The role of APC activators Cdc20p and Cdh1p in regulating mitosis and morphogenesis in *C. albicans*

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Abstract

The role of APC activators Cdc20p and Cdh1p in regulating mitosis and morphogenesis

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Candida albicans is an important fungal pathogen of humans, and its ability to switch between different cell morphologies, including yeast, pseudohyphae and hyphae, is critical for virulence. The cell cycle plays an important, yet poorly understood, role in regulating cellular morphogenesis. Our previous work demonstrated that blocking the yeast cell cycle in mitosis, through depletion of the polo-like kinase Cdc5p, resulted in polarized growth of the yeast bud, producing filamentous cells that were distinct from pseudohyphae or true hyphae. Polarized growth was partially dependent on the spindle checkpoint factor Bub2p, suggesting that checkpoint-activated growth occurs in *C. albicans*. In contrast, similar checkpoint activation leads to cessation of cell proliferation in most other systems. In order to elucidate how mitotic progression and spindle checkpoints are linked to morphogenesis, a better understanding of the basic regulation of mitosis is required. To this end, we characterized homologues of Cdc20p and Cdh1p, which are targets of the spindle checkpoint and activators of the ubiquitin ligase anaphase-promoting complex (APC), a major regulator of mitotic progression in most systems. Cdc20p and Cdh1p were important for the metaphase-to-anaphase transition and mitotic exit, similar to their counterparts in *S. cerevisiae*, but strongly influenced morphogenesis in a different manner. Deletion of *CDH1* resulted in a pleiotropic phenotype, including some enlarged yeast cells, while absence of the same factor in *S. cerevisiae* produced small cells. Absence of Cdc20p produced highly polarized yeast

buds that resembled Cdc5p-depleted cells, in contrast to the large doublets resulting from loss of Cdc20p in *S. cerevisiae*. Overexpression of *CDC20* in Cdc5p-depleted cells partially suppressed the cell cycle defect, suggesting that Cdc20p may be downstream of Cdc5p. However, polarized growth was not abolished. Deletion of the putative Cdc20p-binding and spindle checkpoint factor Mad2p partially compromised polarized growth in Cdc5p-depleted cells, implying that Mad2p may play a role in linking Cdc5p function with Cdc20p. Deletion of *CDH1* had no effect on Cdc5p-dependent polarization, suggesting that Cdh1p is dispensible for the process. While Cdc20p was not required for serum-induced hyphal growth, some cells lacking Cdh1p were not able to form hyphae, yet invaded agar more readily than control cells, suggesting a complex role for this factor in regulating polarized growth. Thus, we provide genetic evidence that Cdc20p and Cdh1p play important roles in regulating mitosis and morphogenesis in *C. albicans*, but in a different manner than their homologues in *S. cerevisiae*. Our results extend our knowledge of the regulatory circuit governing mitosis in *C. albicans* and the potential pathway underlying checkpoint-activated polarized growth.

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Abbreviations

AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
APC Anaphase Promoting Complex
BCIP 5-Bromo-4-chloro-3'-indolyphosphate p-toluidine Salt
BSA Bovine Serum Albumin
BUB Budding Uninhibited in Benzimidazole
cAMP Cyclic Adenosine Monophosphate
CDC Cell Division Cycle
CDK Cyclin-Dependent Kinase
DAPI 4', 6'diamidino-2-phenylindole dihydrochloride
DIG Digoxigenin
DTT Dithiothreitol
EDTA Ethylenediaminetetra acetic acid
EGTA Ethylene glycol tetra acetic acid
FITC Fluorescein-5-isothiocyanate
GAP GTPase-activating protein
GTP Guanosine-5'-triphosphate
gDNA Genomic Deoxyribonucleic Acid
HU Hydroxyrurea
MAD Mitotic Arrest Defective
MAP Mitogen-Activated Protein
MCC Mitotic Checkpoint Complex
MEN Mitotic Exit Network
NBT Nitro-blue tetrazolium chloride
OSB One Step Buffer
PCR Polymerase chain reaction
PEG Polyethylene glycol
PEM PIPES/EGTA/$MgSO_4$
PIPES 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid
SAC Spindle Assembly Checkpoint
SC Synthetic Complete
SDS Sodium dodecyl sulfate
SSC Saline-sodium citrate
TE Tris EDTA
Tris Tris (hydroxymethyl) aminomethane
YPD Yeast Peptone Dextrose

1. Introduction

1.1 *Candida albicans*: pathogenesis

Candida albicans is an opportunistic fungal pathogen of humans and is one of the leading causes of fungal infections worldwide (Sudbery *et al.*, 2004). Although a commensal in healthy individuals, *C. albicans* causes life-threatening infections in immuno-compromised people, such as those undergoing cancer chemotherapy, organ transplantation, or stricken with AIDS (Corner *et al.*, 1997; Lortholary *et al.*, 1997). Superficial infections of mucosal membranes are generally located in the mouth, throat, intestines and genitals but infections become life-threatening when the fungus enters the bloodstream and colonizes organs, such as the heart and brain (Wenzel *et al.*, 1991). Since *C. albicans* is increasingly becoming resistant to common azole-based drug treatments, which often have toxic side effects, there is a need to identify new potential drug targets. To assist these efforts, it is important to have a better knowledge of the biology of *C. albicans*, and to expand our knowledge of the mechanisms that regulate various facets of growth and virulence.

1.2 Cell morphogenesis and virulence

C. albicans is multimorphic and has the ability to grow in a range of cell forms, including budding yeast, pseudohyphae or hyphae (Sudbery *et al.*, 2004). Yeast cells are round/ovoid cells that readily separate from one another after budding. Pseudohyphal cells grow as chains of elongated yeast cells and contain constrictions at the sites of septation. In both cell types, a septin ring forms at the bud neck, where mitosis occurs. Septins are important for determining where mitosis takes place and defining the site of

budding and cytokinesis (Gladfelter *et al.*, 2001). Hyphae are highly polarized cells that continuously grow at the apex and do not show constrictions at septation sites in the germ tube. In addition, the nucleus migrates out of the mother cell and into the germ tube prior to the first mitosis, where the first septin ring is located.

The ability of *C. albicans* to switch between the different cell types is critical for virulence because cells incapable of changing states are avirulent (Lo *et al.*, 1997; Saville *et al.*, 2003). It is believed that the yeast cell form is required for dissemination in the bloodstream, while the filamentous form is more capable of penetrating host tissue and escaping phagocytosis by host immune cells (Lo *et al.*, 1997; Gow *et al.*, 2002; Rooney *et al.*, 2002; Torosantucci *et al.*, 2004). Neutrophils are the first line of defence against *Candida* infections, followed by macrophages and dendritic cells (Fidel, 2002; Richardson, 2005). The different cell forms of *C. albicans* also express different virulence factors, underscoring the importance of all cell types during infection and the ability to switch cell fate based on the host environment.

1.3 Environmental control of morphogenesis

Investigations of the regulation of cell-type switching in *C. albicans* have focused heavily on the involvement of environmental stimuli and associated signalling pathways. External inducers of filamentous growth from yeast cells include different temperature levels, pH, carbon source, nutrient levels and metabolites (Berman *et al.*, 2002). For example, while yeast cells are usually maintained at low pH and at temperatures below 30°C, hyphae can be induced by adding serum in combination with a higher temperature. Neutral pH with a higher temperature of 37°C can also induce hyphal growth, whereas a

temperature of 35°C and a pH of 6.0 can induce pseudohyphal growth. Other environmental factors can influence filamentation, such as iron deprivation, high phosphate or glucose concentrations, low nitrogen, oxidative stress, embedded/microaerophilic conditions and different media substrates (Ernst, 2000).

Various conserved and novel signalling pathways link different environmental signals to changes in cell morphology (Figure 1) (Ernst, 2000; Liu, 2001; Whiteway *et al.*, 2006). For example, a mitogen-activated protein (MAP) kinase pathway and the cAMP-dependent protein kinase pathway with associated downstream transcription factors respond to temperature and various hyphal-inducing factors to influence filamentation. A pH-responsive pathway involving Rim101p is also required for filamentation, while embedded conditions stimulate hyphal growth via the transcription factor Czf1p. Negative regulation of the yeast to hyphal switch involves Tup1p-mediated repression through Rfg1p and Nrg1p; *TUP1* mutants exhibited constitutive filamentous growth in most media tested. Additional pathways impinge on several transcription factors, such as Efgh1p, Rbf1p, Fkh2p, Cph2p and Tec1p, which in turn target the polarization machinery through unknown means and also induce hypha-specific factors, including *HWP1, ECE1, HYR1, ALS3, ALS8, RBT1* and *RBT4*, that are important for virulence, (Sharkey *et al.*, 1999; Staab *et al.*, 1999; Braun *et al.*, 2000).

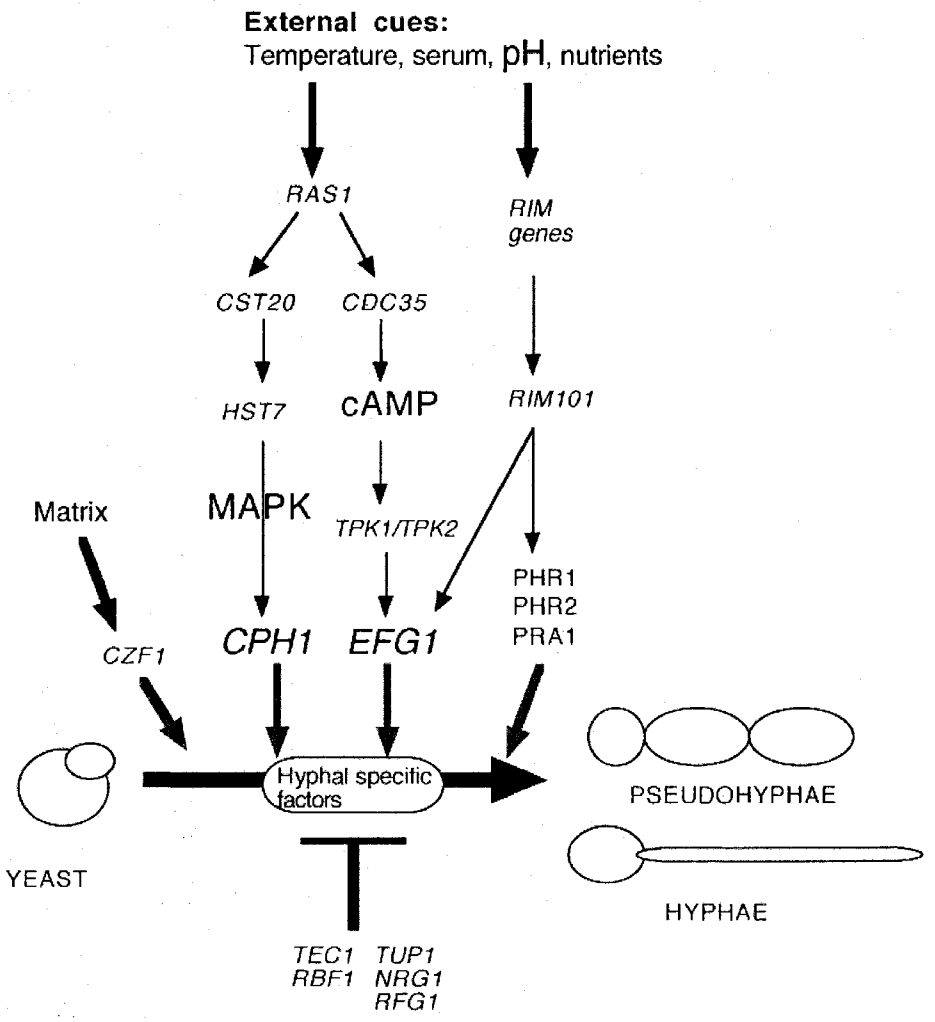


Figure 1: Signaling pathways in *C. albicans*
There are different pathways that regulate cell switching between yeast and pseudohyphal or hyphal growth in *C. albicans*. The positive pathways are the MAP-kinase pathway, cAMP pathway, *RIM101* pH response pathway, *CZF1* matrix pathway and the negative pathways involve *TUP1* and *RBF1* factors. Different external cues act through similar or different pathways for regulating morphogenesis.

