTCTG
CaCDC11 XhoI	CCGCCG***CTCGAG***ATGAATTATTCTACTGAAAATGT
CaCDC10-Tag-SalI	AAA***GTCGAC***GTCAGCGGCCGCATGTCCATCGAAGAACCTAGTAC
CaCDC10-6xHis-BclI	CGA***TGATCA***TTAATGGTGATGATGGTGATGCCCGGGAGATCTTCTAGCAGCAGCAGTACCTGTAG

Note: Sequences in bold italics donate sites of restriction enzymes.

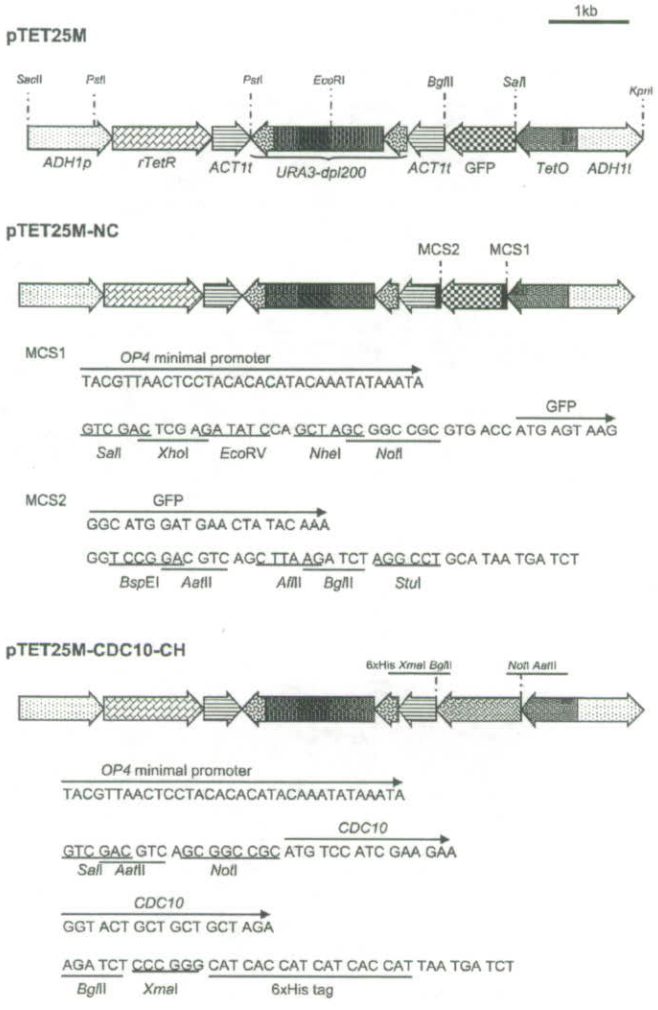

Figure 1. Maps of the pTET25M vector and its derivatives. Only the *Kpn*I/*Sac*II DNA cassettes, which are based on the pBluescript, used to integrate into the *ADH1* locus of *C. albicans* are shown. The restriction sites shown on pTET25M are those that are relevant to its construction. The sequences shown under pTET25M-NC and pTET25M-CDC10-CH are relevant multiple cloning sites, featuring the unique restriction sites, the reading frames, and the flanking sequences. The gene name followed by a small letter "*p*" represents promoter, whereas that followed by a small letter "*t*" donates transcription termination sequence. *URA3-dpl200* contains a functional *C. albicans URA3* gene with an extra downstream sequence upstream of the *URA3* gene. *GFP* and *rTetR* genes use the *C. albicans*-adapted codons. *TetO* contains a fusion of the minimal promoter of *OP4* gene and an operator sequence capable of binding the *rTetR*-encoded protein in the presence of doxycycline to induce gene expression.

