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The Role of Septins in Hyphal Morphogenesis of *Candida albicans*

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Abstract of the Thesis

The Role of Septins in Hyphal Morphogenesis of *Candida albicans*

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Candida albicans is among the most important human fungal pathogens. It resides in bodies of healthy people as a benign commensal but can become pathogenic when the immune system is suppressed. *C. albicans* can adopt several morphologies: bud, pseudohyphae, hyphae and chlamydspore. The ability to grow in different morphologies contributes to the pathogenic potential of *C. albicans.* One class of proteins that play a crucial role in morphogenesis is the family of septin proteins. They are believed to act as both a boundary domain, preventing cellular materials from moving between compartments, and a scaffold, recruiting proteins to their vicinity. To better study septin function in *C. albicans*, septin proteins were fused to the Green Fluorescent

Protein (GFP). In this study, a new version of GFP was identified that is brighter and more photostable in *C. albicans* than the previous version of GFP that was described. To further investigate the role of septins during hyphal morphogenesis, a conditional Ts-Cdc12 septin mutant was constructed. The mutant can respond normally to hyphal stimuli at the restrictive temperature, but forms more pseudohyphae-like cell structures with cell wall defects. These approaches have revealed new insights into septin function during morphogenesis and will be useful tools for future studies.

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Abbreviations

˚C	degree Celsius
mg	microgram
µl	microliter
ml	milliliter
mM	millimolar
nm	nanometer
5-FOA	5-fluoroorotic acid
AIDS	acquired immune deficiency syndrome
BCA	bicinchoninic acid
BCS	bovine calf serum
bp	base pair
Cdc	cell division cycle
Chs	chitin synthase
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid

FITC	fluorescein isothiocyanate
G-6-PD	gluose-6-phosphodehydrogenase
GFP	green fluorescent protein
GST	glutathione-s-transferase
h	hour
Ist	increased sodium tolerance
Lte	low temperature essential
OD660	optical density at 660 nm
PCR	polymerase chain reaction
RFP	red fluorescent protein
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	secretory
Shs	seventh homolog of septin
Spa	spindle pole antigen
SPR	sporulation regulated

SUMO	small ubiquitin-like modifier
Tem	termination of M phase
UV	ultraviolet ray
Vrg	vandate resistance glycosylation
YFP	yellow fluorescent protein
YPD	yeast extract peptone dextrose

Chapter I

Introduction

Candida albicans is a very important human fungal pathogen. In healthy people, *C. albicans* colonizes the gastrointestinal tract at birth or quickly thereafter. It is suppressed by the immune system and persists as an benign commensal in the body. However, in immunocompromised patients, such as cancer, AIDS patients or those with defects in neutrophil function, *C. albicans* can cause severe infections, and sometimes death. The mortalities associated with disseminated candidiasis can be as high as 30%~40%, despite extensive antifungal therapy (Pfaller and Diekema, 2007). *C. albicans* is the fourth most common hospital-acquired infection in the United States, the treatment of which is estimated to cost more than $1 billion annually (Beck-Sague and Jarvis, 1993; Miller *et al*., 2001). Because *C. albicans* and other fungal pathogens are eukaryotes and share many of their biological processes with humans, most antifungal drugs cause deleterious side effects. There are numerous studies on *C. albicans* treatment, but so far no drug has been discovered to provide a consistently effective fungicidal result without serious side effects.

Rapid advances in the understanding of many basic biological processes in *C. albicans* have been made as a result of their similarity to related processes in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae*, which diverged from *C. albicans* 140~841 million years ago (Berbee and Taylor, 2000; Heckman *et al*., 2001), is a crucial guide for studying aspects of cell-cycle progression, signal transduction and metabolism in *C.*

albicans. In addition, *S. cerevisiae* has been used for preliminary testing of hypotheses that were later addressed directly in *C. albicans*.

There are also significant differences between these distant cousins. For example, *S. cerevisiae* grows exclusively by budding off round yeast cells or elongated pseudohyphal cells, whereas *C. albicans* is more morphologically diverse, as it is also capable of forming true hyphae and chlamydospores. This diversity is believed to aid its survival, growth and dissemination in the mammalian host. Such features, which are not shared by *S. cerevisiae,* warrant the study of *C. albicans*, and, in the meantime, make *S. cerevisiae* a model organism where parallel assays of morphogenesis can be carried out.

Studies on *C. albicans* morphogenesis are significant, because the ability to switch between bud and hyphal growth is important for virulence. One interesting class of morphogenesis proteins first identified in *S. cerevisiae* are septins. Septin proteins form filamentous structures that contribute to proper morphogenesis. The septins were first discovered in the budding yeast *S. cerevisiae* and were named for their role in cytokinesis and septum formations. They comprise a family of proteins involved in dynamic membrane events in a wide range of eukaryotic organisms (Cooper and Kiehart, 1996; Longtine et al., 1996; Douglas et al., 2005). The septins function in the formation of septa, mating projections, and spores in *S. cerevisiae,* as well as other physiological processes in animal cells (Longtine et al., 1996; Warenda and Konopka, 2002; Douglas et al., 2005; Gladfelter et al., 2005). The majority of septin studies have been carried out in

the budding yeast *S. cerevisiae*, where five septins, Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p, have been shown to make up a ring of 10-nm filaments on the inner surface of the plasma membrane at the bud neck (Byers and Goetsch, 1976a; Longtine *et al*., 1996). The formation of the septin ring apparently requires interdependent interactions among septin proteins. Shifting of septin mutants to the nonpermissive temperature resulted in the partial or complete disappearance of the septin ring, and can also affect the growth rate of the mutants (Haarer and Pringle, 1987; Ford and Pringle 1991). Deletion of *CDC3* or *CDC12* is lethal. Deletion mutants lacking *CDC10* or *CDC11* are viable only at low temperatures (Flescher *et al*., 1993; Fares *et al*., 1996; Frazier *et al*., 1998). Deletion of another septin, Shs1p/Sep7p, leads to mild defects in cytokinesis (Carroll *et al*., 1998; Mino *et al*., 1998).

The septins have also been implicated in morphogenetic processes other than budding in *S. cerevisiae*. Particularly, septins have been shown to function in mating projection formation, where the septins form a ring at the neck of the polarized growth that forms a conjugation bridge to connect the mating cells (Ford and Pringle, 1991; Kim *et al*., 1991; Giot and Konopka, 1997). The septins are also implicated in sporulation, as indicated by the observation that two *S. cerevisiae* septins, *SPR3* and *SPR28*, are expressed only in cells undergoing meiosis and sporulation (Ozsarac *et al*., 1995; De Virgilio *et al*., 1996; Fares *et al*., 1996; Tachikawa et al., 2001).

The septins carry out at least two major functions. First, they can act as a scaffold at the bud neck, recruiting other proteins to the vicinity. Second, they are also thought to be a bud neck barrier and cytokinesis barrier (Caudron and Barral, 2009). Septins contribute to cell polarization by blocking diffusion between the daughter and mother cells. The evidence for this is that GFP-tagged Ist2 in the plasma membrane of the daughter cell was not able to diffuse across the bud neck to the mother cell in wide type cells, but was able to diffuse to the mother cell in septin mutants (Takizawa *et al*., 2000). Septins were also reported to act as a boundary to restrict to the bud Lte1 that promotes exit from mitosis. In septin mutants, Lte1 can diffuse into the mother cell, where it activates Tem1 and thereby promotes premature exit from mitosis prior to proper nuclear division (Castillon *et al*., 2003). During cytokinesis, the polarisome component Spa2p, exocyst component Sec3p, and Chs2p localize between the split septin rings, as does the actomyosin ring. Cytokinesis then proceeds by actomyosin ring contraction and the fusion of secretory vesicles to form the septum. However, shifting a *cdc12-6* septin mutant to the restrictive temperature to disrupt septin rings revealed that Spa2, Sec3, and Chs2 are rapidly lost from the bud neck, indicating that their diffusion is normally constrained by the split septin rings. In contrast, the actomyosin ring was maintained at the bud neck after the temperature shift (Dobbelaere *et al*., 2003).

Septins are now known to be highly conserved in fungi and animals, although absent in plants and many protozoans (e.g., *Plasmodium fasciculatum* and *Dictyostelium discoideum*). The high degree of homology between septin proteins has led to the

identification of septins in a wide range of eukaryotic cells from yeast to human. Interestingly, septins in multicellular organisms seem to function not only in cytokinesis (Neufeld and Rubin, 1994; Fares *et al*., 1995; Kinoshita *et al*., 1997; Nguyen *et al*., 2000) but also in other processes. For example, septins have been found to associate with the sec6/sec8 exocyst complex in rats (Hsu *et al*., 1998) and also with synaptic vesicles in mice (Beites *et al*., 1999).