1.4 Cell cycle regulation of morphogenesis

In other organisms, cell morphogenesis can also be regulated by internal cues, specifically involving the cell cycle. For example, in the model organism *S. cerevisiae,* which grows as yeast or pseudohyphae, the association of the cyclin-dependent kinase (CDK) Cdc28p with G_1 cyclins *CLN1* and *CLN2* promotes apical growth of the yeast bud, while association with B-type cyclins promotes isotropic growth (Barral *et al.*, 1995; Edgington *et al.*, 1999; Rua *et al.*, 2001). Pseudohyphal growth is associated with a slowing of cell cycle phases. Environment-stimulated pseudohyphae have an extended G_2 phase compared to budding yeast cells (Kron *et al.*, 1994), which is mediated by Swe1p-mediated inhibition of Cdc28p/Clb2p activity (Edgington *et al.*, 1999). Swe1p functions by phosphorylating and inactivating Cdc28p, resulting in a delay in the transition from G_2 to M phase. A morphogenesis checkpoint also exists in this organism, which monitors the state of actin in the developing yeast bud and targets the cell cycle machinery (La Valle *et al.*, 2001). If any perturbation in actin is experienced, this is relayed to activation of Swe1p, which causes a delay in G_2, resulting in a mild polarized elongation of the bud. Pseudohyphal growth can also be induced via a slowing of S phase, which is mediated in part by the DNA synthesis checkpoint factor *RAD53* (Jiang *et al.*, 2003).

The contribution of the cell cycle to the regulation of morphogenesis in *C. albicans* is not well understood, due in part to the fact that very little is known about the cell cycle in this organism. To date, only a few cell cycle homologues have been investigated and we are far from understanding all of the contributing players and their mechanisms of action (Berman, 2006). In addition, evidence supporting a link between

the cell cycle and polarized growth of hyphae, for instance, is conflicting. For example, while there is hyphal versus yeast-specific behaviour in the positioning of the first mitosis, the lengths of cell cycle stages are the same in both yeast and hyphal cells; hyphal growth is not associated with slowing a cell cycle stage like pseudohyphal growth in *S. cerevisiae*. In addition, hyphae can be induced to form in response to serum during any stage of the yeast cell cycle (Hazan *et al.*, 2002; Sudbery *et al.*, 2004). Despite this, an emerging model from more recent evidence suggests that specific cell cycle stages may be linked to different types of polarized morphogenesis in *C. albicans*. G_1 phase appears to be associated with hyphal development, since the G_1 cyclin homologues *HGC1* and *CCN1* are necessary for establishing and maintaining hyphal growth, respectively (Loeb *et al.*, 1999; Zheng *et al.*, 2004), and arresting the cell cycle in G_1 triggers true hyphal and pseudohyphal growth. Depleting the homologue of the G_1 cyclin Cln3p resulted in a G_1 arrest, followed by production of true hyphae and pseudohyphae with active cell cycles (Bachewich *et al.*, 2005a; Chapa y Lazo *et al.*, 2005). In addition, depletion of homologues of other factors believed to play a role in G_1 phase also resulted in constitutive pseudohyphal and hyphal growth (Atir-Lande *et al.*, 2005). Thus, a specific underlying link exists between G_1 phase and hyphal growth.

In contrast, conditions that arrest the yeast cell cycle in S or M phase lead to a different polarized growth response. Depletion of the polo-like kinase Cdc5p resulted in an initial arrest in M phase, followed by a continuous polarized growth of the yeast bud, producing highly elongated cells (Bachewich *et al.*, 2003). These elongated buds were hyphal-like in that they lacked any constrictions along their length, they continuously grew in a polarized manner, and showed migration of the nucleus into the filament

(Bachewich *et al.*, 2003). Microarray analysis also demonstrated that these cells expressed hyphal-specific virulence factors, although at later stages of growth (Bachewich *et al.*, 2005b). Unlike true hyphae, however, the cell cycle was arrested, and the filaments eventually died due to lack of nuclear division. The width of these filaments was wide, like pseudohyphae, but there were no constrictions along the tube length. This elongated bud response thus constitutes a different growth mode than that of true hyphae or pseudohyphae, and may be due to an arrest in M phase since similar filaments were produced when yeast cells were treated with nocodazole (Bai *et al.*, 2002), or depleted of the B-type cyclin *CLB2* (Bensen *et al.*, 2005). Arresting the cell cycle in S phase with high doses of hydroxyurea (HU) caused a similar elongation of the yeast bud (Bachewich *et al.*, 2003). In contrast, slowing the cell cycle in S or M phase results in typical pseudohyphal growth (Shi *et al.*, 2007).

Thus, cell cycle arrest in S or M phase in *C. albicans* results in activation of an unique polarized growth form that is distinct from true hyphae or pseudohyphae. The response is also distinct from when cells are arrested in G_1 of the cell cycle.

1.5 Cell cycle checkpoints and morphogenesis

The fact that yeast cells of *C. albicans* are triggered to grow in a highly polarized manner upon blocking S or M phase is novel, since similar arrests in the cell cycle of other organisms normally cause a cessation in cell proliferation due to cell cycle checkpoint activation (Hartwell *et al.*, 1994). Cell cycle checkpoints are in place to ensure that the cell cycle is blocked in order to allow repair of any damage. In *S. cerevisiae*, for example, depleting cells of Cdc5p or treating them with arresting

concentrations of HU or nocodazole, causes them to arrest in a large-budded state (Slater, 1973; Jacobs *et al.*, 1988).

The elongated bud growth that occurs in *C. albicans* cells that are arrested in S or M phase appears to be mediated by different cell cycle checkpoints. For example, Cdc5p-dependent polarization required the spindle checkpoint factor Bub2p, while HU-induced polarized growth did not (Bachewich *et al.*, 2005b). Nocodazole-induced bud growth required another spindle checkpoint factor, Mad2p (Bai *et al.*, 2002), while the effector kinase of the S-phase checkpoint, Rad53p, was needed for HU-induced polarized growth (Shi *et al.*, 2007). Intriguingly, none of these checkpoint factors, with the exception of Rad53p, were required for true hyphal growth. These results suggest that cell cycle-checkpoint activated growth occurs in *C. albicans*, and reveal a unique level of regulation of polarized cell morphogenesis in this pathogen. Since Mad2p is also required for virulence in *C. albicans*, checkpoint-activated polarized growth may be important for pathogenesis within the host. However, the molecular nature of the checkpoint pathways in *C. albicans* and their link with the polarized growth machinery are not known. The regulatory circuit governing basic mitotic progression in *C. albicans* is also not clear.

1.5.1 The spindle assembly checkpoint pathways and regulation of mitosis

The spindle assembly checkpoint (SAC) pathways have not been characterized in *C. albicans* but are well understood in the model yeast *S. cerevisiae*. The SAC is a conserved system that monitors spindle function, including bipolar chromosome

attachment to microtubules, tension across the kinetochores and spindle orientation. When defects in these processes occur, activation of the system leads to the arrest of cells in mitosis (Lew *et al.*, 2003). The spindle checkpoint includes two distinct pathways, where the function of one is to maintain sister chromatid cohesion and prevent the metaphase-to-anaphase transition, while the other maintains CDK/cyclin activity and thus prevents exit from mitosis.

1.5.2 Metaphase-to-Anaphase transition

The first branch of the SAC is responsible primarily for preventing the metaphase-to-anaphase transition (Figure 2). It is activated via spindle/kinetochore damage, which causes recruitment of checkpoint proteins Mad1p, Mad2p, Mad3p, Bub1p, Bub3p and Mps1p (Sudakin *et al.*, 2001) to the kinetochores. *MAD1-3* (Mitotic Arrest Defective) and *BUB1-3* (Budding Uninhibited in Benzimidazole) gene products are required to induce a metaphase arrest in the presence of disrupted spindles (Hoyt *et al.*, 1991; Li *et al.*, 1991) and were originally isolated based on sensitivity of mutants to spindle-damaging drugs such as benomyl and nocodazole, where cells did not show a cell cycle arrest. Upon activation, Mad2p functions to prevent degradation of an anaphase inhibitor called securin, or Pds1p in *S. cerevisiae*. Pds1p inhibits the seperase Esp1p, which is required to cleave chromosome cohesion subunits, including Scc1p. Normal mitosis proceeds if Pds1p can be degraded, allowing Esp1p to cleave Scc1p and permit anaphase progression (Cohen-Fix *et al.*, 1999; Shirayama *et al.*, 1999). Of these factors, only Mad2p has been investigated in *C. albicans*, where it is required for nocodazole-induced polarized growth and virulence (Bai *et al.*, 2002).

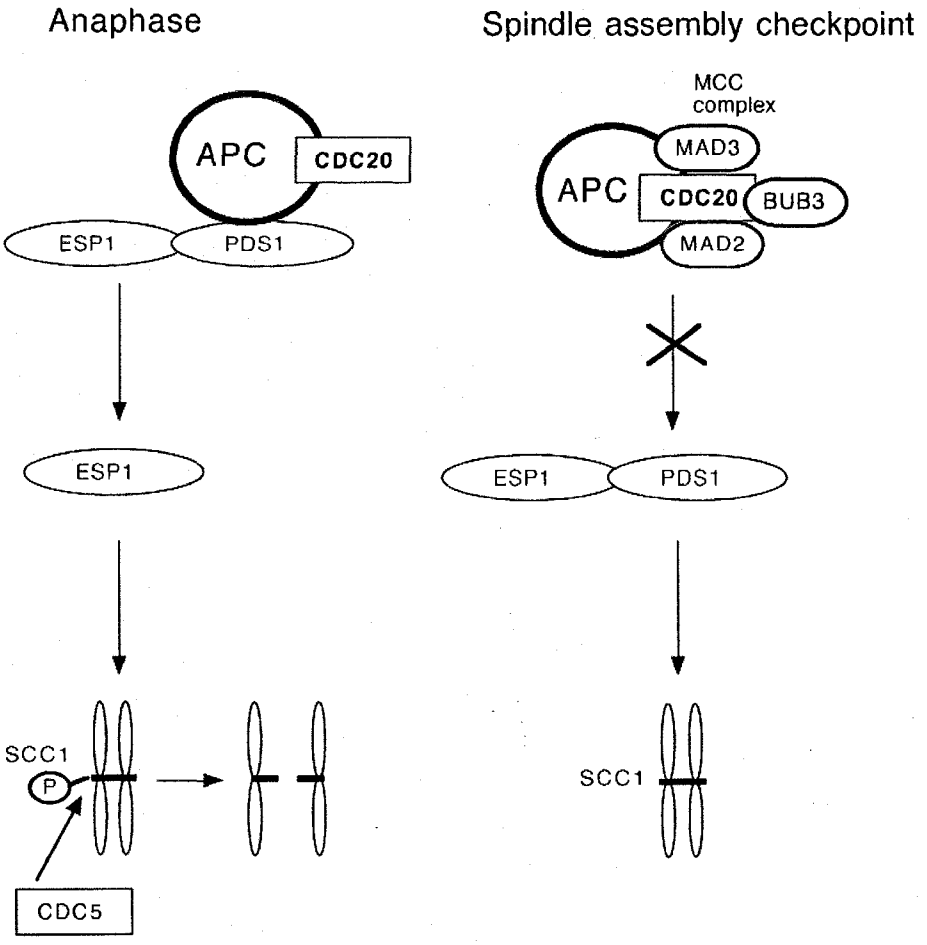


Figure 2: Schematic representation of the spindle checkpoint pathway
Separation of sister chromatids depend on the APC^{Cdc20} ubiquination of securin Pds1p. Upon degradation of Pds1p, seperase Esp1p is released and is responsible for cleavage of cohesion factor Scc1p. The spindle checkpoint factors Mad2p, Mad3p and Bub3p form a mitotic checkpoint complex (MCC) with Cdc20p and inhibiting APC^{Cdc20} from degrading Pds1p, maintaining sister chromatids cohesion and preventing onset of anaphase.