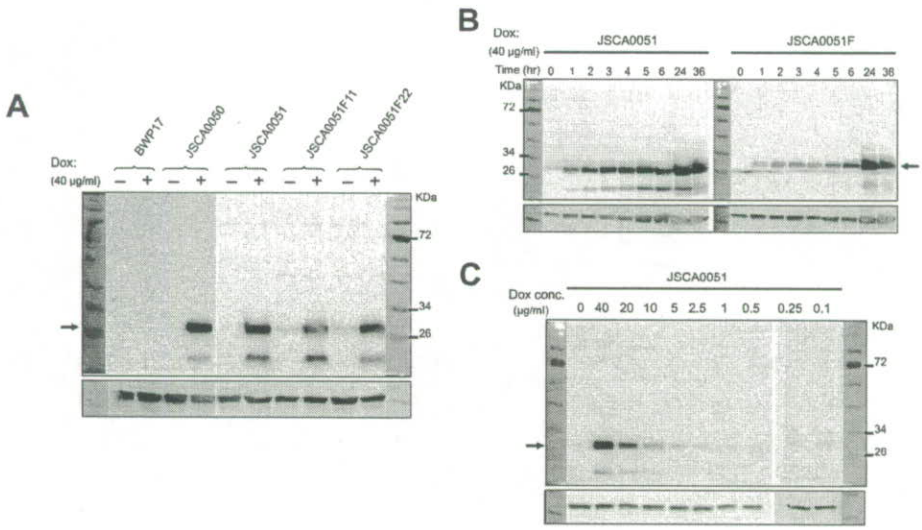

Figure 2. Western blotting analysis to assess protein expression of *C. albicans* strains based on the cassette of the pTET25M vector. (**A**) The doxycycline-induced expression of GFP in the strain with (JSCA0051) or without (JSCA0051F) *URA3*. The cells were grown in YEPD to log phase, and the protein was induced by adding 40 µg/ml doxycycline for 3 h before western blot analysis. The strain JSCA0050 carrying the original cassette of the pTET25 vector was used as a positive control. The doxycycline-induced state is designated as "+"; the non-induced state is designated as "–". Two independent isolates (JSCA0051F11 and JSCA0051F22) of the strains without *URA3* were examined. (**B**) Demonstration of the time-dependent manner of doxycycline-induced protein expression. Both the JSCA0051 and JSCA0051F (without *URA3*) strains were examined in parallel. (**C**) Demonstration of the dose-dependent manner of doxycycline-induced protein expression. JSCA0051 cells were grown in YEPD to log phase before adding the indicated concentration of doxycycline to induce protein expression for 3 h. Arrows indicate the predicted positions to which the GFP proteins migrated.

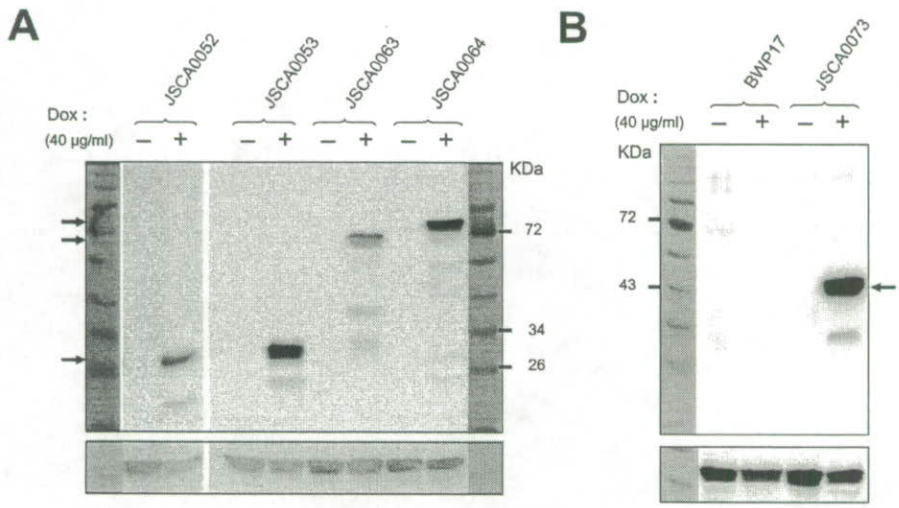

Figure 3. Western blotting analysis to assess protein expression in *C. albicans* strains based on the cassettes of plasmids derived from pTET25M. (**A**) Assessment of ability of the JSCA0063 and JSCA0064 strains to express N-terminal GFP tagged Cdc10 and C-terminal GFP tagged Cdc11, respectively. The strains JSCA0052 and JSCA0053 carrying cassettes from plasmids pTET25M-N and pTET25M-NC, respectively, from which the plasmids for the strains JSCA0063 and JSCA0064 were derived were also assessed. (**B**) Assessment of the JSCA0073 strain in expressing C-terminal 6×His epitope-tagged Cdc10, along with the wild-type strain BWP17. The cells were grown in YEPD to log phase, and protein expression was induced by adding 40 µg/ml doxycycline 3 h before western blot analysis. Arrows indicate predicted positions to which the relevant proteins migrated.