Studies on the septin proteins in *C. albicans* have yielded both similarities and differences compared to septin studies in *S. cerevisiae*. The budding phase in *C. albicans* shows general similarity to *S. cerevisiae* with some key differences. Five septins form the septin ring at the bud neck that are orthologous to Cdc3, Cdc10, Cdc11, Cdc12 and Shs1/Sep7 (Gale *et al*., 2001; Kaneko *et al*., 2004; Tachikawa *et al*., 2001; Warenda and Konopka, 2002; Martin and Konopka, 2004). Deletion analysis indicates that the overall contribution of most septins is similar (Warenda and Konopka, 2002); *CDC3* and *CDC12* are essential, and *SHS1*/*SEP7* plays a relatively minor role. Deletion of *CDC10* and *CDC11* resulted in defects in cytokinesis and spindle orientation but, in contrast to *S. cerevisiae*, *CDC10* and *CDC11* are not essential for growth, even at 42˚C. The high thermostability of *C. albicans* septins may relate to this organism being adapted for growth at 37˚C, the temperature of its host. Septins act as a scaffold to recruit proteins to the bud neck, including the *C. albicans* homologs of Hsl1, Gin4, Int1, and Bni4 (Gale *et al*., 2001; Martin *et al*., 2004; Umeyama *et al*., 2005; Wightman *et al*., 2004). However, the role of the bud neck proteins may be altered. For example, *C. albicans* lacks a

homolog of Hsl7 that in *S. cerevisiae* binds to Hsl1 and is important for its function. Septins are not detectably modified by SUMO (Smt3) as they are in *S. cerevisiae*, but they act as a scaffold to recruit SUMO-modified proteins (Martin et al., 2004). The role of septins in pseudohyphae is thought to be similar to budding, as in both cases a septin ring forms at the junction with the mother cell (Warenda and Konopka, 2002).

C. albicans hypha are commonly seen at sites of infection, and can be induced at 37˚C by a variety of stimuli, such as serum, N-acetylglucosamine, alkaline pH, Lee's medium and phagocytosis by macrophages. Three types of septin localization are observed in hyphae: (i) A diffuse ring of septins is detected at the junction between the mother cell and the germ tube; (ii) A classic septin ring forms further out in the initial protrusions of hyphal growth; (iii) A faint cap of septins is also detected at the leading edge of growth in germ tubes and hyphae (Warenda and Konopka, 2002; Douglas *et al*., 2005). The deletion mutants of *CDC10* and *CDC11* in *C. albicans* display abnormalities in hyphal growth in addition to defects in septum formation. Both mutants are more curved than the relatively straight hyphae formed by wild-type cells. They are also defective in selecting sites of secondary germ tube formation. The *cdc10*Δ and *cdc11*Δ mutants often form a secondary germ tube adjacent to the initial hypha and in some cases from within the hypha whereas wild-type cells form secondary germ tube at a distal site. Although the septin mutants form hyphae, they are defective in invasive growth, both in vitro in agar and in vivo in a mouse model of *Candida* infection (Warenda *et al*., 2003). The *cdc10*Δ and *cdc11*Δ mutants grew to high levels in kidneys of infected mice but did

not cause a disseminated infection, as did the wild-type. Instead, the septin mutants formed large clumps of fungal cells that were surrounded by lymphocytes. Therefore, it may not be necessary to completely block hyphal formation to have significant effects on the treatment of *Candida* infections.

The possible function of the septin proteins as a diffusion barrier could facilitate polarized growth by restricting the localization of plasma membrane proteins and thereby help focus germ tube growth into a small apical region. This could provide advantages to *C. albicans* as a pathogen. One is that the thin filamentous hyphae are presumably better at invasive growth. Also, the smaller zone of apical secretion is expected to concentrate the delivery of secreted hydrolytic enzymes that facilitate invasive growth. Another is that narrow apical growth rapidly elongates cells and enables phagocytosed *C. albicans* to burst out of macrophages and escape destruction. Therefore, studies on the role of septins in forming boundary domain and assays for mutants defective in forming boundary domain for proper hyphal morphogenesis are warranted. Further insights could help drug design and more effective clinical treatment.

The goals of this study are: (i) to identify a GFP variant that has strong fluorescence intensity and greater photostability in *C. albicans* than the currently used GFP variant; (ii) to construct a septin Ts- mutant in one of the essential septin genes to help study the role of septins in hyphal growth and virulence.

Chapter II

Materials and Methods

Strains and Media

The *C. albicans* strains used in this study are derivatives of BWP17 (Wilson *et al*., 1999). The *C. albicans* strains were propagated on either YPD or synthetic medium plus 50 mg/l uridine unless indicated otherwise. Hyphal growth was induced by the addition of 10% BCS to YPD and cells were grown at 37 ˚C. The strains used in the study are described in Table I.

Table I *C. albicans* strains constructed for this study

STRAIN	GENOTYPE
BWP17a	*ura3::imm434/ ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
YAW9 (cdc11)b	BWP17 plus *cdc11*::*ARG4*/ *cdc11*::*HIS1*
YAW12	BWP17 plus *cdc12*::*ARG4*/*CDC12*
CZ1	BWP17 plus *CDC11-YeGFP3::URA3*
CZ2	BWP17 plus *CDC11-mut2::URA3*
CZ3	BWP17 plus *CDC11-CaGFPα::URA3*
CZ4	BWP17 plus *CDC11-CaGFPβ::URA3*
CZ5	BWP17 plus *CDC11-CaGFPγ*::*URA3*
CZ6	BWP17 plus *VRG4-mut2*::*URA3*
CZ7	BWP17 plus *VRG4-CaGFPγ*::*URA3*
CZ8	BWP17 plus *DHFR*::*URA3-DHFR(3')-CDC11*
CZ9	CZ8 plus *cdc11*::*ARG4*
CZ10	YAW12 plus *cdc12-6::URA3*
CZ11	CZ10 plus *CDC10- CaGFPγ*::*CdHis1 (HIS1 from Candida dubliniensis)*
CZ12	BWP17 plus *NOP1-CaGFPγ*::*URA3*
CZ13	YAW12 plus *NOP1-CaGFPγ*::*URA3*
CZ14	CZ10 plus *NOP1- CaGFPγ*::*CdHis1 (HIS1 from Candida dubliniensis)*

a (Wilson et al., 1999)

b (Warenda and Konopka, 2002)

Plasmids

To search for an improved version of GFP for *C. albicans*, we used the plasmid pFa-GFP-URA3 kindly provided by Dr. Jurgen Wenland as a vector (Schaub *et al*., 2006). Restriction enzymes PstI and AcsI were used to cut out the GFP fragment, which was then replaced with new GFP variants described in Chapter 3 (Crameri *et al*., 1996; Shaner *et al*., 2005; Tsien, 1998). We then used the pFa plasmids with the three new GFP variants as a template for PCR to generate the GFP cassettes flanked by regions with homology to *CDC11*. The PCR products were introduced into *C. albicans*, and then the transformants were screened for proper creation of Cdc11-GFP fusion. To tag *CDC11* in *C. albicans* with degron cassette (DHFR followed by URA3 cassette and a small repeat fragment of DHFR), we created plasmid pDDB57PlusLargeAndSmallGAPs and used primers with 70 bp homology with *C. albicans* genome and 23 bp homology with the plasmid to generate linear DNA fragment for homologous recombination to the *C. albicans* genome. To create the truncated *cdc12-6* temperature-sensitive mutant, we inserted the open reading frame and promoter region of *URA3* cassette into the 3' region of *CDC12* open reading frame to truncate the gene. Further details will be provided in Chapter IV.

C. albicans Transformations

Transformations were performed as described previously (Wilson *et al*., 1999). Overnight cultures were diluted 1:200 in 50 ml of medium and grown ~4 h at 30˚C to an OD660 of 0.8~1.0. Cells were harvested by centrifugation, washed in 1/10 volume

LATE buffer (0.1 M lithium acetate, 10 mM Tris-HCl pH 7.5, and 1 mM EDTA) and resuspended in 1/50 volume of LATE buffer. Then 100 µl of the cell suspension was mixed with 50 µg of sheared salmon sperm DNA and 95 µl of a disruption construct PCR reaction. Polyethylene glycol 4000 (40%) in LATE buffer was mixed into the reaction, which was then incubated at 30˚C for 12~15 h. The cells were heat shocked for 15 min at 44˚C, pelleted, and washed in YPD medium before being resuspended in 200 µl of YPD and plated on selective medium.

