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

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Abstract

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

Results: To further elucidate the function of CaCDC4, a C. albicans strain, with one CaCDC4 allele deleted and the other under the repressible C. albicans MET3 promoter (CaMET3p) control, was made before introducing cassettes capable of doxycycline (Dox)-induced expression of various C. albicans Cdc4 (CaCdc4) domains. Cells from each strain could express a specific CaCdc4 domain under Dox-induced, but CaMET3-CaCDC4 repressed conditions. Cells expressing domains without either the F-box or WD40-repeat exhibited filamentation and flocculation similarly to those lacking CaCDC4 expression, indicating the functional essentiality of the F-box and WD40-repeat. Notably, cells expressing the N-terminal 85-amino acid truncated CaCdc4 partially reverse the filament-to-yeast and weaken the ability to flocculate compared to those expressing the full-length CaCdc4, suggesting that N-terminal 85-amino acid of CaCdc4 regulates both morphogenesis and flocculation.

Conclusions: The F-box and the WD40-repeat of CaCdc4 are essential in inhibiting yeast-to-filament transition and flocculation. The N-terminal region (1–85) of CaCdc4 also has a positive role for its function, lost of which impairs both the ability to flocculate and to reverse filamentous growth in C. albicans.

Keywords: Candida albicans, CDC4 domains, Morphogenesis, Flocculation

Background

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

Recently, an F-box protein encoded C. albicans CDC4 (CaCDC4) has been shown to play a role in filamentous development [6,7]. Cdc4, originally identified in the budding yeast Saccharomyces cerevisiae, encodes ubiquitin E3 ligases, which belongs to a member of the Skp1-Cdc53/Cul1-F-box (SCF) complex. This complex is known to play a role in ubiquitin-proteasome dependent degradation of regulatory proteins in eukaryotes [8]. A specific SCF complex is designated by its associated F-box protein. This protein is variable with two interacting domains of F-box for Skp1 and WD40-repeat (or LRR) for specific substrates [9], such that Cdc4 can be named SCFCdc4. To progress through the G1-S transition in S. cerevisiae, SCFCdc4 is required to degrade Sic1 [10] and Far1 [11], which are the cyclin-dependent kinase inhibitors. Therefore, S. cerevisiae CDC4 (ScCDC4) is essential in S. cerevisiae.
Although CaCdc4 is a structural homolog of S. cerevisiae Cdc4 (ScCdc4) and is capable of rescuing the mitotic defect caused by the loss of ScCDC4 in S. cerevisiae [7], the functions of CaCdc4 and ScCdc4 are dissimilar as the null CacaCdc4 mutant is viable and the depletion of CaCdc4 causes the accumulation of Sol1 (Sic1 like) for hyphal development rather than initiation of cell cycle arrest [6]. This verifies that CaCDC4 is nonessential and suppresses filamentation and suggests that controlling the degradation on Sol1 in C. albicans by CaCdc4 is important for inhibition of filamentation. Therefore, while CaCdc4 is required for inhibition of filamentation, and histidine. Selection for the loss of the CaCDC4 URA3 (CaURA3) marker was performed on plates with 50 µg/ml uridine and 1 mg/ml 5-fluoroorotic acid (5-FOA, MD Bio). To repress the CaCDC4 expression that was controlled by CaMET3p, strains were grown on SD medium or on plates with 2.5 mM Met/Cys, which has been shown to optimally switch off the expression of the CaMET3p-driven downstream gene [16]. To induce gene expression under the Tet-on system, 40 µg/ml Dox (Sigma) was added to YEPD or SD media.

**Plasmid DNA manipulation**

Plasmid DNA was extracted routinely from *E. coli* cultures using Gene-Spin™ MiniPrep purification Kit-V2 (PRO TECH, Taipei, Taiwan) and the instructions provided by the manufacturer. *E. coli* was transformed with plasmid DNA by using CaCl2. The DNA cassettes were introduced into *C. albicans* by the lithium acetate method as described previously [17].

**Construction of *C. albicans* strains**

Initially, a strain with repressed CaCDC4 expression was made. A mini-Ura-blaster cassette, flanked with 60-bp sequences homologous to *CaCDC4*, was PCR-amplified using a template of plasmid pDBS7 and long primers of CaCDC4-URA3-F and CaCDC4-URA3-R (Table 1). BWP17 was transformed by integration of the cassette into the *CaCDC4* locus to generate Ura+ strain JSCA0018. The plasmid pFA-HIS1-MET3p-CaCDC4, with a partial *CaCDC4* coding sequence for N-terminal CaCdc4 (1–563), was linearized with BspEI and used to transform JSCA0018 to generate His+ strain JSCA0021 (Figure 1A; Table 1). Cells of JSCA0021 were plated with 5-FOA to induce recombination between two copies of *dpl200* flanking the mini-Ura-blaster for a loss of *CaURA3* to generate JSCA0022.