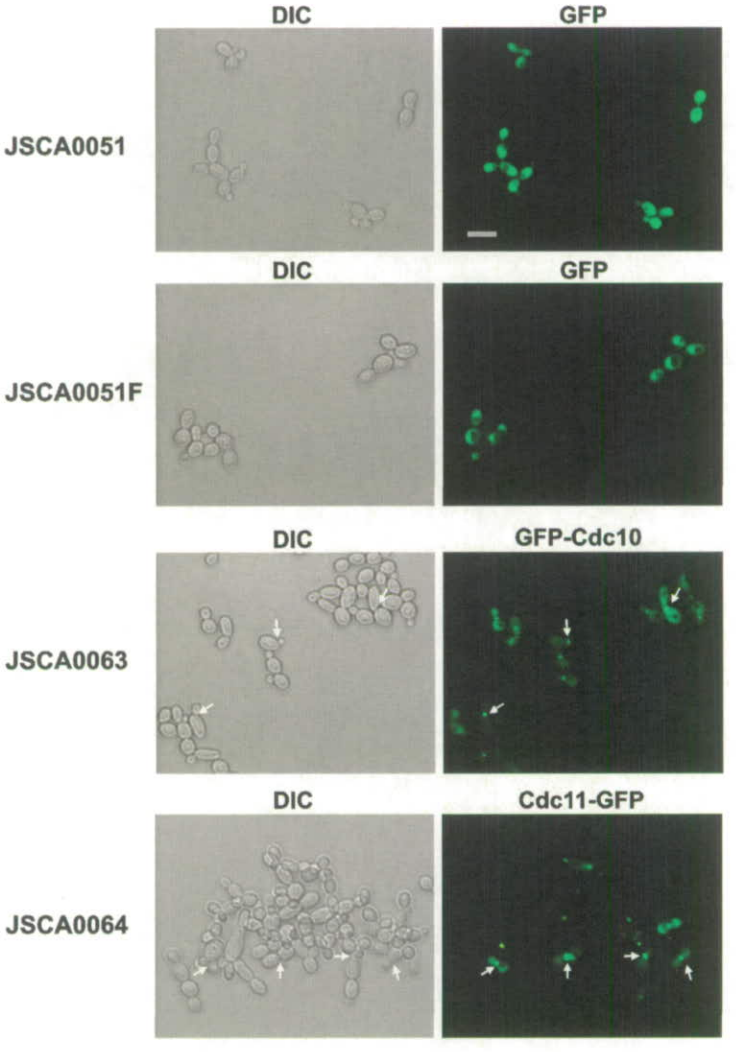

Figure 4. Assessment of protein expression of the representative *C. albicans* strains by fluorescence microscopy. Cells of the strain expressing N-terminal GFP fusion Cdc10 (JSCA0063) and C-terminal GFP fusion Cdc11 (JSCA0064) with those expressing GFP with or without *URA3* (JSCA0051 or JSCA0051F) were grown in YEPD to exponential phase before adding 40 µg/ml doxycycline to induce protein expression for 3 h. The emission of green fluorescence alongside the DIC images was examined microscopically. The representative cells in which the GFP fusion proteins are localized to the septin complex at the mother-daughter junctions are indicated with white arrows. The bar represents 10 µµm.

Dissecting *Candida albicans* Cdc4 protein reveals its essential domains involved in morphogenesis and cell flocculation

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Running title: *Candida albicans* Cdc4 domains instability flocculation

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Abstract

Candida albicans CDC4 (*CaCDC4*), encoding an F-box protein of ubiquitin E3 ligase, suppresses 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)-inducibly 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 the 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 each of these domains in the absence of full-length *Ca*Cdc4 exhibited non-discriminatory phenotypes of filamentation and flocculation. Notably, the Dox-induced expression of *Ca*Cdc4 decreased flocculation rather than completely overturned filamentation in the strain with *CaMET3-CaCDC4* repressed, which is likely the characteristics of systems regulated by the Tet-on and *CaMET3*, and the nature of *Ca*Cdc4 protein. Consequently, we suggest a novel function of *CaCDC4* for negatively controlling flocculation that is independent of filamentation.

Keywords:*Candida albicans*; *CDC4*; *MET3*; domain; Tet-on; flocculation

Introduction

Candida albicans, a natural diploid without complete sexual cycle, exists as yeast, pseudohyphal, and hyphal cells (Whiteway and Bachewich, 2007). It is capable of morphological switch by environmental stimuli (Biswas, *et al.*, 2007), essentially via cAMP-mediated and MAPK signalling pathways (Liu, 2001). Importantly, its ability to alter among cell types is associated with virulence to human (Lo, *et al.*, 1997). Cyclins are also known to control morphogenesis in *C. albicans* (Berman, 2006). Whereas G1 cyclin of Hgc1 expresses in hyphae (Zheng and Wang, 2004), that of Ccn1 is essential for hyphal development (Loeb, *et al.*, 1999). By contrast, Cln3 suppresses filamentation as depleting Cln3 causes germ tube extension (Bachewich and Whiteway, 2005, Chapa y Lazo, *et al.*, 2005).