5-FOA Selection

Colonies were picked from the master plate and streaked out on synthetic media plates with 5-FOA added. The plates were incubated at 30˚C for 24 h until single colonies were clearly visible. The single colonies were then picked and streaked out on another 5-FOA plate. The process was repeated 3 times before genomic DNA was extracted from the single colonies to confirm that the *URA3* cassette has been popped out.

Spot Assay for Analysis of Temperature-Sensitive Growth

Overnight cultures were diluted 1:200 in 50 ml of medium and grown for 4 h at the temperatures indicated to an OD660 of 0.8~1.0. The cultures were then serially diluted to the concentration of $10x10^6$ cells/ml, $5x10^6$ cells/ml, $10x10^5$ cells/ml, $5x10^5$ cells/ml and $10x10^4$ cells/ml. 5 µl from each dilution was then placed on a YPD plate.

The plates were then incubated at the temperatures indicated for 48 h and 72 h respectively.

Microscopy

To achieve the best visualization, cells were grown overnight in log phase at the temperature indicated with all the essential amino acids. The next day the cells were harvested by low-speed centrifugation. Cells were then examined using DIC optics for morphological analysis, and fluorescence microscopy to visualize GFP, Calcofluor White or Filipin. GFP localization and analysis were performed on cultures in logarithmic growth without fixation and viewed on a fluorescence microscope using FITC filters. Chitin staining was performed by adding 0.2 μg/ml Calcofluor White directly to the medium during the last 10~20 min of growth and viewing the cells without fixation using a UV filter set. Filipin staining was performed by adding 0.1μg/ml Filipin stain directly to the medium and the cells were immediately moved to the microscope for visualization using a UV filter set. All microscopy was performed on an Olympus BH-2 microscope. Images were captured with a black-and-white AxioCam (Carl Zeiss, Thornwood, NY) run by Openlab 3.0.1 software from Improvision (Lexington, MA). For quantitation of signal intensity studies, at least 50 cells were counted from three independent experiments. The measurement feature of the software was used to generate the percentages reported.

For GFP time lapse assays, one picture was captured at the time point indicated and the assay was repeated three times on three independent days. For the nuclear movement time lapse assays, solid medium was heated to be liquefied and a small amount (usually 30 μl) was aliquoted onto a special air-permeable glass slide. Then 5 μl of log-phase cell culture was aliquoted onto the solidified medium. 10 μl of serum was mixed with the cell culture if necessary. The slide was then moved to a heating station and the cells on the media were visualized at each time point indicated in the experiment.

Western Blotting

Overnight cultures were diluted 1:200 in 50 ml of medium and grown for 4 h at the temperatures indicated to an OD660 of 0.8~1.0. Cells were harvested by centrifugation, washed in distilled water, pelleted, then resuspended in 200 μl of TNE lysis buffer (10 mM Tris-base, 1 mM EDTA, 100 mM NaCl) with 100 X protease mix (40 mg/ml pepstatin A, 40 mg/ml aprotinin, 20 mg/ml leupeptin) added. 100 μl of washed glass beads were added and tubes were vortexed (3 X 45 s) and then placed immediately on ice. The supernatant was collected after low speed centrifugation at 3000 rpm for 1 min. Protein concentrations were determined by BCA protein assay (PIERCE) before the same volume of sample buffer was added and the sample was incubated at 100˚C for 5 min. The samples, along with protein markers (Invitrogen), were loaded onto a 10% SDS-PAGE and separated proteins were electroblotted to a Protran nitrocellulose membrane (Whatman GmBH). The blot was incubated with mouse anti-GFP (Millipore), mouse anti-G-6-PD and rabbit anti-CDC11 (Santa Cruz Biotechnology)

primary antibody diluted to 1:1,000 and then with goat anti-mouse and goat anti-rabbit secondary antibody conjugated to fluorescence dye (Odyssey) diluted to 1:10,000. The antigen-antibody complexes were visualized with LI-COR fluorescence scanner (Odyssey) run by Odyssey software.

Chapter III

An Improved Version of GFP for Studying Protein Localization in *Candida albicans*

Introduction

A widely used approach for studying protein localization in *C. albicans* during hyphal growth is to fuse GFP to the target genes and investigate the localization of the protein products in the cells. Even though GFP-tagging has been very successful, many fusion proteins, due largely to their low level of expression, are not easily detected in *C. albicans*. Also, to better investigate the localization of a target protein inside the cell, a three-dimensional image, which requires multiple Z-stack images of the same cells, is more preferred than the conventional single-plane image. Thus, a brighter and more photostable GFP would be very helpful detect the exact localization of a target protein.

New GFP variants that improve the fluorescence and protein folding properties have been identified by genetic approaches in other organisms. However, these GFP variants have not been assessed in *C. albicans* and related species, presumably because of the complexity in heterologous expression. The use of GFP and related variants has been hindered by the fact the *C. albicans* reads CTG codon as serine rather than leucine, often rendering heterologous gene expression largely unsuccessful (De Backer *et al*., 2000). To adapt GFP for effective use in *C. albicans*, Cormack *et al* introduced three types of codon changes: the S65G S72A mutations to enhance fluorescence; CTG codon 201 was

changed to TTG; and the other codons were optimized for translation in *C. albicans* (Cormack *et al*., 1997). This variant, known as YeGFP3, was introduced into convenient vectors for creating gene fusions in *C. albicans*. Another version of eGFP known as mut2 (S65A V68L S72A Q80R) was adapted for *C. albicans* by changing the CTG codon, but without further codon optimization (Morschhauser and Hacker, 1998).

Results

Three new GFP variants were constructed by introducing mutations predicted to improve fluorescence properties or protein folding in the cell (Crameri *et al*., 1996). Because of the large number of changes, we gave them the more convenient names of CaGFPα (F64L S65T F99S M153T V163A), CaGFPβ (F64L S65T N149K M153T I167T; also known as emerald), and CaGFPγ (F64L S65C V163A I197T). The new variants were constructed in YeGFP3, and then we subcloned the GFP cassettes into plasmid pFa-GFP-URA3 respectively (Schaub *et al*., 2006) to generate a PCR cassette module. The CaGFPγ was also introduced into the vectors that contain selectable markers *HIS1* and *ARG4*. DNA sequencing confirmed that the correct substitutions were present.

The five GFP variants were compared by fusing them to the C terminus of Cdc11, which was selected because the restricted localization of this septin protein to the bud neck facilitates microscopic analysis (Douglas *et al*., 2005). Septin rings were only

analyzed if they were obviously in focus and at the same stage of the cell cycle (large-budded). The quantitation was carried out at both 30˚C and 37˚C because 30˚C is the temperature at which *C. albicans* is usually grown and 37˚C is the temperature at which hyphae studies are usually conducted. The brightness of GFP variants and background were measured by the Openlab software The relative intensity was calculated by subtracting background from maximum brightness of GFP signal to obtain the true value. Among the five Cdc11-GFP variants, CaGFPγ gave a slightly stronger signal than the other variants at both temperatures (Figure 1A). The assay was repeated three times and statistical analyses indicated that there was difference between any two variants (student's t-test: $P<0.0001$). At least two independent clones were analyzed for each Cdc11-GFP variant, and both gave similar results.

Photobleaching is also an important factor for GFP, especially in time lapse studies or Z-stack analysis. Photostability of the five GFP variants was examined by taking pictures at 4 second intervals during 1 min of continuous exposure to the fluorescence excitation lamp. The fluorescence of YeGFP3, mut2, and CaGFPα decayed to 50% of original intensity within 15-30 sec, and even faster for CaGFPβ. In contrast, CaGFPγ showed extended photostability at both 30˚C and 37˚C ($T_{1/2}$ ~ 2 min) (Figure 1B and 1C).

The levels of the Cdc11-GFP proteins at both 30˚C and 37˚C were compared on two independent Western blots using anti-GFP antibody (Figure 1D). The relative levels of Cdc11-mut2 and -CaGFPα were the lowest, consistent with their lower fluorescence intensity. The Cdc11-YeGFP3 and -CaGFPγ were present at higher levels, and the Cdc11-CaGFPβ was produced at even slightly higher levels, consistent with reports that it has improved folding properties (Shaner *et al*., 2005). The higher levels of Cdc11-CaGFPβ were apparently offset by its increased photobleaching, resulting in no obvious advantage.