To allow the expression of cassettes encoding assorted CaCdc4 domains in *C. albicans*, a Tet-on plasmid, pTET25M [18], which is derived from pTET25 [19] for inducing gene expression with Dox, has been developed. To regulate *CaCDC4* expression by the Tet-on system, the coding sequence of *CaCDC4* was PCR-amplified using plasmid CaCDC4-SBTA bearing *CaCDC4* (Lai WC, unpublished results), primers CaCDC4-Sall and CaCDC4-BglII (Table 2), and *Pfu* polymerase (5 U/µl, MD bio), digested with Sall and BglII for cloning into pTET25M, from which pTET25M-CaCDC4 was generated. Moreover, CaCDC4-6HF, which encodes 6×histidine and FLAG (6HF) tags at the C-terminal of CaCdc4, was PCR-amplified with primers CaCDC4-6HF Sall and CaCDC4-6HF BglII (Table 2), followed by digestion with Sall and BglII and cloning into pTET25M to obtain pTET25M-CaCDC4-6HF.

To define the function of the distinct CaCdc4 domains (Figure 2A), different *CaCDC4* portions were used to replace the full length *CaCDC4* coding sequence

**Methods**

**Strains and growth conditions**

*E. coli* strain DH5α was used for the routine manipulation of the plasmids. They were grown at 37°C in LB broth medium [13] or on plates containing 1.5% agar (Difco, BD Biosciences), with 50 µg/ml ampicillin or 30 µg/ml kanamycin. All *C. albicans* strains (Table 1) were derived from auxotrophic strain BWP17 (arg4/arg4 his1/his1 ural3/ural3) [14]. They were grown at 30°C in either yeast extract-peptone-dextrose (YPD) or supplemented minimal synthetic defined (SD) medium with 2% glucose with or without 2% agar [15]. While *Ura*+ prototrophs were selected on SD agar plates without uridine, His+ prototrophs were selected on SD plates without histidine. Selection for the loss of the *C. albicans URA3* (CaURA3) marker was performed on plates with
pTET25M-CaCDC4-6HF. By using the primer sets listed in Table 2, the following constructs were made: pTET25M-ΔNCaCDC4-6HF (with primers CaCDC4 ΔN AatII and CaCDC4 ΔN Xhol), which encodes the N-terminal truncated CaCdc4; pTET25M-F-6HF (with primers CaCDC4 F-box AatII and CaCDC4 F-box Xhol), which encodes the F-box domain with flanking regions; pTET25M-WD40-6HF (with primers CaCDC4 WD40 AatII and CaCDC4 ΔN Xhol), which encodes eight copies of WD40-repeat; and pTET25M-ΔNF-6HF (with primers CaCDC4 ΔN Xhol), which encodes truncated N-terminal CaCdc4 and the F-box domain. All inserts of the constructs were digested with SacI and KpnI, each of which was transformed into C. albicans for integration at the CaADH1 locus. All strains were verified by colony PCR with specific primers before subjecting to Southern blotting analysis.

**Southern blotting analysis**

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

**Protein extraction and Western blot analysis**

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

**Flocculation assay by low-speed centrifugation**

The cells of strains were streaked on YPD agar plate for 3 days and colonies were picked and inoculated into SD medium with required supplements for 48 hrs. Next, the cultures were diluted into fresh SD medium to 0.1 of an initial OD$_{600}$ with required supplements. To simultaneously repress the expression of CaMET3p-driven CaCDC4 and to induce the expression of various CaCDC4 segments encoding series of CaCdc4 domains, 2.5 mM Met/Cys and 40 μg/ml Dox were also added into the SD medium. After 48 hrs, the cultures were spun down for