Recently, F-box proteins encoded by *C. albicans GRR1* (*CaGRR1*) and *CaCDC4* have been shown to play a role in filamentous development (Atir-Lande, *et al.*, 2005, Butler, *et al.*, 2006, Shieh, *et al.*, 2005). 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 ubiquitin-proteasome dependent degradation of regulatory proteins in eukaryotes (Hochstrasser, 1996). 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 (Willems, *et al.*, 1999), 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 (Feldman, *et al.*, 1997, Skowyra, *et al.*, 1997) and Far1 (Henchoz, *et al.*, 1997), 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 (Drury, *et al.*, 1997) and Gcn4 for control of amino acid biosynthesis(Kornitzer, *et al.*, 1994), 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* (Shieh, et al., 2005), the function of *Ca*Cdc4 and ScCdc4 are dissimilar. While depleting *C. albicans* Grr1 (*Ca*Grr1) stabilizes Ccn1p and Cln3 for pseudohyphal growth (Butler, et al., 2006, Li, *et al.*, 2006), depleting *Ca*Cdc4 causes accumulation of Sol1 (Sic1 1like) for hyphal development (Atir-Lande, et al., 2005), 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 (Atir-Lande, et al., 2005), direct evidence has yet to be established. Additionally, mutants of *Cacdc4* null and *Cacdc4 sol1* double null were comparably filamentous, refuting Sol1 being 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 by *CaMET3* with 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 triggered flocculation, suggesting a novel role of *CaCDC4* for suppressing 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 (Miller, 1972) 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*) (Wilson, *et al.*, 1999). 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 (Sherman, *et al.*, 1986). 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 *CaCDC4* expression controlled by *CaMET3*, 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 DNA cassettes were introduced into *C. albicans* by the lithium acetate method as described (Walther and Wendland, 2003).

Construction of C. albicans strains. A strain whose *CaCDC4* expression is 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 expressing each of cassettes encoding assorted *Ca*Cdc4 domains in *C. albicans*, a Tet-on plasmid, pTET25M, derived from pTET25 (Park and Morschhauser, 2005) 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 for cloning into pTET25M in which pTET25M-CaCDC4 was generated. Moreover, *CaCDC4*-6HF, encoding 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 primer sets 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 (Shieh, *et al.*, 2007). 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 (Wilson, *et al.*, 2000) 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 studying 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, most cells of JSCA0023, JSCA0024, and JSCA0025 still grew as elongated type, even though not o the extent of filament as 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 (Atir-Lande, et al., 2005, Shieh, et al., 2005) and of *cacdc4* null (Atir-Lande, et al., 2005). 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 (Galan and Peter, 1999). 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 (Bertram, *et al.*, 1996).

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 and that N-terminal region (1-85) is unnecessary 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.