To confirm CaGFPγ was truly better than the YeGFP3 that we normally used for tagging proteins in *C. albicans*, we tagged Golgi protein Vrg4 with CaGFPγ and mut2-GFP. Similar results were also obtained from the signal intensity studies (Figure 2A). At both 30˚C and 37˚C, Vrg4-CaGFPγ gave a stronger signal than Vrg4-mut2-GFP in three repeated assays, although the standard deviations were larger because the mobile Golgi frequently moved out of the focal plane during the time course. In the photobleaching assays, the signal of Vrg4-CaGFPγ also lasts significantly longer than Vrg4-mut2-GFP at both 30˚C and 37˚C (Figure 2B). Thus, CaGFPγ had the best overall properties based on protein levels, signal intensity, and photostability in *C. albicans*.

Discussion

There are several fundamental guidelines for using fluorescent proteins in an imaging experiment (Shaner *et al*., 2005). First, the visualized brightness of fluorescent proteins can be affected by several highly variable factors, such as the protein's intrinsic brightness (quantum yield), photostability, optical properties of the imaging setup, camera, and efficiency of GFP folding, and stability of fusion protein *in vivo*.

Second, the fluorescent protein should not deliver any toxic effect to the chosen system and should be able to provide sufficient signal above autofluorescence. According to our study, the GFP-fused Cdc11 does not seem to cause any negative effect on the cells and the cells were able to undergo budding and hyphal growth processes with no detectable change in phenotype.

Third, the fluorescent protein should have sufficient photostability for the duration of the experiment. For experiments requiring a limited number of images, photostability is generally not a concern. But for intensive experiments, such as long-time course studies or three-dimensional construction of protein localization, a large number of images is usually required and the photostability of the fluorescent protein becomes a major factor. Although so far there is no standardized characterization of photostability, our method of continuous exposure to fluorescence excitation lamp mimics the actual situation where a GFP tagged protein is being studied, and therefore the most photostable GFP variant is the one that maintains a detectable signal the longest. Fourth, if the fluorescent protein is to be expressed as a fusion to another protein, it

should not be susceptible to proteolytic cleavage to be released from the protein it was fused to. The Western blot in this study did not show any free GFP band, and only Cdc11-GFP was detected, indicating that the fusion protein is stably produced in the cell (Data not shown). Altogether, the studies in this chapter show that CaGFPγ is a reliable GFP variant for future studies on *C. albicans* under different temperature conditions.

Chapter IV

A Septin Conditional Mutant Strategy to Study Hyphal Morphogenesis in *Candida albicans*

Introduction

The major functions of the septins are thought be their ability to act as a scaffold and as a boundary domain that serves to separate the cell into distinct compartments, where normal cellular operations are performed in a restricted zone, unaffected by the neighboring compartments. Septins can promote a boundary at the plasma membrane, cytoplasm, and nuclear envelope level (Shcheprova *et al*., 2008). During hyphal morphogenesis, the septin proteins can separate the hypha body into several compartments. Deletion of both *CDC10* and *CDC11* septins indicated that some partial growth defects were present and the cells were not able to maintain their normal morphologies. GFP tagged Cdc3 in mutant cells localize largely as expected; that is, in basal bands, cytokinetic rings, and hyphal tips. Mutations in *CDC3* and *CDC12* have not been studied yet, because they are essential for growth. To study these genes, alternative approaches must be used instead of deletion mutations.

Conditional mutants retain the function of a specific gene under one set of conditions, called permissive, and lack that function under a different set of conditions, called nonpermissive. The latter must be still permissive for the wild-type allele of a gene (Dohmen and Varshavsky 2005). Such mutants make possible the analysis of

physiological changes that follow controlled inactivation of a gene or gene product and can be used to address the function of any gene. Temperature-sensitive (Ts-) mutants, first used in functional studies more than half a century ago, remain a mainstay of genetic analyses. The advantage of temperature-sensitive mutants is that the protein of interest can be quickly shut off when the cells are switched to nonpermissive conditions. Ts- mutants are also advantageous compared to alternative approaches that use a regulated promoter to control gene expression (eg. Tetracycline promoter strategy). A promoter shut-off prevents mRNA production but the protein level is not instantly affected. Creating a Ts- mutant will make it possible to examine the efficiency of the initial response to the non-permissive condition at a much faster rate. To better study the septin mutant phenotype and, more importantly, to investigate the relation between loss of function of essential septins during hyphal morphogenesis, a septin temperature-sensitive mutant would be more useful.

A systematic Ts-degron approach for generating thermolabile versions of proteins was first described in *S. cerevisiae* (Dohmen *et al*., 1994). The rationale for this approach is that fusing a Ts-version of dihydrofolate reductase (Arg-DHFR) to the N-terminus of a protein of interest results in that protein being long-lived at 23˚C, but rapidly degraded by the N-end rule pathway at 37˚C, owing to activation of its previously cryptic degron. When fused to the N terminus of a target protein, this portable Ts-degron results in destruction of the target protein at 37˚C. This approach also works in *S. pombe*, so it seemed likely to work in *C. albicans*. Previous studies in *S. cerevisiae* also showed

that N-terminal GST fusions to septins do not affect septin function, so this N-terminal tagging strategy seemed likely to succeed.

Alternatively, a Ts- mutant septin gene could be studied, since they have been used successfully with *S. cerevisiae* septins. Unpublished data indicated that Ts- mutant of *S. cerevisiae, cdc12-6,* has a single base deletion in a stretch of seven adenines 49 bp upstream of the stop codon (Brian Haarer, personal communication). This results in a mutation of lysine to asparagine at position 391 followed immediately by a TAG stop codon, truncating the mutant protein by 16 amino acids, as confirmed by Western blotting. As there is over 70% of homology between the Cdc12 in *S. cerevisiae* and *C. albicans,* it seemed likely that introducing similar mutations to the *CDC12* in *C. albicans* would generate similar phenotype and Ts- mutant.

Results

In this study, *CDC11* was used as a test case, since it is not essential and anti-Cdc11 antibody is readily available. A DHFR-CDC11 strain was constructed through homologous recombination where the DHFR cassette was successfully inserted at the 5' end of *CDC11* in *C. albicans*. This cassette contained DHFR-URA3-DHFR(3') constructed in a manner similar to the URA3 blaster. Using 5-FOA selection, *URA3* was popped out to leave behind the *DHFR-CDC11* fusion. The second copy of wild-type *CDC11* was then deleted.

When the *DHFR-CDC11* mutant was grown at different temperatures, both permissive and nonpermissive, it seemed to behave closer to *cdc11*Δ than to wild-type. In the spot assay at all three temperatures, the mutant showed a partial growth defect as opposed to the wild-type (Figure 3A). At the protein level, only wild-type sized Cdc11 was detected at 23˚C and not the larger molecular weight band expected for DHFR-Cdc11 was shown. Similar results were also seen at both 30˚C and 37˚C (Figure 3B). Thus, there was no evidence for DHFR tagged Cdc11, but Cdc11 was somehow produced, suggesting that the DHFR domain may have been cleared off by proteolysis. The morphologies of the mutant at all three temperatures also show similarity to *cdc11*Δ (Figure 3C). The mutant formed elongated and pseudohyphal cell structures, and the cells were much more likely to aggregate as compared to the wild-type.

The second approach to create a Ts- mutant in *C. albicans* was to construct a Cdc12 Ts- mutant, patterned after the *cdc12-6* mutant in *S. cerevisiae* (Brian Haarer, personal communication). Protein alignment indicated that the lysine at position 391 of Cdc12 in *S. cerevisiae* corresponds to a serine at position 384 of Cdc12 in *C. albicans*. Therefore, in a *CDC12* heterozygote mutant, the serine at position 384 was mutated to asparagine and the lysine at position 385 was replace by stop codon TAG. A *URA3* cassette was subcloned immediately downstream of the stop codon to select for integration of the truncated allele.

Ca-cdc12-6 was grown at 23˚C, 30˚C and 37˚C. It showed no growth defect at 23˚C or 30˚C, whereas at 37˚C it failed to grow (Figure 4A). Microscope examination showed that the cells grew for a few hours and then stopped growing. The morphology of the cells at 23˚C or 30˚C was very similar to that of the wild-type, except for some tendency to aggregate (Figure 4B). At 37˚C, however, the mutant formed elongated cell structures, some of which bore significant resemblance to true hyphae. Others appeared more reminiscent of pseudohyphal structures with constrictions at sites of septation along the parallel cell wall. To investigate whether the *Ca-cdc12-6* mutant was still responsive to normal hyphal induction, serum was added to the medium to 10% final concentration. At 37˚C, serum stimulated more of the mutated cells to form hyphae than without serum. But the hyphal morphogenesis was not as close to true hyphae as in wild-type cells. Thus Cdc12 was required for growth at 37˚C and for efficient hyphal growth.