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

| Strain name of the strain | Parental strain | Name relevant to genotype | Genotype |
|---------------------------|----------------|--------------------------|----------|
| BWP17                     |                | CaCDC4 +/-               | ura3::imm434/ura3::imm434 his1::hisG / his1::hisG arg4::arg4::arg4::hisG |
| JSCA0018                  | BWP17          | CaCDC4 +/-/U3–           | CaCDC4/cdc4::CaURA3::dp200 |
| JSCA0201                  | JSCA0018       | CaCDC4 M3/U3–            | Cacdc4::URA3::dp200/PMET3-CaCDC4::His |
| JSCA0022                  | JSCA0021       | CaCDC4 M3/–              | Cacdc4::dp200/PMET3-CaCDC4::His |
| JSCA0023                  | JSCA0022       | CaCDC4 M3/– | Tet-CaCDC4 | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/
| JSCA0024                  | JSCA0022       | CaCDC4 M3/– | Tet-CaCDC4-6HF | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/CaURA3 |
| JSCA0025                  | JSCA0022       | CaCDC4 M3/– | Tet-ΔN-6HF | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/ΔN-6HF/CaURA3 |
| JSCA0026                  | JSCA0022       | CaCDC4 M3/– | Tet-F-box-6HF | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/ΔN-6HF/CaURA3 |
| JSCA0027                  | JSCA0022       | CaCDC4 M3/– | Tet-WD40-6HF | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/ΔN-6HF/CaURA3 |
| JSCA0030                  | JSCA0022       | CaCDC4 M3/– | Tet-ΔNF-6HF | Cacdc4::dp200/PMET3-CaCDC4::His | CaADH1/adh1::PMET3-CaCDC4::HIS1/ΔN-6HF/CaURA3 |

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1 minute at 500 rpm, and the suspensions of the cultures were sampled to determine their optical density at OD600. Three independent assays were conducted and each sample was assayed in duplication. A paired Student t test with p < 0.05 was considered significant.

Ca²⁺-initiated flocculation assay

The FLO-encoded flocculins are known to be essential for flocculation in S. cerevisiae [21]. Functional homologues of FLO genes have been found in C. albicans. In particular, the important S. cerevisiae gene FLO11 responsible for flocculation has C. albicans functional counterpart ALS1 [22]. Since FLO11-associated flocculation is dependent on the presence of Ca²⁺, we adopted an alternative flocculation assay in which the rate of flocculation is initiated by Ca²⁺ and the optical density was assessed within a short time-frame [23]. Briefly, to initiate flocculation, an aliquot of 800 μl deflocculated cell suspension was transferred into a 1-ml cuvette, followed by addition of 200 μl of 100 mM CaCl₂. The cuvette was mixed robustly by pipetting and the absorbance (OD600) was assessed instantly at 30-s intervals for 5 minutes using a spectrophotometer (DU800, Beckman Coulter, Inc.). All assays were conducted in triplicate.

Figure 1 Construction of a C. albicans strain for repressibly expressing CaCDC4. (A) Strain construction (detailed in the Methods). The first CaCDC4 allele on BWP17 was deleted by mini-Ura-blaster to obtain JSCA0018. Plasmid pFA-HIS1-MET3p-CaCDC4 containing partial CaCDC4 coding sequence was linearized at a unique site for introducing into strain JSCA0018 to generate JSCA0021. 5-FOA was used to counter-select CaURA3 removal to obtain JSCA0022 for re-introducing the Tet-on plasmid with a CaURA3 marker. (B) Verification of constructed strains by Southern blotting analysis. Organization of the CaCDC4 locus with respect to NdeI sites is shown. The relative positions of the probe used and the predicted NdeI-digested pattern of the CaCDC4 locus are indicated. Two NdeI-fragments of 14 kb and 8.5 kb, specific to CaCDC4, could be detected in genomic DNA from BWP17 digested with NdeI; two NdeI-fragments of 14 kb and 8.5 kb, specific to CaCDC4 and Cacdc4::URA3-dpl200, respectively, could be detected in JSCA0018; two NdeI-fragments of 13.5 kb and 7.4 kb specific to Cacdc4::URA3-dpl200 and Cacdc4::PMET3-CDC4:HIS1, respectively, could be detected in JSCA0021; and two NdeI-fragments of 13.5 kb and 7.4 kb specific to Cacdc4::dpl200 and Cacdc4::PMET3-CDC4:HIS1, respectively, could be detected in JSCA0022. A non-specific NdeI-fragment is indicated as "*" and can be detected in all strains tested.
Tet-on plasmid cassette is introduced into the parent strain JSCA0018 to generate the JSCA0021 strain (Figure 1A; Table 1). This strain was further used to delete the second allele of the CaCDC4 gene directly in C. albicans BWP17 by mini-Ura-blaster [24]. To allow the introduction of Tet-on plasmid cassettes capable of expressing assorted genes directly in C. albicans, the JSCA0022 strain was created that had one CaCDC4 allele deleted and the other under CaMET3 control that was Met/Cys repressible. To allow the introduction of genes to their respective locations in the genome, the Tet-on system, allows us to study the function of expected organizations in their genome.