1.5.3 Mitotic Exit

The second branch of the spindle checkpoint is also activated upon spindle damage or spindle misorientation, and prevents mitotic exit through regulating the mitotic exit network (MEN). The MEN is a signal transduction pathway that involves many factors but ultimately regulates the release of the phosphatase Cdc14p from the nucleolus, which is a key event for mitotic exit (Figure 3). Upon activation of MEN, the guanine nucleotide exchange factor Lte1p modifies the Ras-like GTPase Tem1p from its inactive to active form. The active form of Tem1p leads to a protein kinase signalling cascade including Cdc15p, Dbf2p and Mob1p, which in turn allow release of Cdc14p phosphotase from the nucleolus (Bardin *et al.*, 2001). The release of Cdc14p from the nucleolus leads to degradation of the mitotic cyclin Clb2p and down-regulation of Cdc28p/Clb2p CDK activity, ultimately leading to mitotic exit (Visintin *et al.*, 1999). Tem1p is negatively regulated by the Bub2p/Bfa1p GTPase-activating protein (GAP) complex. During normal mitotic progression, Cdc5p antagonizes Bub2p/Bfa1p, allowing Tem1p activation. In the presence of spindle errors, Cdc5p is negatively regulated by Mad2p and Mps1p, thus allowing activation of the Bub2p-containing complex, and inhibition of Tem1p, preventing mitotic exit (Wang *et al.*, 2000; Hu *et al.*, 2001; Hu *et al.*, 2002; Kim *et al.*, 2006). Of these factors, Cdc5p, Bub2p and Cdc14p have been investigated in *C. albicans*, where Bub2p is required for Cdc5p-depleted polarized growth (Bachewich *et al.*, 2005b), and Cdc14p is required for mitotic exit (Clemente-Blanco *et al.*, 2006).

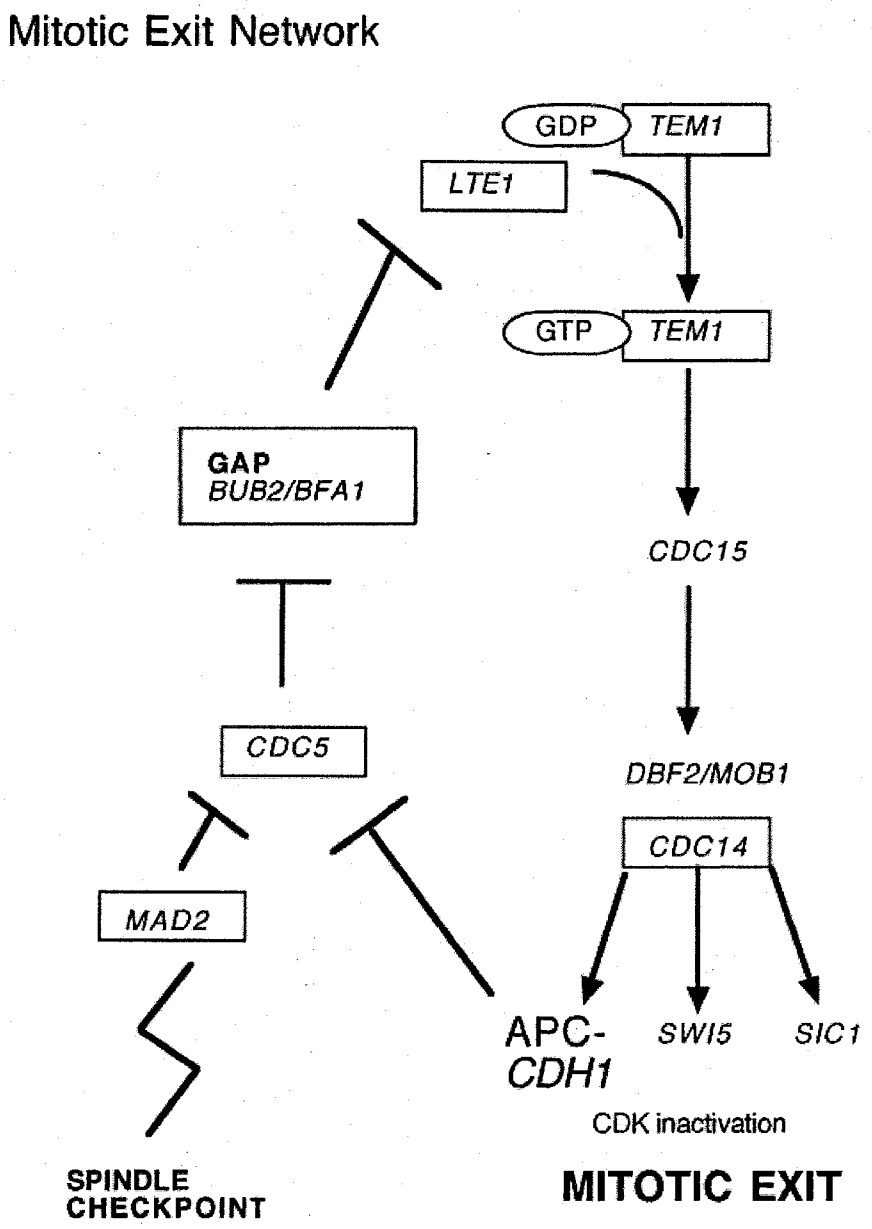


Figure 3: Mitotic exit network pathway
Ras-like GTPase Tem1p is activated by guanine nucleotide exchange factor Lte1p. Tem1p activates Cdc15p kinase which phosphorylates and activates the inhibitory Dbf2p/Mob1p complex of phosphatese Cdc14p, leading to eventual release of Cdc14p from the nucleolus. Cdc14p is responsible for dephosphorylation and stabilization of CDK inhibitor Sic1p and its transcription factor Swi5p. Cdc14p is also responsible for dephosphorylation of Cdh1p, which then becomes activated and degrades polo kinase Cdc5p, which normally helps activation of Tem1p by relieving the inhibitory effect of the GAP complex. The spindle checkpoint will reinforce GAP complex inhibition on Tem1p and prevent mitotic exit by preventing Bub2p/Bfa1p phosphorylation.

1.5.4 APC activators: major regulators of mitosis and targets of the spindle checkpoint pathway

Ubiquitin-mediated protein degradation plays a critical role in regulatory cell cycle progression. The ubiquitin ligase complex that is responsible for mitotic progression, the anaphase promoting complex (APC), is also a major target of the SAC. The APC is a conserved multiprotein E3 ubiquitin ligase complex that is important for controlling cell cycle progression through ubiquination of different substrates, including mitotic cyclins (Harper *et al.*, 2002; Baker *et al.*, 2007). Cells with mutations in various APC subunits arrest in metaphase and have undegraded B-type cyclins (Zachariae *et al.*, 1996). The APC has two main activator factors, Cdc20p and Cdh1p, which are responsible for degradation of the proteins necessary for the onset of anaphase and mitotic exit (Harper *et al.*, 2002).

Cdc20p is a main target of the spindle checkpoint, where Mad2p, Mad3p and Bub3p form an inhibitory mitotic checkpoint complex (MCC) with Cdc20p, thus preventing activation of the APC and the onset of anaphase (Sudakin *et al.*, 2001; May *et al.*, 2006; Burton *et al.*, 2007). APC^{Cdc20} in turn targets Pds1p, Clb2p and Clb5p (B-type cyclin) for destruction (Shirayama *et al.*, 1999; Baumer *et al.*, 2000; Irniger,2002; Wasch *et al.*, 2002). Although *CDC20* mutants arrest in metaphase, Cdc20p is also important for late mitosis because deletion of *PDS1* can suppress the *CDC20* mutant phenotype, but cells still fail to exit mitosis and arrest with elongated spindles (Lim *et al.*, 1998). Overexpression of *CDC20* allowed cells to exit mitosis in the presence of DNA damage or spindle defects (Hwang *et al.*, 1998).

Cdc20p is regulated at multiple levels. Cks1p, a protein that interacts with CDKs, promotes mitosis by modulating transcriptional activation of *CDC20* (Morris *et al.*, 2003). Cdc20p protein levels fluctuate during the cell cycle like mitotic cyclins, and destruction of Cdc20p in late mitosis/G_1 is initiated by activation of the APC via another factor, Cdh1p (Visintin *et al.*, 1997; Lim *et al.*, 1998; Shirayama *et al.*, 1998; Huang *et al.*, 2001; Morris *et al.*, 2003). Cdc20p may also be regulated by phosphorylation, but the mechanism is not clear (Harper *et al.*, 2002).

Cdh1p is a second APC activator, which has a role in degradation of late mitotic cyclins and lowering CDK activity in order for cells to exit mitosis. Cdh1p is a downstream effector of the MEN, where it becomes dephosphorylated and thus activated by Cdc14p, leading to degradation of Clb2p, Cdc5p, as well as various proteins necessary for spindle functions (Figure 2). Cdh1p activity is targeted by the spindle checkpoint pathway, since it is important for mitotic exit. Unlike *CDC20*, *CDH1* is not essential in *S. cerevisiae*, and cells lacking Cdh1p are small, and grow slightly slower than the wild-type cells (Visintin *et al.*, 1997; Jorgensen *et al.*, 2002). Cdh1p levels are constant throughout the cell cycle, and its activity and ability to bind to the APC is negatively regulated by phosphorylation via CDK activity (Prinz *et al.*, 1998; Jaspersen *et al.*, 1999; Yeong *et al.*, 2000; Huang *et al.*, 2001). To date, APC activity and its role in regulating mitosis in *C. albicans* have not been investigated.

1.6 Overview

The aim of this study was to help define the mitotic regulatory circuit in *C. albicans*, as well as determine the pathway linking checkpoint activation and mitotic

progression to elongated bud growth in *C. albicans*. Specifically, we investigated the function of putative targets of the spindle checkpoint and major regulators of mitosis, including the APC activators Cdc20p and Cdh1p, as well as the checkpoint protein, Mad2p. We show that Cdc20p and Cdh1p are important for regulating mitosis and morphogenesis in *C. albicans*, but in a different manner than their counterparts in *S. cerevisiae*. The results extend our knowledge of the mitotic regulatory circuit that is emerging in *C. albicans*, which had some unique characteristics, and the pathway underlying checkpoint- activated polarized growth.

2. Materials and Methods

2.1 Strains, oligonucleotides, culture conditions

Strains, oligonucleotides and plasmids used in this study are listed in Tables 1, 2 and 3, respectively. For conditional strains, cells were grown at 30°C in synthetic complete (SC) medium containing 0.67% yeast nitrogen base, 2% glucose and amino acids supplemented with or without 2.5 mM methionine and 0.5 mM cysteine for repression and induction of the *MET3* promoter, respectively (Care *et al.*, 1999). Alternatively, cells were gown at 30°C in SC medium containing 2% glucose or 2% casamino acids to repress or induce the *PCK1* promoter, respectively (Leuker *et al.*, 1997). Strains carrying deletions of genes were grown in rich medium (YPD) containing 1% yeast extract, 2% peptone and 2% dextrose. To study cells under hyphal-inducing conditions, 10% fetal bovine serum (Hyclone) was added to growth medium, and cells were incubated at 37°C. All media were supplemented with 100 mg/L of uridine, histidine or arginine to allow optimal growth of *URA3+*, *HIS1+* and *ARG4+* prototrophs, except when being selected (Bensen *et al.*, 2002). Most strains were grown overnight, then diluted into fresh medium to an $O.D._{600nm}$ of 0.2. In the case of determining growth rate of *MAD2*-deleted strains, cells were serial diluted and grown overnight such that they were in exponentional growth the next day, prior to diluting into fresh medium to an $O.D._{600nm}$ of 0.2. For colony size comparison of *MAD2*-deleted cells, cells were grown overnight and diluted to an $O.D._{600nm}$ of 0.05. For agar invasion assays, *CDH1*-deleted and control strains were grown overnight in rich YPD media, diluted to an $O.D._{600nm}$ of 0.2, and 50 μl were spotted on solid YPD medium. After 24 or 72 h of growth, cells were washed off.