The *Sc-cdc12-6* mutant fails to form a septin ring at high temperature (Frazier *et al*., 1998). In order to see how *Ca-cdc12-6* affects septin ring formation, GFP was tagged to another septin protein Cdc10. After the cells grew into log phase stage at 23˚C, the temperature was shifted up to 37˚C. The wild-type and *CDC12* heterozygote both showed normal-looking Cdc10-GFP septin structures at all hourly time points, whereas *Ca-cdc12-6* displayed similar Cdc10-GFP septin structures at early time point and abnormal structures at later time point (Figure 5). In later hours, septin structures were only found near the tip of the hypha and seemed to extend further into the hypha while keeping a fixed distance with the hyphal tips. The higher temperature seemed to cause

the abnormal localization of the septins, perturbing their ability to remain at the fixed position at certain stages of cell cycle.

It was thus reasonable to hypothesize that the negative effect of *Ca-cdc12-6* on the integrity of the septin structures could lead to the instability of the cell wall and plasma membrane. To test this, two types of fluorescent staining were performed. Calcofluor staining, which specifically stains chitin in the cell wall, indicated that, at 37˚C, bright spots and patches appeared and remained by the side of the cell wall (Figure 6A). This also happened when serum was added to the medium. This indicates that there was significant structural defects in the cell wall, which could be caused by the instability of septin filaments in *Ca-cdc12-6*. Filipin stains ergosterol in the plasma membrane and shows predominant staining at the tip of the true hyphae (Martin and Konopka, 2004). When the mutant was induced with serum, the tip of the true hyphae was stained. However, tips of pseudohyphae, which would normally not be stained by filipin, were also stained by filipin in *Ca-cdc12-6* (Figure 6B). Therefore, the plasma membrane seemed to be able to respond normally to hyphal-inducing conditions (e.g. serum), but acted abnormally when no hyphal inducing stimuli were present.

To find out whether the *Ca-cdc12-6* septin protein could jeopardize the role of septins as a boundary domain, we tagged a small nucleolar ribonucleoprotein, Nop1, with GFP. In the wild-type cells, Nop1-GFP localized constitutively to a bright, round structure in yeast and hyphal cells, as indicated in a previous report (Finley and Berman,

2005). Previous studies also showed that during hyphal morphogenesis, the nucleus in the mother cell would move into the hypha before it divides into two nuclei. A time-lapse assay was performed to visualize movement of the nucleus of *Ca-cdc12-6* during hyphal morphogenesis. Inconsistent with what has previously been reported (Finley and Berman, 2005), nuclei in both the wild-type and *CDC12* heterozygote mutant stayed in the mother cell until the hyphae grew to a certain length, and then separated inside the mother cell, with one nucleus moving quickly into the hyphae and the other remaining. We did not observe that the nucleus would move into the germ tube before the division took place (Figure 7). The mutant, however, showed a very similar nucleus-movement pattern, except that, when no serum was added, nuclear division took much longer than in the wild-type, suggesting that a cell cycle checkpoint might occur in the absence of hyphal stimulus. Perhaps septin perturbation affects nuclear events by failing to act as a boundary domain or as a scaffold to recruit protein components crucial to nuclear division and movement.

Discussion

Conditional mutants allow the analysis of physiological changes caused by inactivation of a gene or a gene product and can be used to address the function of any gene (Dohmen *et al*., 1994). Conventional Ts- mutations require an extensive search for a Ts- mutation in the gene of interest, and are often too leaky to be useful. The degron approach, however, is based on portable, heat-inducible N-degron, which is an intracellular degradation signal that can target its attached protein to proteosomal

degradation. Such a strategy in one species will not necessarily be equally effective in its relatives. Attaching DHFR at the N-terminal end of a protein proved to yield satisfactory Ts- mutant phenotypes in *S. cerevisiae* and *S. pombe*, while in *C. albicans*, this strategy has not yet been demonstrated. A *DHFR-CDC11* mutant was not temperature-sensitive, apparently because DHFR domain was proteolytically cleaved off, leaving behind stable Cdc11 protein.

The homology between *S. cerevisiae* and *C. albicans* helped us generate a Ts- mutant through truncating an essential septin protein. A previously proposed model (Bertin *et al*., 2008) indicated that the septins form an octameric nonpolar rod-like structure. It has the order Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11, in which the C terminus of Cdc12 presumably associates with another Cdc12 from another octamer, forming a stable filamentous structure. The mutations in *Ca-cdc12-6* truncate the C terminus of Cdc12, and may cause a weakening of the septin octamer that leads to septin destabilization at higher temperatures. Loss of Cdc12 function would then destabilize the septin complex, causing the abnormal phenotypes.

As the septin structure in *Ca-cdc12-6* mutant did not completely break down, it is likely that the mislocalized septins are still able to perform part of their function as a boundary domain and a scaffold. Based on the data of nuclear movement, the nucleus was still able to separate in a pattern similar to the wild-type, except that nuclear migration took longer, indicating that septins, though mislocalized during later stages of

hyphal morphogenesis, could still function during the early stages. Future research will focus on discovering a Ts- mutant that will preferably cause complete septin mislocalization under nonpermissive conditions, which likely indicates a complete loss of function of septins. This strain would be ideal for studying the role of septins in hyphal morphogenesis and localization of virulence proteins.

Chapter V

Conclusions

C. albicans is an important human fungal pathogen. It is among the most common hospital-acquired infectious pathogens. Every year, a large amount of money is spent on studying the physiology of *C. albicans* and discovering potential drugs with less side effects.

In this study, five GFP variants were examined and one of them appeared to be the brightest and most photostable in *C. albicans*. Future studies will examine other GFP variants with improved fluorescence or better folding features. Also, to study multiple protein localization, a common strategy is to tag proteins with fluorescent proteins with different colors. We will study a red fluorescent protein variant, developed by Dr. Neta Dean (Keppler-Ross *et al*., 2008), as well as cyan and yellow variants in *C. albicans* in order to obtain more choices of fluorescent proteins when studying protein localization.

Conditional mutants are a powerful tool for studying physiological processes and molecular mechanisms in biological research. In *C. albicans*, the degron DHFR approach did not obviously yield a satisfactory result as the protein of interest did not disappear at the nonpermissive temperature and no fusion protein was detected. It is unclear whether or not the degron approach might work when the DHFR cassette is

tagged to other proteins in *C. albicans*. The *Ca-cdc12-6* has a strong temperature-sensitive phenotype, but septin structures are still present, albeit abnormal, and could still serve as a boundary domain or scaffold. Thus, *Ca-cdc12-6* might not be an optimal Ts- mutant to study complete loss of function of septin proteins. Further research will be carried out to identify a Ts- mutation in *CDC3* or a different mutation in *CDC12* that would have a complete loss of septin structure under nonpermissive conditions. One possibility is to take advantage of genetic accessibility of *S. cerevisiae* and screen for Ts- mutations in *C. albicans* septin genes expressed in *S. cerevisiae*.

Figures and Legends

Figure 1. Properties of different Cdc11-GFP fusion proteins.

Cells were grown to log phase overnight in BYNB plus amino acids and dextrose overnight at 30˚C or 37˚C. (A) Signal intensity for each mutant was compared in three independent assays. In each assay 50 septin rings were quantified. The average fluorescence intensity was normalized to 100 for the YeGFP3 version. The CaGFPγ variant gave a consistently stronger signal than the other variants ($P<0.001$). (B) Relative fluorescence intensity of the GFP variants at 4 second intervals over a time course of 1 min of continuous exposure to the fluorescence excitation lamp after growth at 30˚C and 37˚C as indicated. CaGFPγ showed the best photostability ($T_{1/2} \sim 2$ min). The results represent the average of three independent assays in which three septin rings were analyzed for each mutant. Error bars indicate SD. (C) Cells carrying Cdc11 fused to YeGFP3 or CaGFPγ were continuously exposed to the fluorescence excitation lamp and then images were captured at the indicated times. (D) Total cell lysate was extracted from five Cdc11-GFP variants and a western blot shows Cdc11 and GFP tagged Cdc11 of all five variants. G-6-PD was used as a loading control.