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

It has been shown that Ura” auxotrophic mutants are avirulent [25] and other virulence-associated features can be influenced by the level of CalURA3 gene expression [26]. To assess presence of CalURA3 having effect on yeast-to-filament transition, the yeast-to-filament transitions between strain JSCA0021 and JSCA0022 were compared, cells of those strains were assessed under CalMET3p repressed or de-repressed conditions. Cells of both strains on SD plates without Met/Cys grew as circular colonies with smooth surfaces (Figure 2). Under the microscope, these strains exhibited equivalent filamentous forms, suggesting a comparable ability to deplete CaCDC4 for expression and inability of CaCDC4 interfering with yeast-to-filament transition in C. albicans. Subsequently, JSCA0022 was used as a parental strain to introduce the Tet-on cassettes (with CalURA3 marker) that encoded assorted CaCdc4 domains.

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

The filamentous development of JSCA0022 under CalMET3p-CaCDC4 repressed conditions, with Met/Cys and the Tet-on system, allows us to study the function of the CaCdc4 domains. A set of Tet-on cassettes (obtained from pTET25M-CaCDC4-6HF, pTET25M-DN-6HF, pTET25M-F-box-6HF, pTET25M-WD40-6HF, and pTET25M-ΔNF-6HF) that encoded each of the
assorted domains of CaCdc4 (Figure 3A) were used to transform JSCA0022 (which contained a CaMET3p-repressible CaCDC4) to Ura⁺ by integration at the CaADH1 locus (Figure 3B). The correctness of the strains was confirmed by yeast colony PCR with specific primers before Southern blotting analysis. The CaADH1 locus from strain JSCA0022 could detect a SpeI-digested fragment with size of 3.3 kb (Figure 3C). The CaADH1 locus from strains JSCA0023 and JSCA0024 detected an increased SpeI-digested fragment of 9.4 kb due to the integration of Tet-on cassettes of either pTET25M-CaCDC4 or pTET25M-CaCDC4-6HF (Figure 3C). The CaADH1 locus from other strains also showed expected alteration in size according to the size of different CaCDC4 domains (Figure 3C). These results confirmed the correctness of the strains.

The JSCA0022 strain, which expressed the non-tagged and repressible CaCdc4, was used as a negative control. The sample obtained from JSCA0022 contained two prominent proteins of approximately 55 kDa and 72 kDa (Figure 4A) which were presumably a result of cross-reactivity to the anti-FLAG antibody. Those two proteins were used as an internal control. The F-box and WD40-repeat proteins from strains JSCA0026 and JSCA0027 migrated to their expected positions of approximately 19 kDa and 43 kDa (Figure 4A), respectively. However, the full-length CaCdc4 and the N-terminus truncated CaCdc4 (ΔN) from strains JSCA0024 and JSCA0025 exhibited signals at positions corresponding to 100 kDa and over 100 kDa (Figure 4A), respectively, as opposed to 86 kDa and 77 kDa, respectively. Three distinctive signals (Figure 4A) were observed for strain JSCA0030 expressing ΔNF of CaCdc4, but none of them matched the expected size of 34 kDa; however, the signal at the lowest position could be meaningful. These patterns of expression were similar to strains expressing each of the domains, with either BWP17 or JSCA0021 as a parental strain (Lai WC, unpublished results). Therefore, even though some of the strains expressed domains with unexpected size, they were unique from the negative control of JSCA0022. We concluded that the Tet-on system functions in JSCA0022 and that CaCdc4 might be undergoing undefined modifications.