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

Strains	Genotype	Source
SC5314	Prototrophic	Fonzi *et al.* 1993
BWP17	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG*	Wilson *et al.* 1999
HCCA1	BWP17 *CDC20/cdc20::URA3*	This study
HCCA5	BWP17 *CDH1/cdh1::URA3*	This study
HCCA7	BWP17 *CDC5/cdc5::hisG*	This study
HCCA16	BWP17 *CDC20/cdc20::URA3*, BWP17 *MET3::HIS1*	This study
HCCA23	BWP17 *cdc20::URA3/MET3::CDC20:HIS1*	This study
HCCA26	BWP17 *cdh1::URA3/MET3::CDH1:HIS1*	This study
HCCA34	BWP17 *cdc5::hisG/MET3::CDC5:ARG4*	This study
HCCA118	BWP17 *cdc5::hisG/MET3::CDC5:ARG4*	This study
HCCA45	BWP17 *cdh1::URA3/cdh1::HIS1*	This study
HCCA47	BWP17 *mad2::URA3/MAD2*	This study
HCCA56	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, mad2::URA3/mad2::HIS1*	This study
HCCA67	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, CDH1/cdh1::URA3*	This study
HCCA68	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, mad2::URA3/MAL2::MAD2:HIS1*	This study
HCCA75	BWP17 *MAD2/mad2::URA3*	This study
HCCA100	BWP17 pRM100 (*URA3, HIS1*)	This study
HCCA109	BWP17 *cdc20::URA3/MET3::CDC20:HIS1*	This study
HCCA110	BWP17 *mad2::URA3/mad2::HIS1*	This study
HCCA216	BWP17 *mad2::URA3/mad2::HIS1*	This study
HCCA114	BWP17 *CDH1/cdh1::HIS1*	This study
HCCA126	BWP17 *cdc5::hisG/MET3::CDC5:ARG4*, pRM100 (*URA3, HIS1*)	This study
HCCA131	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, CDH1/cdh1::URA3*	This study
HCCA143	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, cdh1::URA3/cdh1::HIS1*	This study
HCCA153	BWP17 *cdh1::URA3/cdh1::ARG4*	This study
HCCA164	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, PCK1::CDC20:URA3*	This study
HCCA180	BWP17 *cdc5::hisG/MET3::CDC5:ARG4, PCK1:URA3* (pJA24)	This study
HCCA184	BWP17 *yke2::URA3/yke2::HIS1*	This study
HCCA194	BWP17 *cdc5::hisG/MET3::CDC5:ARG4,*	This study

	yke2::URA3/yke2::HIS1	
HCCA211	BWP17 *cdh1::URA3/cdh1::ARG4,*	This study
	CLB2/CLB2-3HA:*HIS1*	This study
HCCA218	BWP17 *mob1::URA3/MET3::MOB1:HIS1*	This study
HCCA223	BWP17 *CDC5/CDC5, CDC20/PCK1::CDC20:URA3*	This study
HCCA228	BWP17 *CDH1/CDH1, CLB2/CLB2*-3HA:*HIS1*	This study

Table 2: Oligonucleotides used in this study

CDC20

GSCDC20F	GACCCAGGACTAACAATTTCTT
HC6R	CAGTTGCGCGTTCGTGTAAT
HCGS1	AGTCATTTCCATCCATCAGTCTAATCAACT
HCGS1R	GGATTGTAGTTGATCAATGATATGGATCTT
HC2F	GCTTATTTCCATTCAACTATAATACTTATTCAACCCCTAA CATTATGTCATTGGTATCTCCCAACAGTAAACCAACAAT TTATAGGGCGAATTGGA GCT C
HC2R	ATATGGTTTGCATTAAGTAAAATCGTTTGGTAGTGACCA CTCTTTGGTGGTTTTACAATGCCAAAATCGTTATTATAGA GGACGGTATCGATAAGCTTGA
HC4F	GCTTATTTCCATTCA ACTATAATACTTATTCAA CCCCTAA CATTATGTCATTGGTATCTCCCA ACA GTA AACCAACAAT TGGATCCTGGAGGATGAGGAG
HC4R	TGATGGTCTTTTCAACGATTGCTGTTTCAATGGAGTATCAT TTGGTAAATGGACTGTTCTGTTAGATATATTCGGACTCATG TTTTCTGGGGAGGGT
HCGS21F	ATGTCATTGGTATCTCCCAACAGTAAACCA
HCGS4B	TATATGTATTTCTGGTGCCGCACTAGGTAA
HCGS22F	AAGATCCATATCATTGATCAACTACAATCCGGATCCTGGA GGATGAGGAG
HCGS22R	TGGTTTACTGTTGGGAGATACCAATGACATCATGTTTTCTG GGGAGGGTA
HC12FB	CGGGATCCTCATTGATCAACTACAATCC
HC12RB	CGGGATTCCGTAGTGACCACTCTTTGGTG
PCK1F	CAGATCGATAATTGGTGCGA
PCK1R	ACAGCAGTAGGAGGAGCCAT

CDH1

GSCDH1F	CTACAATATTGGAGTAAGGG
HCGS16R	ATGACCAGGCCAATGGCTATATAATTCGAG
HC3F	CTGTTTGAGACTCCTAGGTCGCCATCACGATCAACCAGA AGTCTAAATCCTCCCAAGTTGAACGAAATGGGTGCTATA

	CATATAGGGCGAATTGGAGCTC
HC3R	CCCGTTTCAAAGAAGACATTTGGGTTCATTGTTGATTGAATT TCTGTATCAATTGGCTGCAGTTACTCCAGGACAACTTAGACG GTATCGATAAGCTTGA
HC5F	CTGTTTGAGACTCCTAGGTCGCCATCACGATCAACCAGA AGTCTAAATCCTCCCAAGTTGAACGAAATGGGTGCTATA CAGGAT CCTGGAGGATGAGGAG
HC5R	GATTTGCTTCCTCTTCTCTTCTAATTTCTATTTCATTATCAG CGTTTCTAGTGTTTCTCAAATCCGGTAGTATTTCCTCAT GTTTTCTGGGGAGGGTA
HCGS7F	TCTATCAGCAGGTCATGAAGACTACAAACT
HCGS7R2	AGATGTTCAACTCTTGATTAGTATGGATTG
HCGS23F	ATGGACAAAAGAGATTCTAATAGACCTTCT
HCGS9R	GGTGACAATGAGTACAATTCTTGTTGCAAG
HCGS16F	TTCAAGTCGTTCAAGTGAAAGTGTCACATT
HCGS16R	ATGACCAGGCCAATGGCTATATAATTCGAG
HCGS17F	AGACCTTCTAAGGACAATGCTATCCGTAATTATAGGGCGA ATTGGAGCTCTATAGGGCGAATTGGAGCTC
HCGS17R	AATGTGACACTTTCACTTTCACTTGAACGACTTGAAGACG GTATC GAT AAG CTTGA

MAD2

HCSF	TCTATTCCTGGGAGATCACC
HCSR	GATGATCCTCATACTATAGC
HC8F	CCCAAATTCATATTTCCAATTAACTTTCCCAAGTTTAACTA ATTCGAATAATAACCAACGACGATGATTCCAAATCCAAT
HC8R	TCCTCATTGTATTGCTTTGGCTATTATAATTATTAGTTTGAA TTTAAATCCAATTGAAATCAAATTAAACCATGAACCACGA CGGTATCGATAAGCTTGA
HCGS10F	TTAATATTTCTCGACGATAATTCCAAATGG
HCGS10R	GAAGGGATAGATAGGGTTGTGTGGAAACTG
HCGS11F	CAGTTTCCACACAACCCTATCTATCCCTTCTATAGGGCGA ATTGGAGCTC
HCGS11R	GGATTATACAATTAGTTCTAAAGGTTCAAC/GACGGTATC GATAAGCTTGA
HCGS12F	GTTGAACCTTTAGAACTAATTGTATAATCC
HCGS12R	AATGTCTACTCCATTAAGTTTGGTTGTACC

CDC5

HC11	ACTATGAATAGAGAAAGCAG
CB87R	TGCCTCGACGATAGTCTCGTCC

HC10F	CGAGCAGGACCAATTGCGATGTAATCA AAATTGTTAACAT GAGTCTGTGTCTATTCGCCTACTACTAACCTTAGAGTGTTGG ATCCCCCCTTTAGTAAGA
HC10R	GACAGGAGTAATGTTATTAGCTCTAGCATTGAGCTG GCCA CTATTCAATGGCTGTAA AGGTTGTGAACGAAGCGCCGACA TGTTTTCTGGGGAGGGTA
HCGS13F	TCGAGCAGGACCAATTGCGATGTAATCAAA
HCGS13R	GGTTAAACCTCTTTAATAATCAATGCTGGT
HCGS13R	GGTTAAACCTCTTTAATAATCAATGCTGGT
HCGS14F	ACCAGCATTGATTATTAAAGAGGTTTAACCGGATCCCCCCT TTAG TAAGA
HCGS14R	CTGTAAAGGTTGTGAACGAAGCGCCGACATGTTTTCTGGG GAGGGTA
HCGS15F	ATG TCG GCG CTT CGT TCA CAA CCT TTA CAG
HCGS15R	TAA AGA ATC TAA CCT CTG GTT CAG ACA CTC

***CLB2*-HA**

HC18F	TTGGCAGTCAATGTAATGGA
HC18R	TTTCGGTGGGTCCATATAAT
HCGS32F	CAGAAGAAGTGGTACCAAGC
HCGS32R	CTCTTCTGCTTCTGCTACCA
HCGS33F	TGGTAGCAGAAGCAGAAGAGCGGATCCCCGGGTTAATTAA
HCGS33R	ACCTAGATCCAATAGTCATCGAATTCCGGAATATTTATG AGAAAC
HCGS34F	GATGACTATTGG ATCTAGGT
HCGS34R	TCTGAAACATGATTG AGTAG

Southern probes

HCSP7	GTAGTAGTTACCAGCATGCA
HCSP8B	ATTGGGTCAGAGTGTGGGTT
HCSP3B	GGTGGTGGCAATTCTATATC
HCSP4	GTAACAGTTTCCACATGTAC
HCSP5B	TAATTATGAACAAGGCGACG
HCSP6	GGTGATCTCCCAGGAATAGA
HCSP13	CGATGCTCTTCGCAAGAAAG
HCSP14	CCAGAATCTGCCGTTGAAGC

For gene sequencing

SQ1	CCACCATCAAATTCCCTTGA
SQ2	CCCTCATAAGTCAAGGCGGT
SQ3	HCCATTGGGTTAGAAGATGC

SQ4	GCTGCTGTTAAGGCATTATG
SQ5	GACGGTGGTCATGATAGTAA
SQ11	AGTGTTTGATTCGATATCCC

Table 3:	**Plasmids used in this study**	
Plasmid	Description	Source
pHC4	pJA24 *PCK1::CDC20:URA3*	This study
pJA24	pUC18 *PCK1:URA3*	J. Ash
pRM100	pUC19 *URA3,HIS1*	J. Pla
pBS-*cURA3*	pBluescript *URA3*	A. J. P. Brown
pBS-*cHIS1*	pBluescript *HIS1*	C. Bachewich
pFA-*HIS1*-Met3p	pFA *HIS1*-Met3p	Gola et al. 2003
pFA-*ARG4*-Met3p	pFA *ARG4*-Met3p	Gola et al. 2003
pFA-*HIS1*-Mal2p	pFA *HIS1*-Mal2p	Gola et al. 2003
pFA-GFP-*HIS1*	pFA GFP-*HIS1*	Gola et al. 2003
pMG2091	pUC18 *HIS1*-3HA-tag	J. Berman

2.2 Transformation of *C. albicans*

To transform deletion or promoter replacement constructs, a lithium acetate one-step transformation protocol was utilized (Chen *et al.*, 1992). Briefly, one-step-buffer (OSB) was prepared, where 25 µl of salmon sperm DNA (10 mg/ml stock, Invitrogen) were first boiled, and snap-cooled on ice for 5 min prior to adding 25 µl of 4M DTT (Fischer). Then, 200 µl of 1M Lithium Acetate and 800 µl of 50% PEG 4000 (Sigma) were added to the mixture. This OSB solution (100 µl) was added to 200-300 µl of washed stationary phase cells and vortexed for 1 min prior to adding 5-10 µg of transforming DNA in a maximum volume of 10 µl. The reaction mix was vortexed for 1 min and incubated overnight at 30°C. The following day, the mixture was heat-shocked for 60 min at 43°C and plated on selective solid medium. Transformants were streaked to single colonies three times on fresh selective medium prior to screening. For increased transformation efficiency in the regulated *CDC5* strains, cells were grown overnight in

minimal inducing medium lacking methionine and cysteine, then transferred into rich YPD medium for at least 1 h prior to collection for transformation.