Figure 1

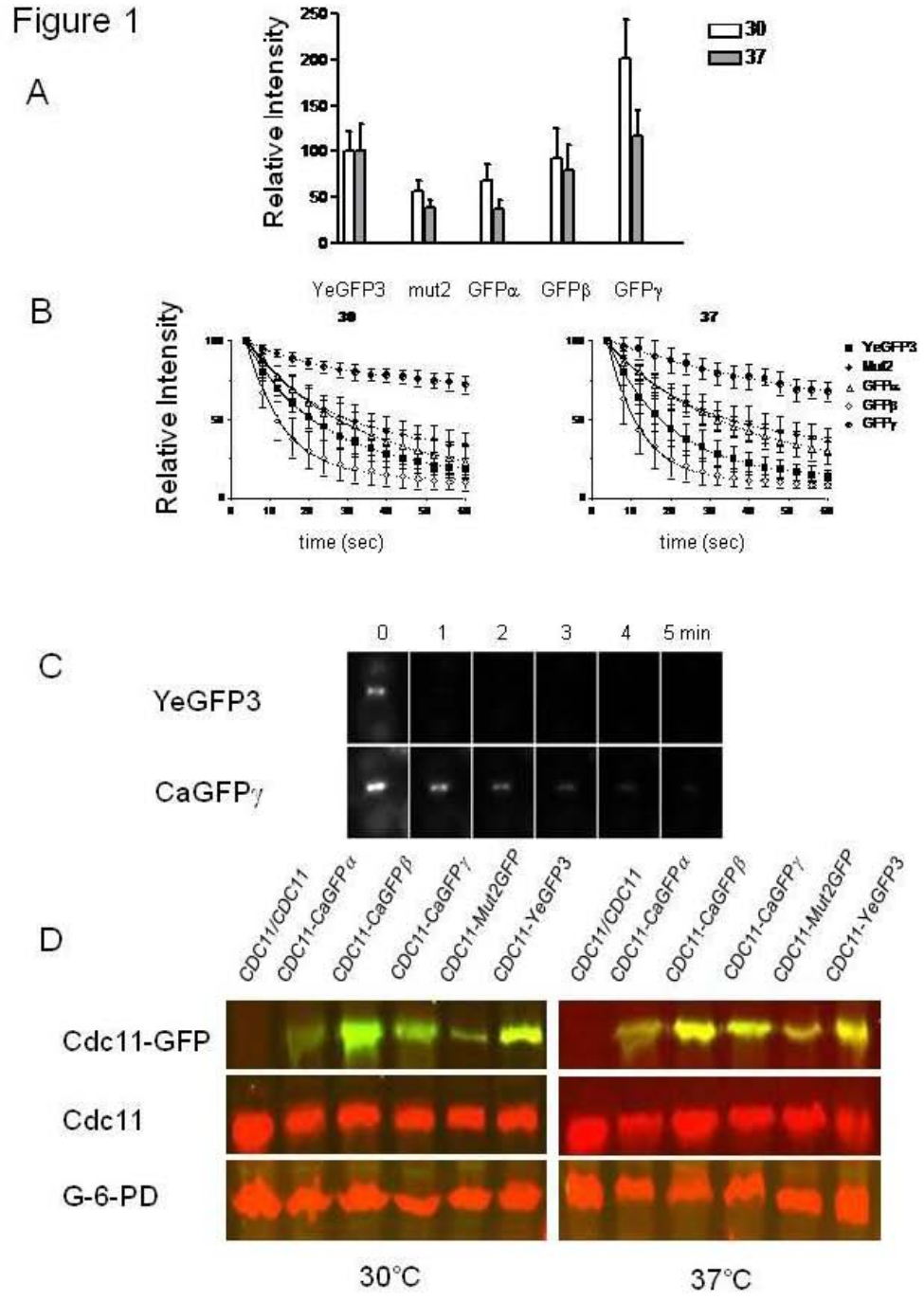
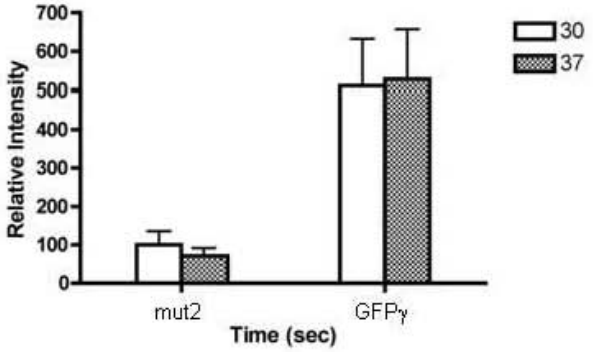

A
Relative Intensity
250
200
150
100
50
0
30
37
YeGFP3
mut2
GFPα
GFPβ
GFPγ
B
Relative Intensity
30
37
YeGFP3
Mut2
GFPα
GFPβ
GFPγ
time (sec)
time (sec)
C
0
1
2
3
4
5 min
YeGFP3
CaGFPγ
D
CDC11/CDC11
CDC11-CaGFPα
CDC11-CaGFPβ
CDC11-CaGFPγ
CDC11-Mut2GFP
CDC11-YeGFP3
Cdc11-GFP
Cdc11
G-6-PD
30°C
37°C

Figure 2. Properties of different Vrg4-GFP fusion proteins.

Cells were grown to log phase overnight in BYNB plus amino acids and dextrose at 30˚C or 37˚C. (A) Signal intensity for each mutant was compared in three independent assays. In each assay 50 Vrg5-GFP golgi spots were quantified. The average fluorescence intensity was normalized to 100 for the YeGPF3 version. (B) Relative fluorescence intensity of the GFP variants at 5 second intervals over a time course of 1 min of continuous exposure to the fluorescence excitation lamp after growth at 30˚C and 37˚C as indicated. The results represent the average of three independent assays were analyzed for each mutant. Error bars indicate SD.

Figure 2

A


700
600
500
400
300
200
100
0
Relative Intensity
30
37
mut2
GFPγ
Time (sec)

B

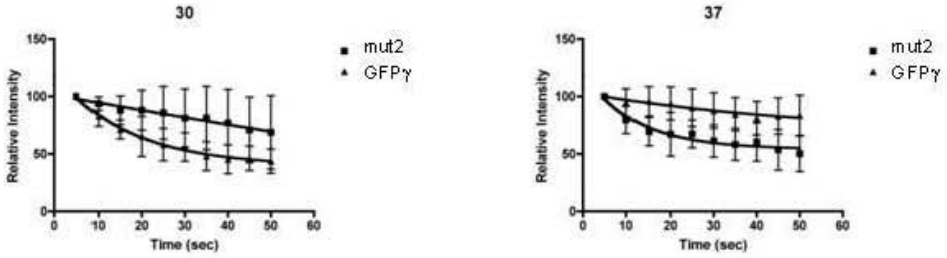
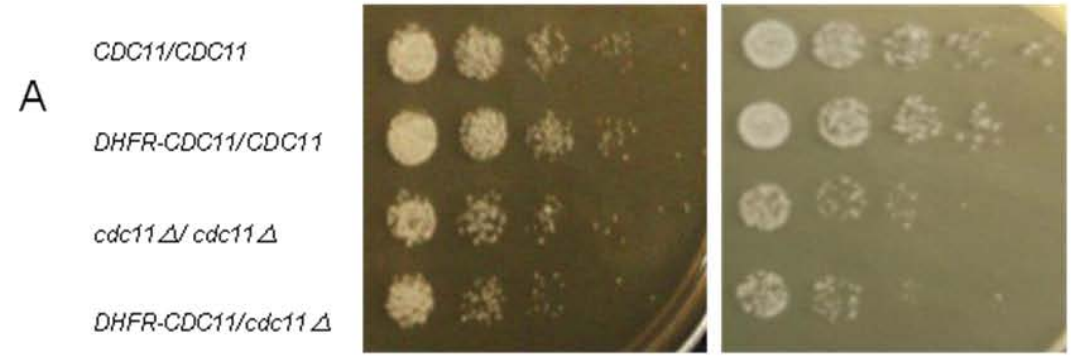
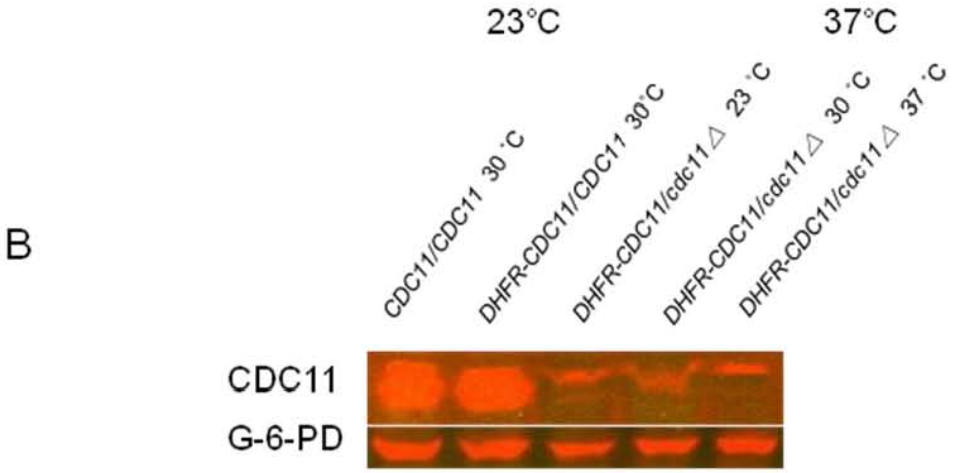