To determine the function of the assorted CaCdc4 domains, JSCA0022-based strains capable of repressing CaCDC4 and inducing expression of assorted CaCdc4 domains were grown in SD medium with or without Met/Cys and in the presence or absence of Dox. Cells from strains in SD medium without Met/Cys grew as yeast in the presence or absence of Dox. Cells from strains in SD medium without Met/Cys grew as yeast in the presence or absence of Dox (Figure 4B). As expected, cells of JSCA0023 and JSCA0024 growing on medium with Met/Cys and Dox and that expressed the full-length CaCdc4 with or without tag grew as yeast. Disregarding the full-length CaCdc4, cells from all strains, except JSCA0025 expressing assorted domains, still grew as filaments (Figure 4B). Under Met/Cys and Dox conditions, cells from JSCA0025 expressing the N-terminal 85-amino acid truncated CaCdc4 seemed to have an ability to suppress filamentation but not complete back to the yeast form (Figure 4B). This is in consistent with our previous observation in which, comparing with cells capable of expressing the full-length CaCdc4 under the CaMET3p repressible control, those cells expressing the N-terminal 85-amino acid truncated CaCdc4 lagged behind in reaching exponential stage (Additional file 1: Figure S1) and converted to filamentous form earlier (Additional file 2: Figure S2) in the repressed condition.
C. albicans CDC4 negatively regulating cell flocculation

Significant differences in the ability among strains to form suspensions (to resist flocculation) were observed. The extent of flocculation among strains was observed after resuspending the cells in cuvettes, where they remained for 30 seconds. When cells were grown under the Met/Cys and Dox conditions, only those from JSCA0023 and JSCA0024 were somewhat easier to maintain as a suspension. To exclude the possibility that this was a result of increases in cell density, cells from all strains were initially grown to saturation, and the cultures were subsequently diluted to the same initial optical density and grown exponentially to similar optical density. The extent of flocculation among strains was observed after spinning the cells for 1 minute at 500 rpm. The suspended cells were sampled for determination of their optical density. Cells resisted in flocculation would remain in suspension upon centrifugation. Under the CaMET3p de-repressed condition and in the presence or absence of Dox, all strains exhibited a similar degree of suspension. However, under the CaMET3p repressed condition, JSCA0026, JSCA0027, and JSCA0030 displayed flocculation similar to JSCA0022 regardless of the presence or absence of Dox (Figure 5A). While more cells of strains JSCA0023, JSCA0024 maintained as suspension, those of JSCA0025 with some filamentous cells, showed comparable extent of flocculation to JSCA0022 under CaMET3p repressed but Tet-on induced conditions (Figure 5).

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

Discussion
In this study, we aimed to dissect the function of Ca\(Cdc4\) domains by introducing a Tet-on system with cassettes that encoded for a variety of Ca\(Cdc4\) domains in a \(\text{C. albicans}\) mutant of \(\text{CaCdc4}\) null. However, the \(\text{CaCdc4}\) null mutant with a filamentous form could not be easily used to introduce the Tet-on cassettes; therefore, we constructed the JSCA0022 strain, where \(\text{CaURA3}\) was released from the strain JSCA0021, and \(\text{CaCDC4}\) expression was repressible. Under repressed conditions, the JSCA0022 strain showed similar filamentous morphology (Figure 2) to those from previous reports of cells with \(\text{CaCDC4}\) repressed strain [6,7] and of \(\text{cacdc4}\) null mutant [6] (Tseng TL, Hsu WH, and Shieh JC, unpublished results). We confirmed that the JSCA0022 strain under repressed conditions was equivalent to a strain that had completely lost \(\text{CaCDC4}\) function. Hence, by introduction of the Tet-on cassettes into JCSA0022 strain, each of the strains was capable of expressing individual \(\text{CaCdc4}\) domains in the presence of Met/Cys and Dox for functional comparisons.

To verify the ability of the Tet-on cassettes in \(\text{C. albicans}\), each of the cassettes encoding various \(\text{CaCdc4}\) domains was transformed into BWP17 and JSCA0021 before introducing them into JSCA0022 at the \(\text{CaADH1}\) locus. Individual \(\text{CaCdc4}\) domains from relevant strains were all detectable, suggesting that the Tet-on system functions in \(\text{C. albicans}\). However, while cells expressing the F-box and the WD40 repeat could be detected as their expected sizes, those expressing the full-length \(\text{CaCdc4}\), the N-terminus truncated \(\text{CaCdc4}\) (\(\Delta N\)), and the \(\Delta NF\) of \(\text{CaCdc4}\) could be detected at positions higher than anticipated (Figure 4A). In particular, the sample from strain JSCA0030 expressing the \(\Delta NF\) could be detected three signals (Figure 4A), all of which were greater than the predicted sizes. These results suggest that the N-terminal \(\text{CaCdc4}\) from residue 85 to 241 (Figure 3A) might be undergoing post-translational modification during the Tet-on-induced expression,
although its functional significance is unknown. Interestingly, the region between residue 85 and 241 of CaCdc4 contains abundant serine and threonine residues, the majority of which are homologous to S. cerevisiae Cdc4 [7]. This implies possible phosphorylations or other modifications on these residues that is specific to C. albicans. However, the genuine nature of these residues remains to be determined, and their functional significance of this N-terminal CaCdc4 requires further study.