2.3 Strain construction

cdc5/MET3::CDC5

A *CDC5* conditional strain (Bachewich *et al.*, 2003) was re-created by placing *CDC5* under the control of the *MET3* promoter in strain BWP17, which is auxotrophic for *ARG4, URA3* and *HIS1*. The first copy of *CDC5* was deleted using the *URA3* blaster method (Fonzi *et al.*, 1993; Bachewich *et al.*, 2003), followed by looping out of the *URA3* marker and selection of *URA3*-prototrophs on 5'-Fluoro-otic acid (Sigma) (Bachewich *et al.*, 2003), resulting in strain HCCA7. The second copy of *CDC5* was then placed under the regulation of the *MET3* promoter, creating strains HCCA34 and HCCA118. A 2-step fusion PCR method was employed (Yang *et al.*, 2004). Oligonucleotides HCGS13F and HCGS13R were used to amplify 640 bp of the 5' flanking sequence of *CDC5*, while HCGS15F and HCGS15R were used to amplify 518 bp including downstream of the start site of the *CDC5* ORF (orf19.6010). PCR conditions included 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 41-49°C for 30 sec, 68°C for 55 sec and a final 7 min elongation at 68°C. Oligonucleotides HCGS14F and HCGS14R were used to amplify an *ARG4-MET3* fragment from plasmid pFA-*ARG4*-Met3p (Gola *et al.*, 2003) with running conditions consisting of 94°C for 4 min, followed by 25 cycles of 94°C for 30 sec, 41°C for 30 sec, 68°C for 3 min and finally with a 7 min elongation at 68°C. The 3 resulting fragments were combined and amplified using oligonucleotides HCGS13F and HCGS15R to make the promoter

replacement construct. The PCR conditions included 94°C for 2 min, followed by 10 cycles of 94°C for 10 sec, 50°C for 30 sec, 68°C for 4 min 30 sec, 15 cycles of the same with 20 sec added to the extension/elongation time after each cycle, and a final 7 min elongation at 68°C. Expand Long Template Polymerase with Buffer 3 (Roche) was used for the PCR reaction. To generate the prototrophic strain HCCA126, strain HCCA118 was transformed with pRM100 (J. Pla), which contains both *Candida URA3* and *HIS1* markers.

cdc20/MET3::CDC20

In order to construct a strain containing a single copy of *CDC20* under the control of the *MET3* promoter, the first copy was replaced with a deletion construct containing a *URA3* marker and 80 nucleotides of flanking sequences of *CDC20*. The construct was created using oligonucleotides HC2F and HC2R with pBS-c*URA3* (A. J. P. Brown) as a template. The PCR conditions for amplification of the fragment included 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 41°C for 1 min, 68°C for 1 min 30 sec and a final elongation step at 68°C for 7 min. The PCR construct was then transformed into strain BWP17 to generate strain HCCA1. To place the remaining copy of *CDC20* under the control of a *MET3* promoter, oligonucleotides HC4F and HC4R, which contained 80 nucleotides identical to the sequences upstream and downstream of the *CDC20* start site, respectively, were used with plasmid pFA-*HIS1*-Met3p (Gola *et al.*, 2003) to create a *HIS1-MET3* promoter replacement construct. The PCR conditions included 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 41°C for 1 min, 68°C for 3 min and a final elongation time of 7 min at 68°C. The final product was transformed into strain HCCA1, generating strain HCCA23. Since the *C. albicans* genome annotation was updated and

version 19 indicated a new start site for *CDC20* while this work was in progress, we created a second *CDC20*-regulated strain with the new start site, but using the 2-step PCR fusion method, which allowed for longer identical sequences and a higher yield of positive transformants. Oligonucleotides HCGS1 and HCGS1R were used to amplify 515 bp of the 5' flanking sequence of *CDC20* and oligonucleotides HCGS21F and HCGS4B were used to amplify a fragment containing the new start site of *CDC20* and 720 bp of downstream sequence. PCR conditions included 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 41°C for 30 sec, 68°C for 55 sec and a final 7 min elongation at 68°C. A third fragment containing the *HIS1-MET3* promoter sequence was amplified from plasmid pFA-*HIS*-Met3p using oligonucleotides HCGS22F and HCGS22R, with running conditions consisting of 94°C for 4 min, followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, 68°C for 3 min and finally with a 7 min elongation at 68°C. All 3 fragments were then combined in a final fusion PCR reaction in a 1:3:1 (50:150:50 ng) ratio of DNA, using oligonucleotides HCGS1 and HCGS4B. PCR conditions included 94°C for 2 min, followed by 10 cycles with 94°C for 10 sec, 48°C for 30 sec, 68°C for 4 min 30 sec, 15 cycles of the same followed by an additional 20 sec extension, and a 7 min final elongation at 68°C. Transformation of the final construct into strain HCCA1 resulted in strain HCCA109.

cdc5/MET3::CDC5, PCK1::CDC20

In order to construct a strain where *CDC20* could be overexpressed in the *CDC5*-regulated background, we first cloned *CDC20* into plasmid pJA24 (Bachewich *et al.*, 2003), which contained the *PCK1* promoter and the *hisG-URA3-hisG* cassette (Fonzi *et al.*, 1993). Oligonucleotides HC12FB and HC12RB were used to PCR amplify a

fragment containing the entire *CDC20* ORF along with 300 bp of 3' untranslated terminator region, flanked by *Bam*HI sites. The PCR conditions included 94°C for 3 min followed by 10 cycles at 94°C for 30 sec, 36°C for 30 sec and 94°C for 2 min, 15 cycles of the same with a 20 sec extension after each cycle, and a final elongation step at 68°C for 7 min. The product was restriction digested with *Bam*HI and the plasmid was cut with *Bgl*II. Removal of 5' phosphate residues from the linearized plasmid was performed by incubating in 0.5 units of Calf Intestinal Alkaline Phosphatase (NEB) per μg of plasmid DNA at 37°C for 1 h. The PCR product was then cleaned and cloned into pJA24. For the ligation process, 10 ng of plasmid DNA, 0.5 μl T4 DNA ligase (NEB) with 1 μl of T4 buffer (NEB) and different ratios of insert DNA (1:1, 1:2, 1:4, 1:8) were combined in a final volume of 10 μl. The ligation mixture was incubated at room temperature for 3 h and transformed into 50 μl of competent *E. coli* DH5α cells. Cells were chilled on ice for 30 min, subjected to heat shock at 37°C for 20 sec and cooled on ice for 2 min. Pre-warmed YT media (950 μl) was added and cells were incubated at 37°C for 1 h. Following incubation, cells were centrifuged at 15 700 x g, the supernatant was removed, the cells were spread onto YT plates containing ampicillin (100 μg/ml) and grown overnight at 37°C. Plasmids were extracted from ampicillin-resistant *E. coli* colonies using a plasmid mini-prep kit (Quiagen). Restriction enzyme digests with *Kpn*I and *Eco*RI were used to identify the positive clones. Positive clones were sequenced at the McGill University/Genome Quebec Innovation Centre, using sequencing oligonucleotides SQ1, SQ2, SQ3, SQ4 and SQ5. Plasmid pHC4 was obtained and subsequently transformed into strain HCC118 to create strain HCCA164 (*cdc5/MET3::CDC5, PCK1::CDC20*). An empty pJA24 plasmid was also transformed

into strain HCCA118 to generate control strain HCCA180 (*cdc5/MET3::CDC5,* pJA24). Strain HCCA223 (BWP17 *PCK1::CDC20*) was created by transforming pHC4 into strain BWP17.

cdh1/cdh1

To create the *CDH1* deletion strain, the first copy of *CDH1* was replaced with a *URA3* marker to generate strain HCCA5. Oligonucleotides HC3F and HC3R were used with plasmid pBS-*cURA3* to generate a deletion construct containing a *URA3* marker with 80 nucleotide flanking sequences of *CDH1*. The PCR conditions for amplification of the fragment included 94°C for 4 min, followed by 25 cycles with 94°C for 1 min, 40°C for 1 min, 68°C for 1 min 35 sec and a final elongation step at 68°C for 7 min. The second copy of *CDH1* was replaced with a *HIS1* marker created with the 2-step fusion PCR method, to generate strain HCCA45. Oligonucleotides HCGS7F and HCGS7R2 were used to amplify a 620 bp fragment homologous to the 5' flank of *CDH1* and oligonucleotides HCGS16F and HCGS16R were used to amplify a 560 bp fragment homologous to the 3' flank of the gene. PCR conditions for the flanking fragments included 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 46-48°C for 30 sec and 68°C for 55 sec and a final 7 min run at 68°C. Oligonucleotides HCGS17F and HCGS17R were used to amplify a fragment containing the *HIS1* marker from plasmid pBS-*cHIS1*. The PCR conditions were the same as above with an annealing temperature of 40°C and an extension time of 1 min 30 sec. The 3 individual fragments were then combined in a 1:3:1 (50:150:50 ng) ratio and amplified using oligonucleotides HCGS7F and HCGS16R to create a 2.6 kb deletion construct. The PCR conditions included 94°C for 2 min, followed by 10 cycles at 94°C for 10 sec, 49°C for 30 sec, 68°C for 2 min 35

sec, and 15 cycles of the same with an additional 20 sec extension/elongation, followed by a final elongation of 7 min at 68°C. To determine if *CDH1* was required for Cdc5p-depleted polarized growth, both *CDH1* alleles were deleted from strain HCCA34 (*cdc5/MET3::CDC5*) using the 2-step fusion PCR method as described. In order to confirm the *CDH1* deletion phenotype, a strain carrying a single copy of *CDH1* under control of the *MET3* promoter was created. Oligonucleotides HC5F and HC5R were used to generate a *HIS1-MET3* promoter-containing fragment with 80 nucleotides identical to the sequences upstream and downstream of the *CDH1* start site, which was transformed into *CDH1* heterozygous strain HCCA5, creating strain HCCA26. The PCR conditions included 94°C for 4 min, followed by 25 cycles with 94°C for 1 min, 45°C for 1 min, 68°C for 1 min 35 sec and a final elongation step at 68°C for 7 min.

mad2/mad2

In order to determine if Cdc5p-dependent polarized growth required Mad2p, the first copy of *MAD2* in a *CDC5*-regulated strain HCCA34 was replaced with a *URA3* marker using a deletion construct containing 80 nucleotides of flanking sequences. The fragment was produced by PCR with oligonucleotides HC8F and HC8R and pBS-*cURA3* plasmid, and transformed into strain HCCA34, generating strain HCCA47. The PCR conditions included 94°C for 4 min, followed by 25 cycles with 94°C for 1 min, 41°C for 1 min, 68°C for 1 min 35 sec and a final elongation step at 68°C for 7 min. The second copy of *MAD2* was replaced with a *HIS1*-containing deletion construct produced via 2-step fusion PCR, which allowed for longer flanking sequence and facilitated the replacement process. Oligonucleotides HCGS10F and HCGS10R were used to amplify a 500 bp fragment identical to the 5' flank of *MAD2* and oligonucleotides HCGS12F and

HCGS12R were used to amplify a 450 bp fragment homologous to the 3' flank. PCR conditions for the flanking fragments included 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 45°C for 30 sec and 68°C for 55 sec followed by a 7 min run at 68°C. Oligonucleotides HCGS11F and HCGS11R were used to amplify the *HIS1* marker from plasmid pBS-*cHIS1*. The PCR conditions, using HGS11F and HCGS11R, were the same as above except with an extension time of 1 min 30 sec at 68°C. The 3 individual fragments were amplified together using oligonucleotides HCGS10F and HCGS12R to make the final deletion construct. The PCR conditions included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 48°C for 30 sec, 68°C for 3 min 50 sec, 15 cycles of the same with additional 20 sec extension/elongation, and a final 7 min elongation at 68°C. Transformation of the final construct into strain HCCA47 resulted in strain HCCA56. An additional control was constructed which consisted of both copies of *MAD2* deleted from strain BWP17. Oligonucleotides HCGS10F, HCGS10R, HCGS12F and HCGS12R were used to amplify a 500 bp or 450 bp fragments that were homologous to the to the 5' and 3' flanks of *MAD2*, respectively. PCR conditions for the flanking fragments included 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 45°C for 30 sec and 68°C for 55 sec followed by a 7 min run at 68°C. Oligonucleotides HCGS11F and HCGS11R were used to amplify the *URA3* or *HIS1* markers from pBS-*cURA3* or pBS-*cHIS1* plasmids. The PCR conditions, using HGS11F and HCGS11R, were the same as above except with a longer extension time of 1 min 30 sec at 68°C. The 3 individual fragments were amplified together using oligonucleotides HCGS10F and HCGS12R to make the final deletion construct. The PCR conditions included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 48°C for 30 sec, 68°C for 2 min 30 sec,

15 cycles of the same with additional 20 sec extension/elongation after each cycle, and a 7 min final elongation at 68°C. After transformation, strains HCCA110 and HCCA216 were created.