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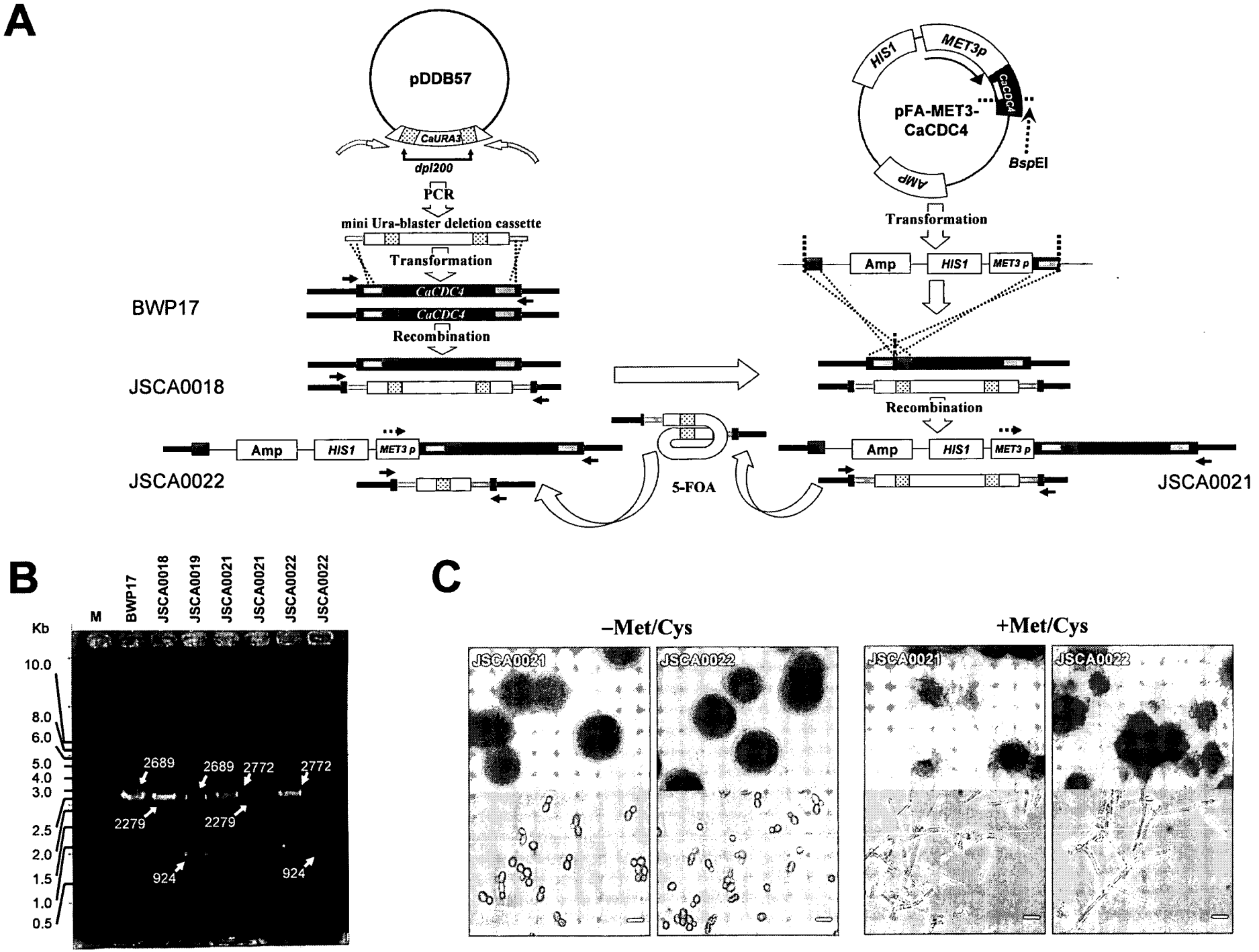
Figure legends

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 *CaURA3* removal 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 MET-F and CaCDC4 locus R were used to PCR-generate a 2772-bp amplicon for verifying 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 (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 the constructed strains. Cells of strains JSCA0021 and JSCA0022 were grown on SD medium or plates with (+) or without (–) Met/Cys. Colonies were photographed with MEIJI stereoscopic microscope EMZ5 at 40× magnification (top panel). Cells in liquid culture were recorded with Nikon 50i microscope at 400× magnification (bottom panel). Bars represent 10 μm.