30
37
mut2
GFPγ
Relative Intensity
Time (sec)
0
10
20
30
40
50
60
50
100
150

Figure 3. Properties of DHFR tagged Cdc11 mutant

(A) Cells were grown to log phase overnight in YPD plus amino acids at 23˚C or 37˚C. After serial dilutions, 5μl of cell culture from each dilution group was aliquoted on YPD plates. The plates were incubated at the indicated temperatures for 48 hours. (B) Total cell lysates were extracted from BWP17, DHFR tagged *CDC11* mutant with a normal copy of *CDC11*, and DHFR tagged *CDC11* mutant without normal copy of *CDC11* at three different temperatures indicated. The lysates were run on a western blot, detected by anti-Cdc11 antibody. G-6-PD was used as a loading control. (C) Cells were grown to log phase at the indicated temperatures before they were visualized under the microscope.

Figure 3


A
CDC11/CDC11
DHFR-CDC11/CDC11
cdc11Δ/ cdc11Δ
DHFR-CDC11/cdc11Δ


B
23°C
37°C
CDC11/CDC11 30°C
DHFR-CDC11/CDC11 30°C
DHFR-CDC11/cdc11Δ 23°C
DHFR-CDC11/cdc11Δ 30°C
DHFR-CDC11/cdc11Δ 37°C
CDC11
G-6-PD

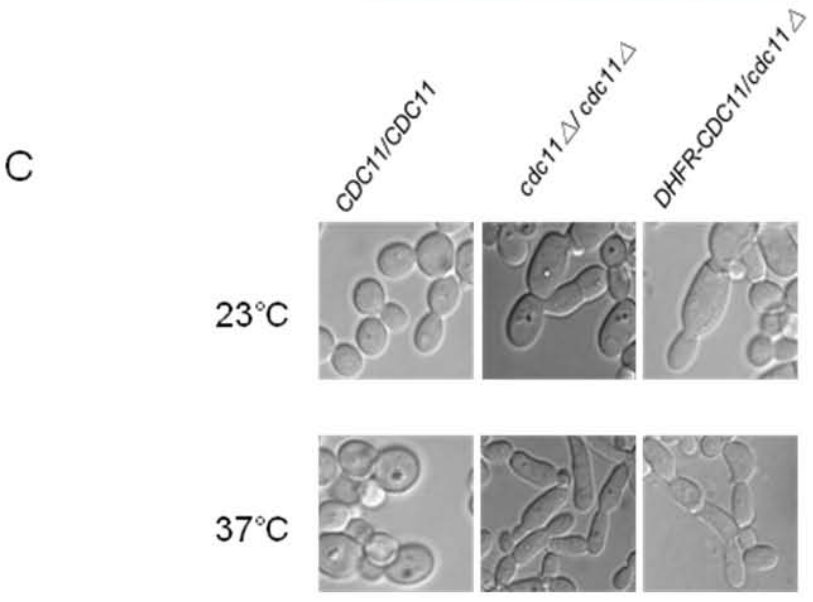

C
CDC11/CDC11
cdc11Δ/ cdc11Δ
DHFR-CDC11/cdc11Δ
23°C
37°C

Figure 4. Growth defects of *Ca-cdc12-6*

(A) Cells were grown to log phase overnight in YPD plus amino acids at 23˚C or 37˚C. After serial dilutions, 5µl of cell culture from each dilution group was aliquoted on YPD plates. The plates were incubated at the indicated temperatures for 48 and 72 hours. (B) Cells were grown to log phase overnight in YPD plus amino acids at 23˚C or 37˚C. Next day, cells were diluted to 1:10 and grown for 2 to 5 hours. At each time point, the cells were visualized under the microscope.

Figure 4

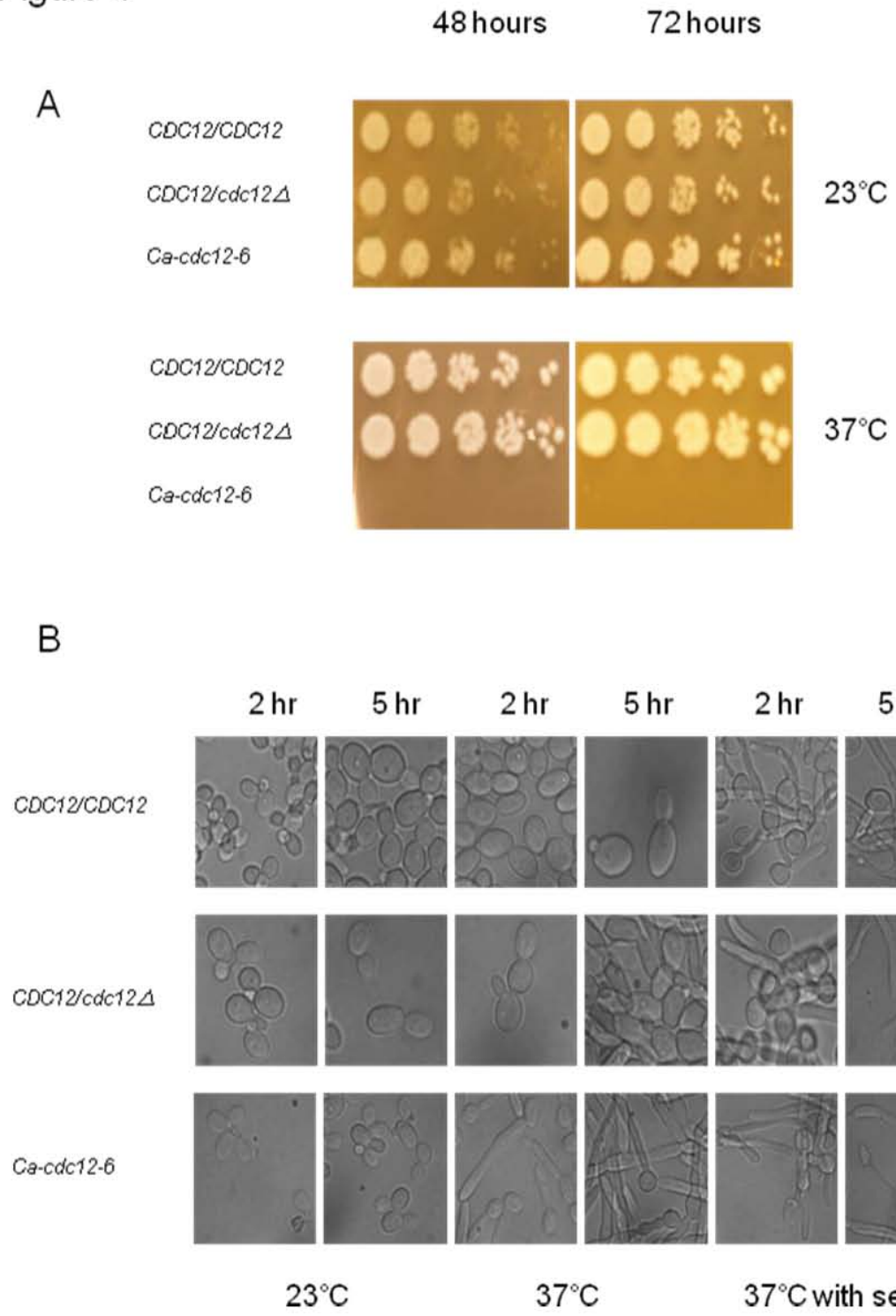

48 hours
72 hours
A
CDC12/CDC12
CDC12/cdc12Δ
Ca-cdc12-6
23°C
CDC12/CDC12
CDC12/cdc12Δ
Ca-cdc12-6
37°C
B
2 hr
5 hr
2 hr
5 hr
2 hr
5 hr
CDC12/CDC12
CDC12/cdc12Δ
Ca-cdc12-6
23°C
37°C
37°C with serum

Figure 5 Septin localization in *Ca-cdc12-6*

Cells were grown at 23˚C in BYNB plus amino acids and dextrose to log phase before the temperature was shifted up to 37˚C. The pictures were taken at each hourly time point.