2.4 Genomic DNA (gDNA) extraction

To extract gDNA, cells were inoculated in 5-10 ml of minimal medium, grown overnight, and then centrifuged for 5 min at 2 095 x g. The supernatant was discarded and the pellet was washed with sterile water. The cell pellet was resuspended in 1 ml of sorbitol buffer (1M sorbitol, 0.1M EDTA) mixed with 10 µl of stock lyticase (10U/µl) (Sigma) and 2 µl of 4M DTT. The mixture was incubated at 37°C for 1-2h. Cells were centrifuged at 15 700 x g and the supernatant was discarded. The pellet was resuspended in 200 µl Tris-EDTA solution (50 mM Tris, 20 mM EDTA), 2% SDS and incubated at 65°C for 30 min. After incubation, 100 µl of 5M potassium acetate (KAc) was added and the solution was gently mixed to avoid shearing DNA. The mixture was incubated on ice for 60 min, centrifuged for 10 min at 15 700 x g, and the supernatant was transferred to a new microtube. An equal amount of 100% isopropanol was added to the supernatant, and after mixing for 1 min, the DNA pellet was collected by centrifuging at 15 700 x g for 1 min. The DNA pellet was then washed with 70% ethanol, air-dried, and resuspended into 150 µl of TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0) with 2 µl of RNaseA (10 mg/ml, Molecular Bioproducts). DNA was incubated at 37°C for 30 min and stored at 4°C. Genomic DNA was quantified with a fluorometer (Hoefer DQ300) using Hoechst Dye (Invitrogen).

2.5 Colony PCR screening

For PCR screenings of transformants, cells were used as template (Ling *et al.*, 1995). Briefly, average size yeast colonies on solid medium or cell pellets from liquid medium were touched with a sterile pipette tip and the cells were transferred to 10 µl of Zymolase solution (2.5 mg/ml of Zymolaze (MP Biomedicals, LLC), 1.2 M sorbitol, 0.1M $NaPO_4$). After mixing by pipetting up and down several times, the cells were incubated at 43°C for 60 min. A template of 2 µl of spheroplasted cells was used in 50 µl PCR reactions. PCR utilized 15 µM of oligonucleotides, gDNA template, 100 mM of dNTPs (Fermentas), and Expand Long Template Polymerase with Buffer 3. For PCR screening of transformants, Buffer 1 was used instead of Buffer 3.

CDC20 transformants were initially screened by PCR using flanking oligonucleotides GSCDC20F and HC6R to verify proper integration. PCR conditions included 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 40-42°C, 3 min at 68°C, and a final elongation step of 7 min at 68°C. To screen *PCK::CDC20*-containing strains, oligonucleotides PCK1F and HC12R were used and PCR conditions included 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 41°C, 3 min 35 sec at 68°C, and a final elongation step of 7 min at 68°C. The *CDH1* strains were verified by PCR using oligonucleotides GSCDH1F and HCGS16R. PCR running conditions included 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 38°C, 1 min at 68°C, and a final elongation step of 7 min at 68°C. For screening *MAD2* strains, oligonucleotides HCSF and HCSR were used. PCR conditions included 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 38°C, 3 min 30 sec at 68°C, and

a final elongation step of 7 min at 68°C. In cases where colony screening was not successful, PCR screening was repeated using gDNA.

2.6 Southern blotting

Strain construction was validated using Southern blot analysis with the DIG Hybridization System (Roche Diagnostics). Restriction digests included 4 μg of gDNA, 10X BSA, the appropriate 10X Buffer (NEB), 2-4 units of restriction enzyme per μg of DNA (NEB) and sterile water to make a final volume of 70 μl. Genomic DNA from *CDC20* conditional strains was digested with *Xba*I, while that of *CDH1*-deletion strains was digested with *Kpn*I and *Xho*I. Genomic DNA (gDNA) from *CDH1* conditional strains was cut with *Xho*I and *Xba*I, and that of *MAD2* deletion strains with *Bam*HI. DNA was digested overnight at 37°C and precipitated the following day by the addition of 5 μl of 5M NaCl, 500 μl of 95% ethanol, and incubated at –80°C for 1 h or –20°C for 6 h to overnight. The DNA was pelleted by centrifugation at 4°C for 10 min at 17 000 x g, washed with 70% ethanol, pelleted by centrifugation for 5 min, and air-dried for 15 min. DNA was resuspended in 30 μl of TE buffer. gDNA was separated on a 0.7% agarose gel at 25 mV overnight. The agarose gel was denatured by incubation twice for 15 min in 1.5M NaCl and 0.5M NaOH. After washing with water, the gel was neutralized by incubating in 3M NaCl, 0.5M TRIS-HCl at pH 8.0 twice for 15 min. DNA was transferred to a positive-charged nylon membrane (Roche) in 20X Saline-Sodium Citrate (SSC) (3M NaCl, 0.3M Na citrate, pH to 7.0 with HCl). The membrane was then UV-cross-linked (Stratagene), air-dried and washed with 20X SSC. The membrane was incubated at 65°C for 1 h in prehybridization solution consisting of 5X SSC, 1% blocking

agent, 0.1% Sarkosyl (Sodium Lauryl Sarcosinate), and 0.02% Sodium Dodecyl Sulfate (SDS). The membrane was then transferred to hybridization solution, consisting of prehybridization solution with 50 ng of probe. The blot was incubated overnight at 65°C. The membrane was washed twice the following day with 2X wash (2X SSC, 0.1% SDS) for 5 min at room temperature followed by 2 washes in 0.1X wash (0.1X SSC, 0.1% SDS) for 15 min at 65°C. Chemiluminescence utilized CSPD (Roche).

Probes were constructed by PCR amplification followed by DIG-labelling. For *CDC20*, oligonucleotides HCSP3B and HCSP4 were used to amplify a 1 kb product. For *CDH1*, oligonucleotides HCSP7 and HCSP8B were used to amplify a 1.1 kb product. For *MAD2*, oligonucleotides HCSP9 and HCSP10 or oligonucleotides HCSP5B and HCSP6 were used to make 1 kb probes at different ends of the gene. PCR conditions using Short Expand Template Polymerase (Roche) included 94°C for 2 min, followed by 10 cycles at 94°C for 15 sec, 36-44°C for 30 sec, 72°C for 1 min, and 20 cycles of the same with an additional 5 sec added to the extension/elongation time, and 7 min final elongation at 68°C. When PCR products could be not be amplified, Long Expand Template Polymerase was used. PCR conditions included 94°C for 3 min, followed by 10 cycles with 94°C for 30 sec, 36-44°C for 30 sec, 68°C for 1 min, and 15 cycles of the same with an additional 20 sec extension/elongation time and a 7 min final elongation at 68°C. For labelling of Southern DNA probes, 500 ng of PCR product in a total volume of 10 µl was boiled for 10 min, snap cooled on ice for 5 min, then mixed with stock reagents to give a final concentration of 1X hexanucleotide mix, 1X dNTP labelling reaction, and 5 µl of Klenow enzyme (50 U/µl, NEB). The mixture with a total volume of 100 µl was incubated overnight at 37°C and the reaction was stopped the next day by

adding 4 µl of 0.5M EDTA. DNA was precipitated with 1 µl of 20 mg/ml glycogen, 7.92 µl of 5M lithium chloride, 330 µl of 95% ethanol and placed at –80°C for 1 h. DNA was centrifuged at 4°C for 10 min at 17 000 x g, washed with cold 70% ethanol, centrifuged again for 5 min and air-dried before resuspension in 50 µl of TE buffer. The labelled probe was quantified by comparing to a DIG-labelled control DNA sample of known concentration (5 ng/µL). Control and experimental probes were serially diluted to 1/10, 1/100, 1/1000, 1/10 000 and 1/100 000 times in Solution 1 (0.1M maleic acid, 0.15M NaCl, 0.175M NaOH, pH 7.5). An aliquot of each dilution (1 µl) was spotted onto a positively-charged nylon membrane (Roche) and UV-crosslinked. The membrane was pre-wetted with Solution 1 for 1 min and blocked in Solution 2 (1% blocking solution in Solution 1) for 30 min. The membrane was then incubated with anti-DIG antibody conjugated to alkaline phosphatase (1:5000 final dilution) (Roche) for 30 min in Solution 1. Following 2 washes with Solution 1 for 15 min each, the membrane was equilibrated for 2 min in Solution 3 (0.1M NaCl and 0.1M Tris-HCl pH 9.5) and incubated with NBT/BCIP (1:50 dilution in Solution 3) for 20 min in the dark. The reaction was inhibited by adding Solution 4 (0.01M Tris-HCl pH 8.0 and 0.001M EDTA) for 10 min.

2.7 Cell staining and microscopy

To visualize nuclei, cells were fixed with 70% ethanol for a minimum of 1h, stained with 1 µg/ml of 4', 6'diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) for 20 min, and washed with sterile water. To visualize septa, fixed cells were subsequently stained with 2 µg/ml calcofluor white (Sigma) for 10 min. For immuno-localization of tubulin, cells were grown overnight in minimal or rich medium, diluted to

an $O.D._{600nm}$ of 0.2 for the desired incubation period in minimal or rich medium, and collected by centrifugation. The cell pellets were suspended in 500 µl of fresh medium and fixed for 20 min with an equal volume of 2X fixative, containing 8% paraformaldehyde (Fisher), 10 mM MgSO4, 50 mM EGTA, 4 mM AEBSF (MP Biomedicals, LLC), 10 µg/ml leupeptin (MP Biomedicals, LLC) and 1 µM of aproptinin (MP Biomedicals, LLC). Fixed cells were centrifuged at 3 300 x g for 3 min, and the pellet was washed twice with chilled PEM buffer (40 mM PIPES, 25 mM EGTA pH 7.0). Cell walls were digested with digestive solution (10 µg/ml Zymolaze 100T, 1.2M Sorbitol, 2% BSA, 10 µg/ml of leupeptin, 50 µg/ml of AEBSF, 4 µg/ml of aprotinin) for 30 min at 37°C. Cells were centrifuged gently at 2 300 x g for 2 min and rinsed twice with ice cold PEM buffer. Cell walls were permeabilized with ice cold 0.1% Nonidet P-40 detergent (Fluka) for 5 min and gently rinsed with PEM buffer. Cells were incubated overnight at 4°C in 1:100 dilution of monoclonal anti-α-tubulin (Sigma) in antibody dilution buffer (0.05% sodium azide, 2% BSA, PEM Buffer), washed with PEM buffer, and incubated in 1:100 dilution of sheep anti-mouse FITC conjugate (Sigma) in antibody dilution buffer in the dark for 2 h at room temperature. Cells were then rinsed with PEM buffer and stained with 1 µg/ml DAPI for 20 min. Cells were examined under a Leica microscope (model DM6000B) using a 63X and 100X immersion oil objective. The DAPI (460 nm) filter was used to visualize nuclei and septa and the FITC (520 nm) filter was used to visualize tubulin.