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

Under the Met/Cys and Dox conditions, cells expressing F-box, WD40 repeat, and the ΔNF of CaCdc4 exhibited filamentous forms similar to those of JSCA0022, whose CaCDC4 was repressed, compared to those expressing the full-length CaCdc4 without or with tag (JSCA0023 and JSCA0024), which exhibited yeast forms

Figure 5 Analysis of cell flocculation by low-speed centrifugation. Cultures of the indicated strains were grown in SD medium with histidine, arginine, uridine for 2 days before diluting into the SD medium to an initial OD_{600} = 0.1 with addition of 2.5 mM Met/Cys to repress the expression of CaMET3p-driven CaCDC4 and 40 μg/ml Dox to induce the expression of CaCdc4 domains tested for 48 hrs to OD_{600} = 1.6. Cultures were photographed before and after centrifugation. (A) A representative of the cultures. Upper panel: two-day culture. Bottom panel: cultures being spun down with 500 rpm for 1 minute. (B) Quantitative results. Data are represented as means with standard deviation from three independent experiments, each sample was in duplication. The data from JSCA0022 were compared with those of other strains. **: P < 0.01. The designations of strains are the same as in Table 1.
acid truncated CaCdc4 were unable to totally overturn filamentous-to-yeast cells, suggesting that N-terminal 85-amino acid is required for full activity of CaCDC4 function in C. albicans to inhibit filamentation. However, if flocculation is tightly associated with filamentation, we expect to see the extent of flocculation in JCSA0025 (ΔN 6HF) being greater than that of JSCA0022 but less than that of JSCA0023 and JSCA0024 in the presence of Met/Cys and Dox. This was not revealed by the low speed-centrifugation method but by the Ca2+-initiation assay. Importantly, both JSCA0025 and JSCA0027 expressing CaCdc4 lacking N-terminal 85-amino acid (Figure 3A) exhibits similar extent of flocculation. Moreover, JSCA0025 that expressing CaCdc4 lacking N-terminal 85-amino acid could only partially suppress filamentation yet JSCA0027 that expressing CaCdc4 lacking N-terminal 85-amino acid and F-box with flanking regions completely lose the ability to inhibit filamentation (Figure 3A and Figure 4B). These results imply that N-terminal 85-amino acid of CaCdc4 has a role in inhibition of cell flocculation in C. albicans and that the F-box and its flanking region in addition to the N-terminal 85-amino acid of CaCdc4 might be associated with proper control of both morphogenesis and flocculation.

Conclusions

Therefore, we conclude that F-box and WD40-repeat are important in suppressing yeast-to-filament transition and flocculation and that the N-terminal region (1–85) has a positive role in CaCDC4 function, lost of which impairs reverse of filament-to-yeast and reduces the ability to flocculate in C. albicans. Moreover, the function of CaCdc4 for suppressing flocculation that is related to cell-cell adhesion [21] implies a role of CaCDC4 in biofilm formation [28] that is under investigation.

Additional files

Additional file 1: Figure S1. N-terminal 85-amino acid of CaCdc4 is required for normal growth of C. albicans. Strains: BWP17, heterozygous null mutant CaCDC4 +/-, M3CaCDC4 +/- carrying CaMET3-full-length CaCDC4, and M3NTCaCDC4 +/- carrying CaMET3-partial CaCDC4 (capable of expressing N-terminal 85-amino acid of truncated CaCdc4). Cells of the strains were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration. Cells were grown for 12 hrs in SD either with or without 2.5 mM Met/Cys (−Met/Cys or + Met/Cys) and at each 2 hr interval the cells were sampled to determine the optical density of 595 nm (O.D. 595) in which the growth curves could be plotted.

Additional file 2: Figure S2. N-terminal 85-amino acid of CaCdc4 is required for suppression of yeast-to-filament transition in C. albicans. Cells of the strains were grown initially in SD medium without Met/Cys to saturation and were diluted to the same initial concentration. Cells were grown for 8 hrs in SD either with or without 2.5 mM Met/Cys (−Met/Cys or + Met/Cys). The images were visualized and recorded with a Nikon 50i microscope at 400x magnification. Bars represent 10 μm. The designations of strains are the same as in Additional file 1: Figure S1.
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