Figure 5

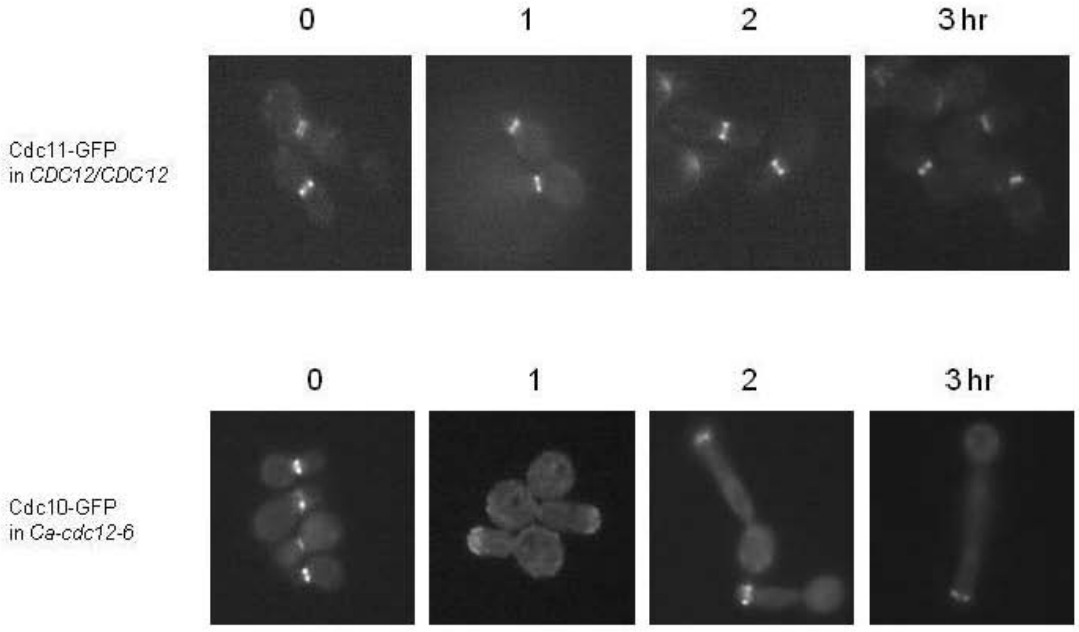

0
1
2
3 hr
Cdc11-GFP
in CDC12/CDC12
0
1
2
3 hr
Cdc10-GFP
in Ca-cdc12-6

Figure 6 Cell wall and plasma membrane of *Ca-cdc12-6*

Cell were grown at 37˚C in YPD plus amino acids to log phase before Calcofluor staining (A) and Filipin staining (B) were performed.

Figure 6

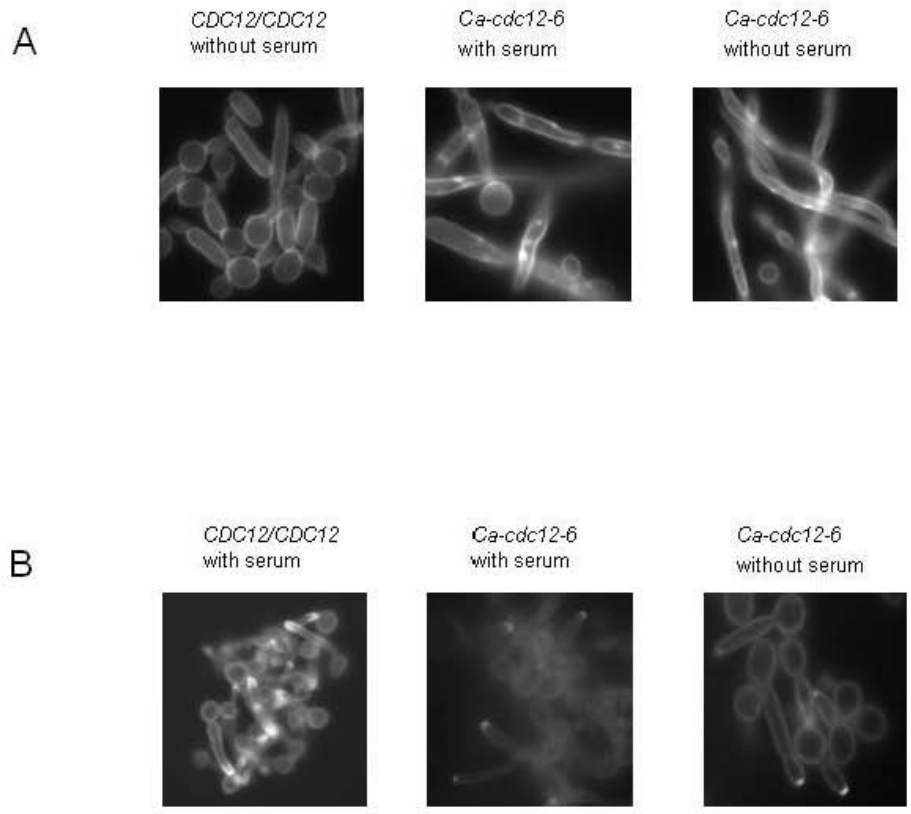
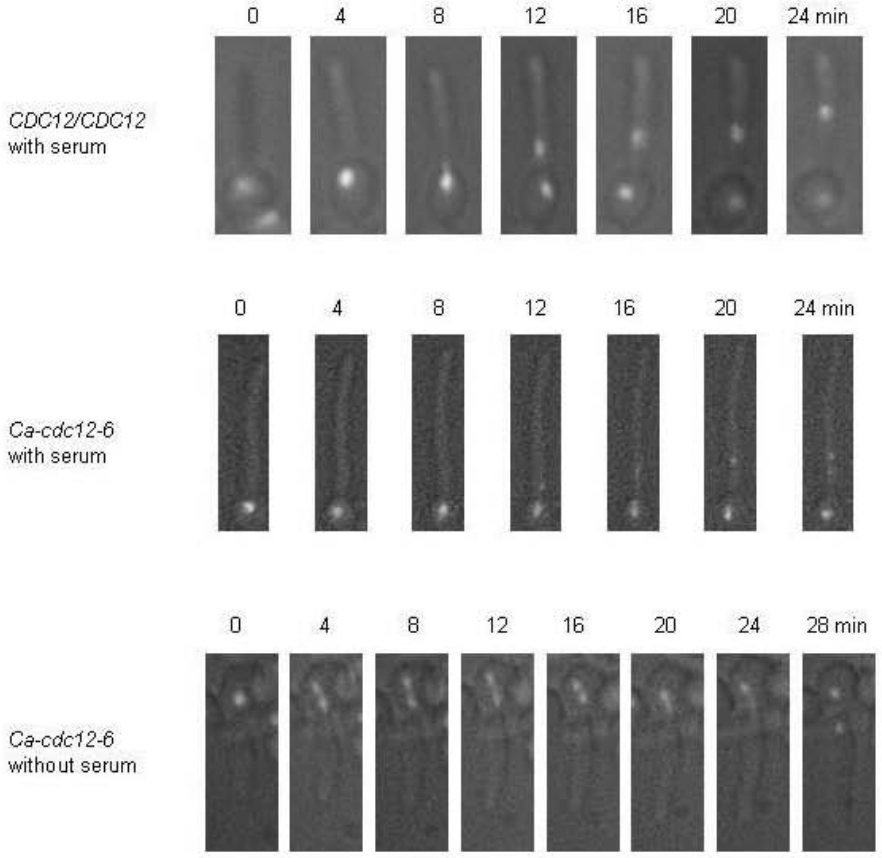

A
CDC12/CDC12
without serum
Ca-cdc12-6
with serum
Ca-cdc12-6
without serum
B
CDC12/CDC12
with serum
Ca-cdc12-6
with serum
Ca-cdc12-6
without serum

Figure 7. Nuclear movement in *Ca-cdc12-6*

Cells were grown at 23˚C before temperature was shifted up to 37˚C. Serum was added as indicated. Every 4 minutes, an image was taken. Images were selected when nuclear division took place. When no serum was added, *Ca-cdc12-6* mutant took significantly longer time to separate the nucleus than with the serum and wild-type.

Figure 7


0
4
8
12
16
20
24 min
CDC12/CDC12
with serum
0
4
8
12
16
20
24 min
Ca-cdc12-6
with serum
0
4
8
12
16
20
24
28 min
Ca-cdc12-6
without serum

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