Cells were sonicated (Marcil *et al.*, 2002) using a Branson model 1510 (Fischer) sonicator. Cells were sonicated for less than 1 min to limit cell death.

3. Results

3.1 *C. albicans* contains a homologue of the APC activator *CDC20*

Previous work in *C. albicans* demonstrated that blocking mitosis through different means, including depleting the polo-like kinase Cdc5p, resulted in polarized growth that was partially dependent on spindle checkpoint factors (Bai *et al.*, 2002; Bachewich *et al.*, 2005). In order to further our understanding of how mitosis itself is regulated and in turn linked to polarized morphogenesis, we initially focused on the APC activator Cdc20p. The APC is a major regulator of mitosis in most systems, and Cdc20p was shown to be modulated in transcription profiles of Cdc5p-depleted, polarized cells (Bachewich *et al.*, 2005).

The *C. albicans* homologue of *CDC20*, identified in CGD (http://www.candidagenome.org/), is 51 % identical to Cdc20p in *S. cerevisiae*. In order to determine the function of Cdc20p and its potential contribution to polarized morphogenesis, we constructed strain HCCA23, where one copy of *CDC20* was replaced with the *URA3* marker and the second copy was placed under the control of the *MET3* promoter (Care *et al.*, 1999) (Figure 4), which is induced in absence of methionine and cysteine and repressed in presence of 2.5 mM methionine and 0.5 mM cysteine. While this work was in progress, however, assembly 19 was published, which demonstrated a different start site for *CDC20*. We therefore constructed another strain, HCCA109, which contained the updated start site. PCR screening and Southern analysis confirmed the proper construction of both strains (Figure 5). Since strain HCCA23 grew in a similar manner as strain HCCA109, and a comparison of the start sites revealed that strain HCCA109 contained a more optimal start site based on the Kozak sequence rules

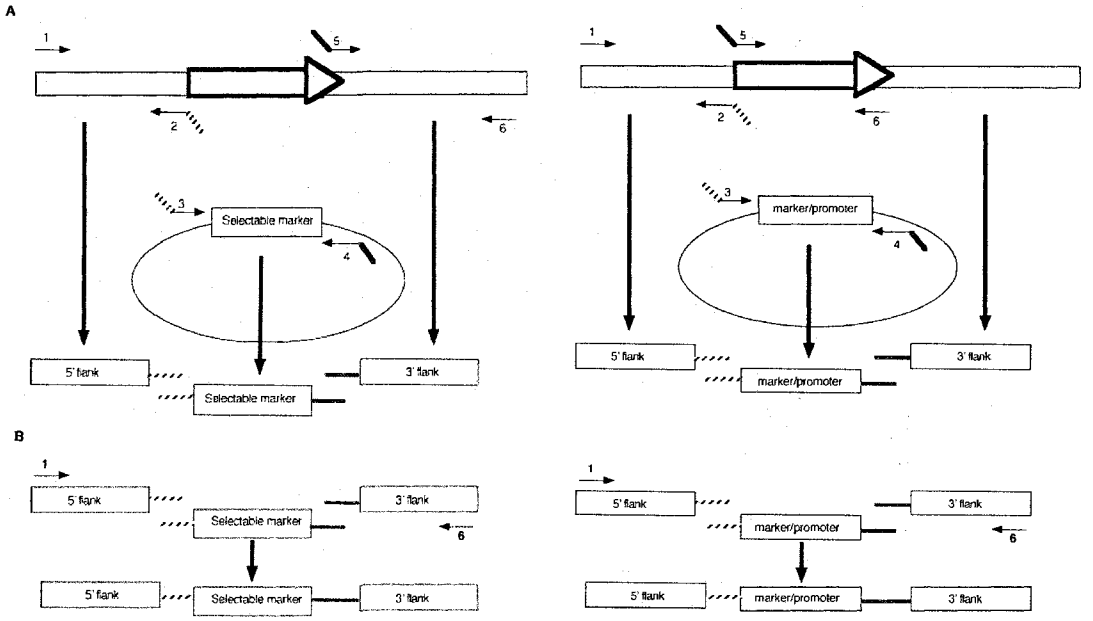


Figure 4: Strategy for gene deletion and/or promoter replacement using a 2-step PCR fusion protocol

(A) Single PCR reactions to amplify 500 bp of homologues sequences for gene deletion or promoter replacement. Oligonucleotides 3 and 4 contained reverse complement sequence to oligonucleotides 2 and 5, respectively. (B) Final fusion PCR using all 3 templates of previous reactions to create final construct.

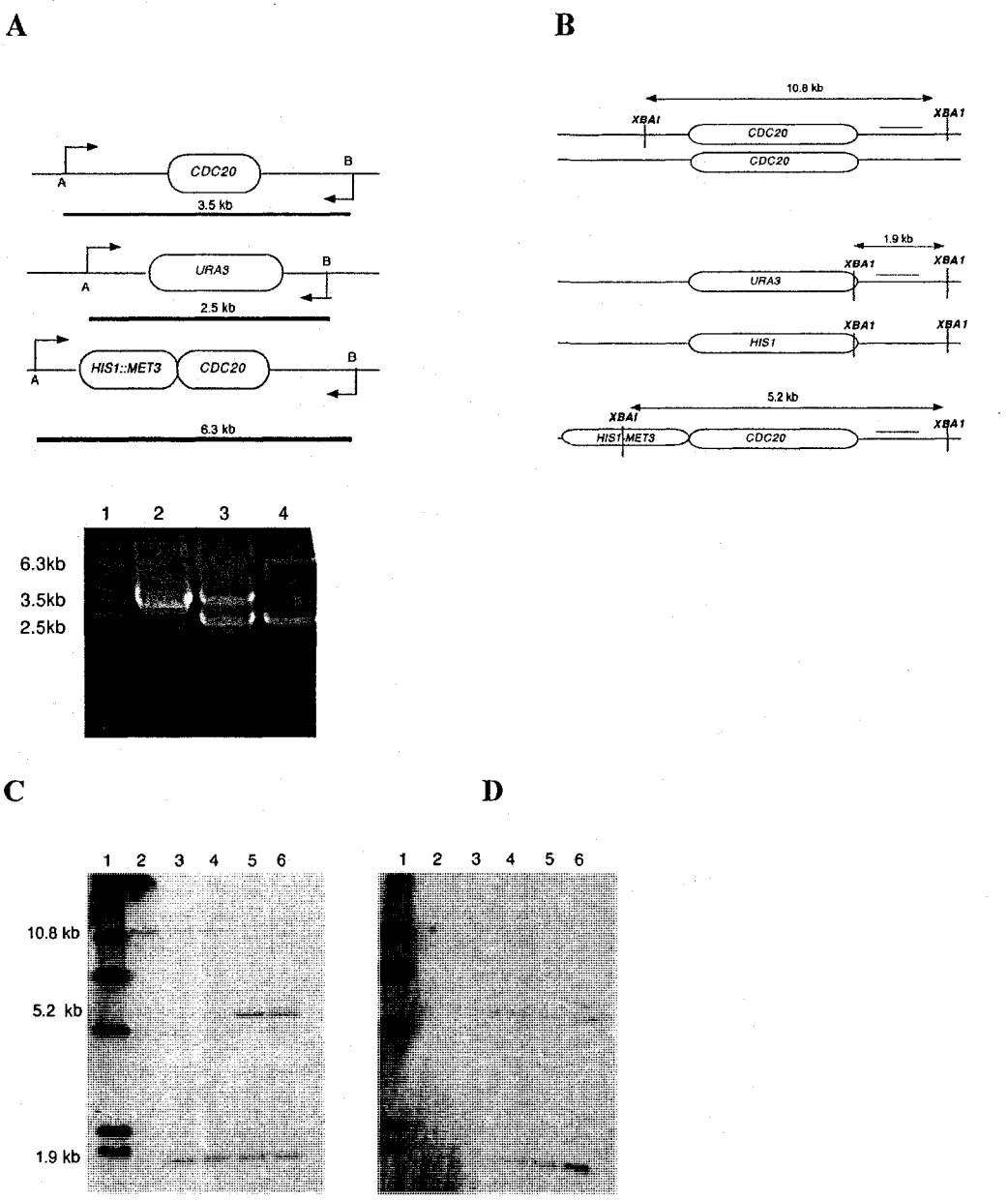


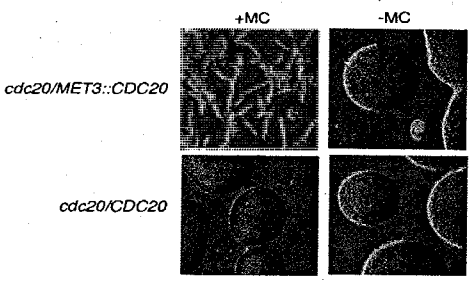
Figure 5: PCR and Southern screens for conditional *CDC20* strains
(A) Map for PCR screening of *CDC20* conditional strains, and resulting band sizes. Lane 1: MWM; lane 2: wild-type; lane 3: *cdc20/CDC20*; lane 4: *cdc20/MET3::CDC20*. (B) Map for Southern analysis. Genomic DNA was digested with *Xba*I. (C, D) Blots showing strains utilizing start sites from assembly 21 and 19, respectively. Lane 1: MWM, lane 2: Wild-type, lanes 3-4: *cdc20/CDC20*, lanes 5-6: *cdc20/MET3::CDC20*.

(Kozak,1986), we used strain HCCA109 for subsequent investigations. Strain HCCA16, which was heterozygous for *CDC20* and contained a randomly integrated *HIS1-MET3* construct, was used as a control.

3.2 Depletion of Cdc20p results in highly polarized growth of yeast buds under yeast growth conditions, similar to Cdc5p-depleted cells

In order to demonstrate the effects of Cdc20p depletion, cells of strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were inoculated on solid Met3p-inducing medium lacking methionine and cysteine, or solid repressing medium containing 2.5 mM methionine and 0.5 mM cysteine at 30°C (Figure 6A). On inducing medium, cells from strain HCCA109 formed normal yeast colonies. However, on repressing medium, the yeast cells failed to form colonies and instead generated filaments. In contrast, the control strain HCCA16 formed smooth colonies, indicating that cells remained in the yeast cell form when grown on either inducing or repressing medium. When cells of strain HCCA109 were incubated in liquid repressing medium for 3 h at 30°C, many cells were large-budded, with a significant proportion containing a short polarized extension (Table 4, Figure 6B). At 6 h, most cells contained highly elongated buds that did not contain constrictions along their length, indicating a maintained polarized growth mode. By 24 h, cells continued to elongate but some contained constrictions, branches and appeared more pseudohyphal (Figure 7). In addition, many cells were no longer viable, as shown with propidium iodide staining (Figure 7), suggesting that *CDC20* may be an essential gene. Intriguingly, the elongation response only occurred when cells under inducing conditions were at an $O.D._{600nm}$ of 5.0

A



B

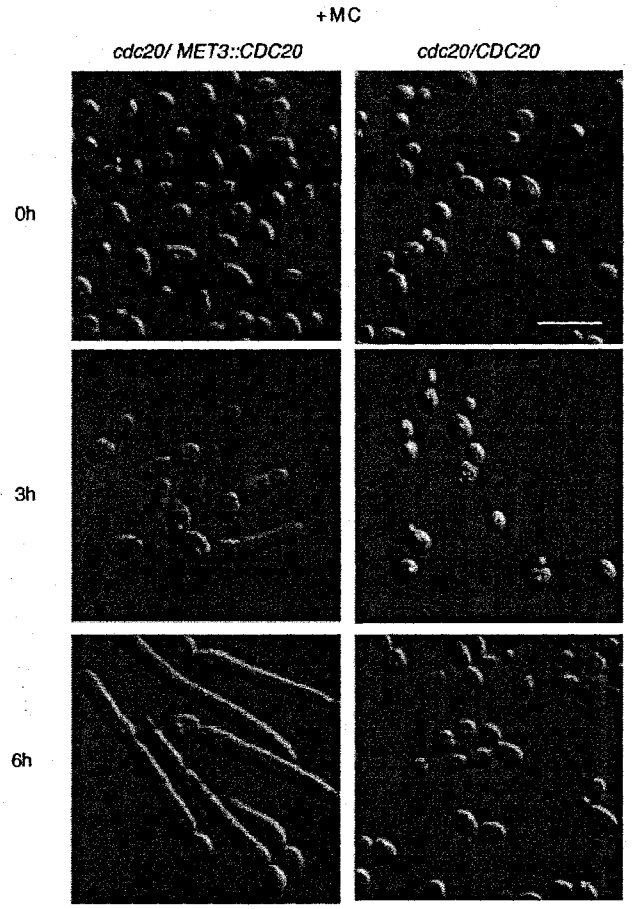


Figure 6: Depletion of Cdc20p results in filament formation under yeast growth conditions