Fig. 2. Construction of *C. albicans* strains for Dox-inducibly expressing assorted *CaCDC4* domains. (**A**) Schematic representation of *Ca*Cdc4 domains expressed from the Tet-on system. (**B**) Generation of Tet-on cassettes for expressing assorted CaCdc4 domains. Different portions of *CaCDC4* were PCR-generated with primer sets (Table 2) containing common *Aat*II and *Xho*I sites for replacing full-length *CaCDC4* on pTET25M-CaCDC4-6HF as described in the Material and Methods. By digestion with *Sac*II and *Kpn*I, each cassette 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-inducibly expressing 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-inducibly expressing 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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CaURA3
dpl200
PCR
mini Ura-blaster deletion cassette
Transformation
BWP17
CaCDC4
CaCDC4
Recombination
JSCA0018
Amp
HIS1
MET3 p
JSCA0022
5-FOA
pFA-MET3-CaCDC4
HIS1
MET3p
CaCDC4
BspEI
AMP
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
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924
924
C
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+Met/Cys
JSCA0021
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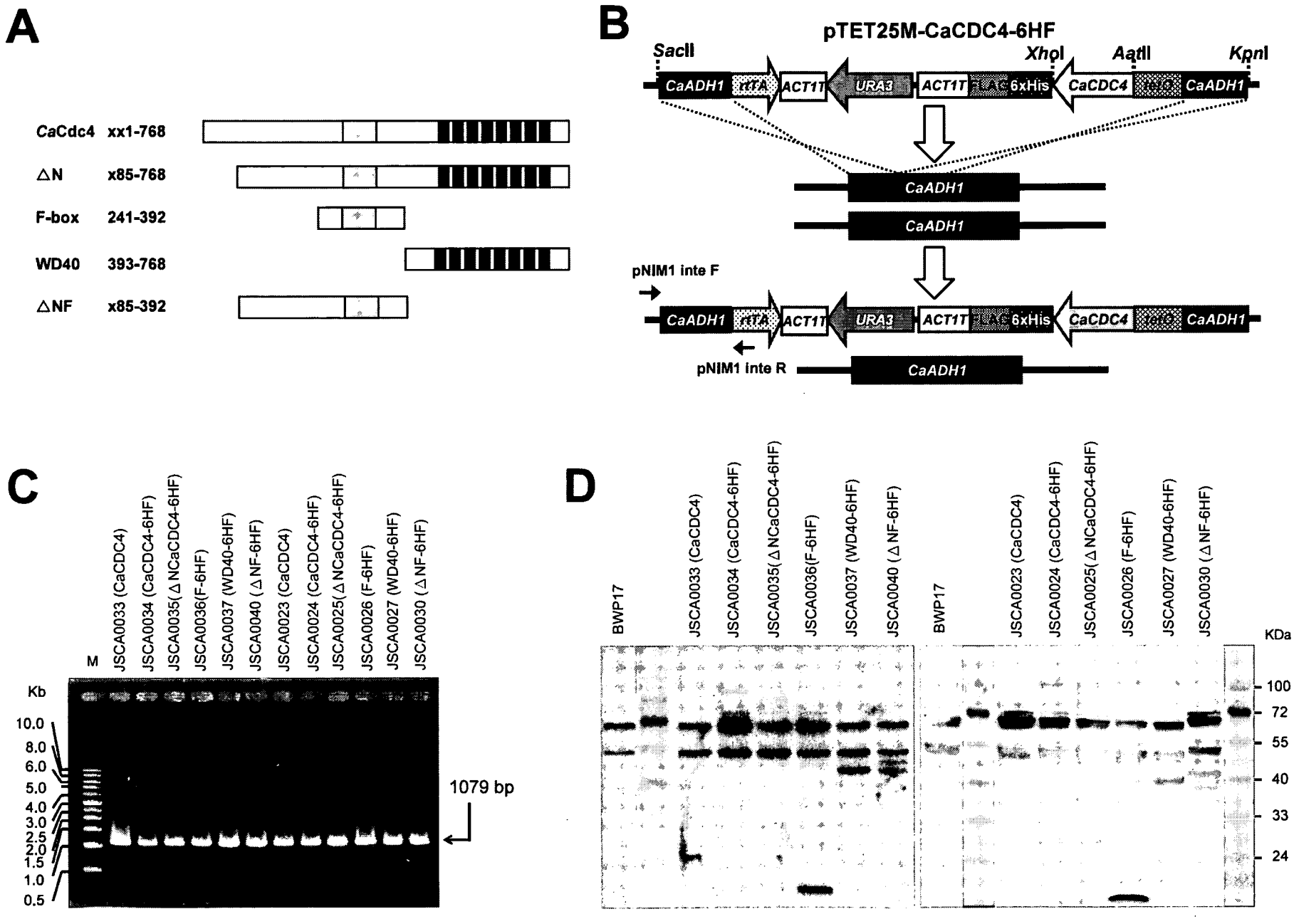
JSCA0021
JSCA0022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F-box 241-392
WD40 393-768
ΔNF x85-392
B
pTET25M-CaCDC4-6HF
SacII
XhoI
AatII
KpnI
CaADH1
rtTA
ACT1T
URA3
ACT1T
FLAG
6xHis
CaCDC4
tetO
CaADH1
CaADH1
CaADH1
pNIM1 inte F
CaADH1
rtTA
ACT1T
URA3
ACT1T
FLAG
6xHis
CaCDC4
tetO
CaADH1
pNIM1 inte R
CaADH1
C
M
JSCA0033 (CaCDC4)
JSCA0034 (CaCDC4-6HF)
JSCA0035(ΔNCaCDC4-6HF)
JSCA0036(F-6HF)
JSCA0037 (WD40-6HF)
JSCA0040 (ΔNF-6HF)
JSCA0023 (CaCDC4)
JSCA0024 (CaCDC4-6HF)
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JSCA0026 (F-6HF)
JSCA0027 (WD40-6HF)
JSCA0030 (ΔNF-6HF)
Kb
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8.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5
1079 bp
D
BWP17
JSCA0033 (CaCDC4)
JSCA0034 (CaCDC4-6HF)
JSCA0035(ΔNCaCDC4-6HF)
JSCA0036(F-6HF)
JSCA0037 (WD40-6HF)
JSCA0040 (ΔNF-6HF)
BWP17
JSCA0023 (CaCDC4)
JSCA0024 (CaCDC4-6HF)
JSCA0025(ΔNCaCDC4-6HF)
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KDa
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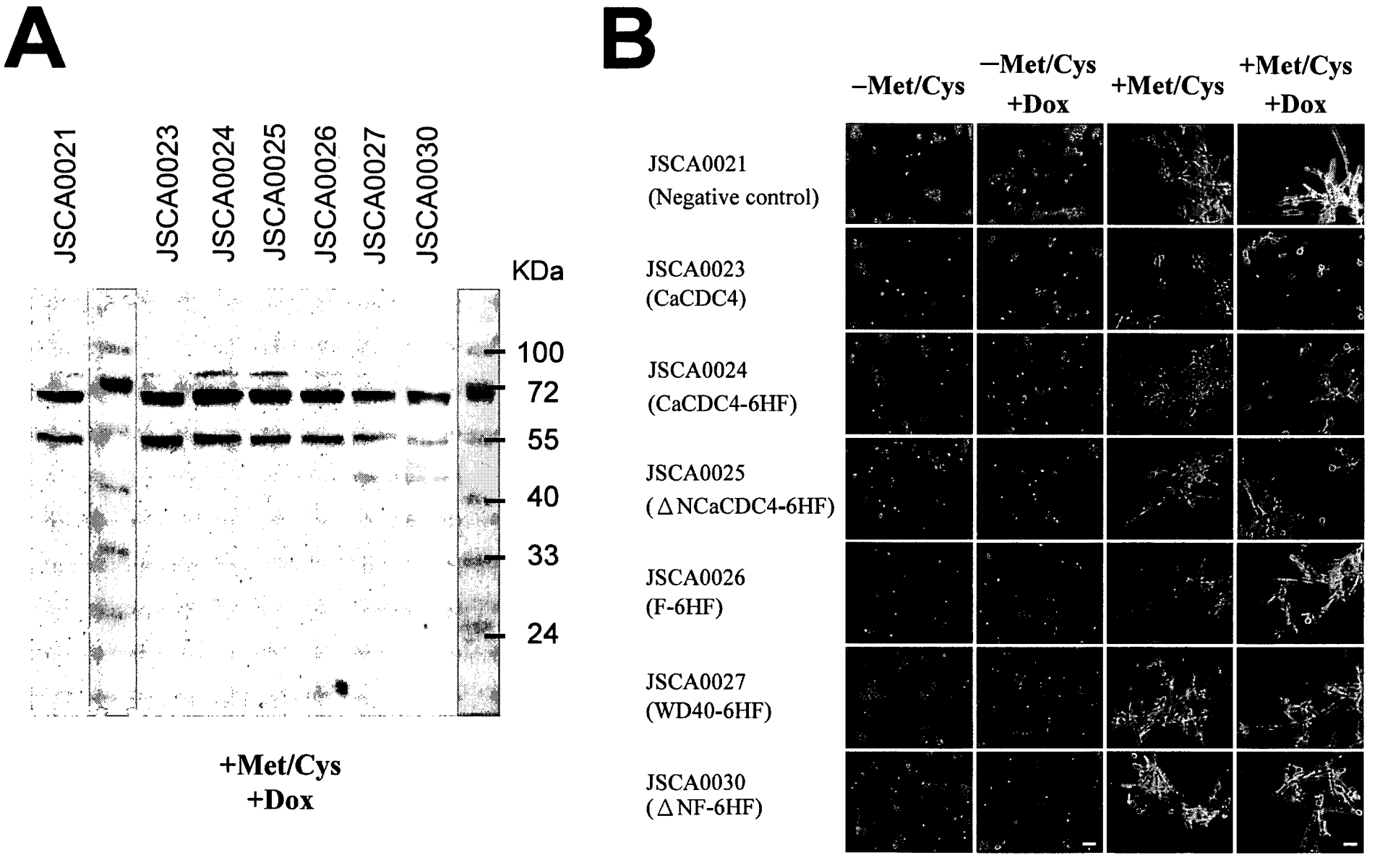
55
40
33
24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JSCA0023
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JSCA0025
JSCA0026
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KDa
100
72
55
40
33
24
+Met/Cys
+Dox
B
–Met/Cys
–Met/Cys
+Dox
+Met/Cys
+Met/Cys
+Dox
JSCA0021
(Negative control)
JSCA0023
(CaCDC4)
JSCA0024
(CaCDC4-6HF)
JSCA0025
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(WD40-6HF)
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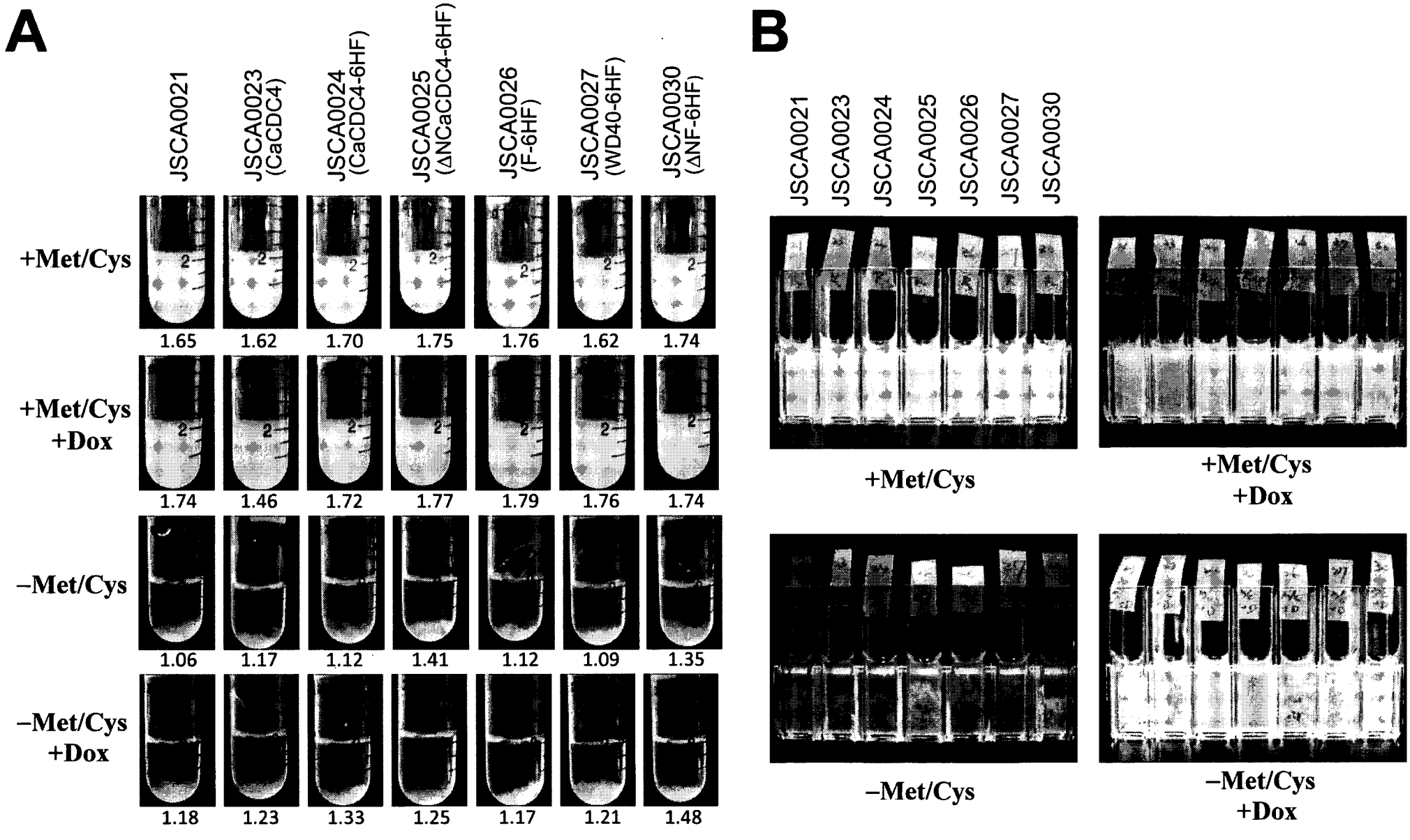
(ΔNF-6HF)


A
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JSCA0023 (CaCDC4)
JSCA0024 (CaCDC4-6HF)
JSCA0025 (ΔNCaCDC4-6HF)
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