(A) Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were grown overnight in inducing medium lacking methionine and cysteine, diluted, streaked onto solid inducing medium lacking cysteine and methionine and repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and incubated at 30°C for 24 h. (B) Overnight cultures of the same strains were diluted in liquid repressing medium, and incubated for the indicated times at 30°C. Bar: 10 µm

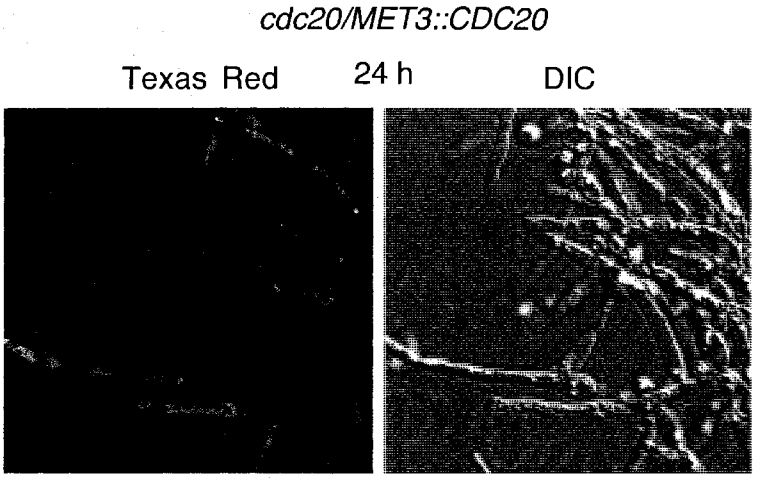


Figure 7: Cells depleted of Cdc20p die by 24h
Strain HCCA109 (*cdc20/MET3::CDC20*) was grown overnight in liquid inducing medium lacking methionine and cysteine at 30°C and diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine. Cells were collected at 24 h and incubated in propidium iodide for 10 min. Bar: 10 μm

Table 4: Number of nuclei[1] and cell morphology[1] in Cdc20p-depleted cells[2]

	Number of nuclei					Cell morphology	
	1	2	3	Frag[3]	Unbudded/ small	Large bud	Elongated bud
cdc20/ MET3::CDC20							
0 (n=236)	81.8	18.2	0	0	91.1	8.9	0
3 (n=278)	63.7	36.3	0	0	5.0	29.5	65.5
6 (n=216)	22.2	54.2	2.8	20.8	3.0	2.0	95
cdc20/CDC20							
0 (n=137)	78.8	21.2	0	0	71.6	28.4	0
3 (n=134)	53.7	46.3	0	0	36.6	61.2	2.2
6 (n=201)	68.7	31.3	0	0	66.2	30.8	3.0

[1] Values are expressed in %

[2] Cells from strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were incubated in repressing medium at 30°C and collected at indicated time points. Cells were fixed and stained with DAPI.

[3] Fragmentation of chromosomes prevented accurate quantification of total nuclei per cell at later time points.

or lower before transferring to repressing medium; cells taken from cultures with a high $O.D._{600nm}$ did not polarize or proliferate as yeast when diluted into repressing medium (data not shown). In contrast, cells from control strain HCCA16 grew in the yeast form at all time points in either inducing or repressing medium (Table 4, Figure 6B). The Cdc20p-depleted phenotype differs from that of *CDC20* mutants in *S. cerevisiae*, which arrested as large doublets (Hartwell *et al.*, 1973). However, the phenotype closely resembles that of Cdc5p-depleted cells, suggesting that Cdc20p may lie in the same pathway as Cdc5p leading to elongated bud growth.

3.3 Cdc20p is required for early and late stages of nuclear division

Since Cdc20p is required for the metaphase to anaphase transition and mitotic exit in *S. cerevisiae*, and Cdc5p-depleted cells in *C. albicans* were blocked in mitosis prior to forming similar filaments, we next examined nuclear division in Cdc20p-depleted cells. Cells from strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were inoculated overnight in inducing medium, diluted in repressing medium, collected at 0, 3 and 6 h, fixed, and then stained with DAPI and calcofluor to visualize nuclei and septa, respectively. At 3 h, when the majority of the cells in strain HCCA109 were either large doublets or slightly elongated, 63.7% (n=278) of the cells contained 1 nucleus with unsegregated chrosomomes, suggesting an early block in mitosis, while the remaining 36.3% contained 2 nuclei (Table 4, Figure 8). At 6 h, when most cells were highly elongated, 22.2% (n=216) contained 1 nucleus, 54.2% contained 2 nuclei, and 2.8% had 3 nuclei. The remaining cells contained degraded chromatin, making it difficult to quantify the number of nuclei. The frequency of degradation was higher after overnight

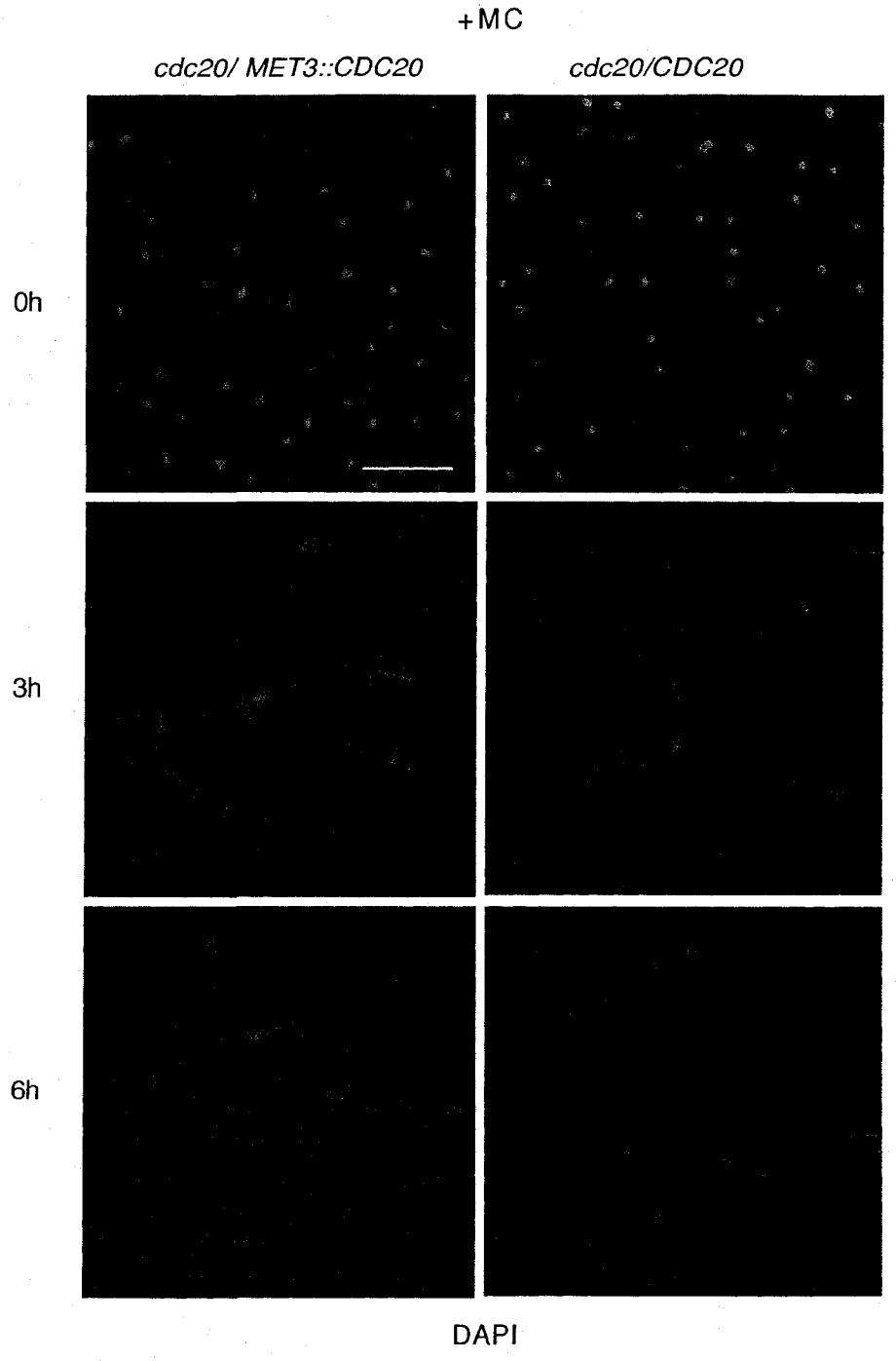


Figure 8: An early block in nuclear division is associated with repression of *CDC20*
Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were grown in inducing medium lacking methionine and cysteine at 30°C, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine, fixed and stained with DAPI and calcofluor white at indicated times. Bar: 10 μm

incubation in repressing medium (data not shown). In contrast, nuclear division in control strain HCCA16 at 3 h and 6 h was normal as cells were budding and undergoing mitosis.

The fact that more nuclei were present in strain HCCA109 at 6 h versus 3 h of repression could be due to either leakiness of the *MET3* promoter, or that cells were in telophase, with separated chromatin. In comparison, *CDC20* mutants in *S. cerevisiae* arrest in metaphase with a single nucleus positioned at the bud neck (Hartwell *et al.*, 1973; Alexandru *et al.*, 1999). To confirm the precise phase of mitosis in which Cdc20p is required in *C. albicans*, we analyzed spindle patterns in *CDC20*-repressed cells by immunolocalizing α-tubulin (Table 5, Figure 9). Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were incubated in repressing medium and collected at 3 and 6 h as previously described. Cells were then fixed and processed for immunofluorescence. At 3h, 32.6% (n=546) of cells from strain HCCA109 showed only spindle pole bodies staining, which was visualized as single or double spots. These cells were thus in interphase. In contrast, 39.4% of cells contained short rod-like spindles, consistent with a metaphase arrest. The remaining 28% of cells contained medium to long spindles, suggesting cells were in anaphase/telophase. Thus, approximately 67% of cells were in mitosis, consistent with the fact that most cells were either large doublets or contained elongated buds at this time point (Table 4). At 6 h, however, the proportion of cells with long spindles increased to 70.6% (n=320). Thus, many cells with 2 nuclei at 6 h were likely in telophase. In contrast, cells of control strain HCCA16 at 3 and 6 h contained predominantly interphase tubulin patterns (Table 5). These results suggest that a delay initially occurs at metaphase and then in telophase upon

Table 5: Spindle patterns[1] in Cdc20p-depleted cells[2]

		Interphase[3]	Mitosis[4]	
Strain	hr		early	late
HCCA109	3 (n=546)	32.6	39.4	28
(cdc20/MET3::CDC20)	6 (n= 320)	16.3	12.8	70.9
HCCA16	3 (n= 230)	58.3	16	25.7
(cdc20/CDC20)	6 (n= 217)	61.8	15.6	22.6

[1] Values are expressed in %.
[2] Cells from strain HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were inoculated in repressing medium at 30°C and processed for immunofluorescence of α tubulin.
[3] Cells containing single or double spots of tubulin representing spindle pole bodies without spindles indicated cells were in interphase.
[4] Cells containing short rod-like spindles were in early mitosis, while those with longer spindles were in later stages of mitosis.

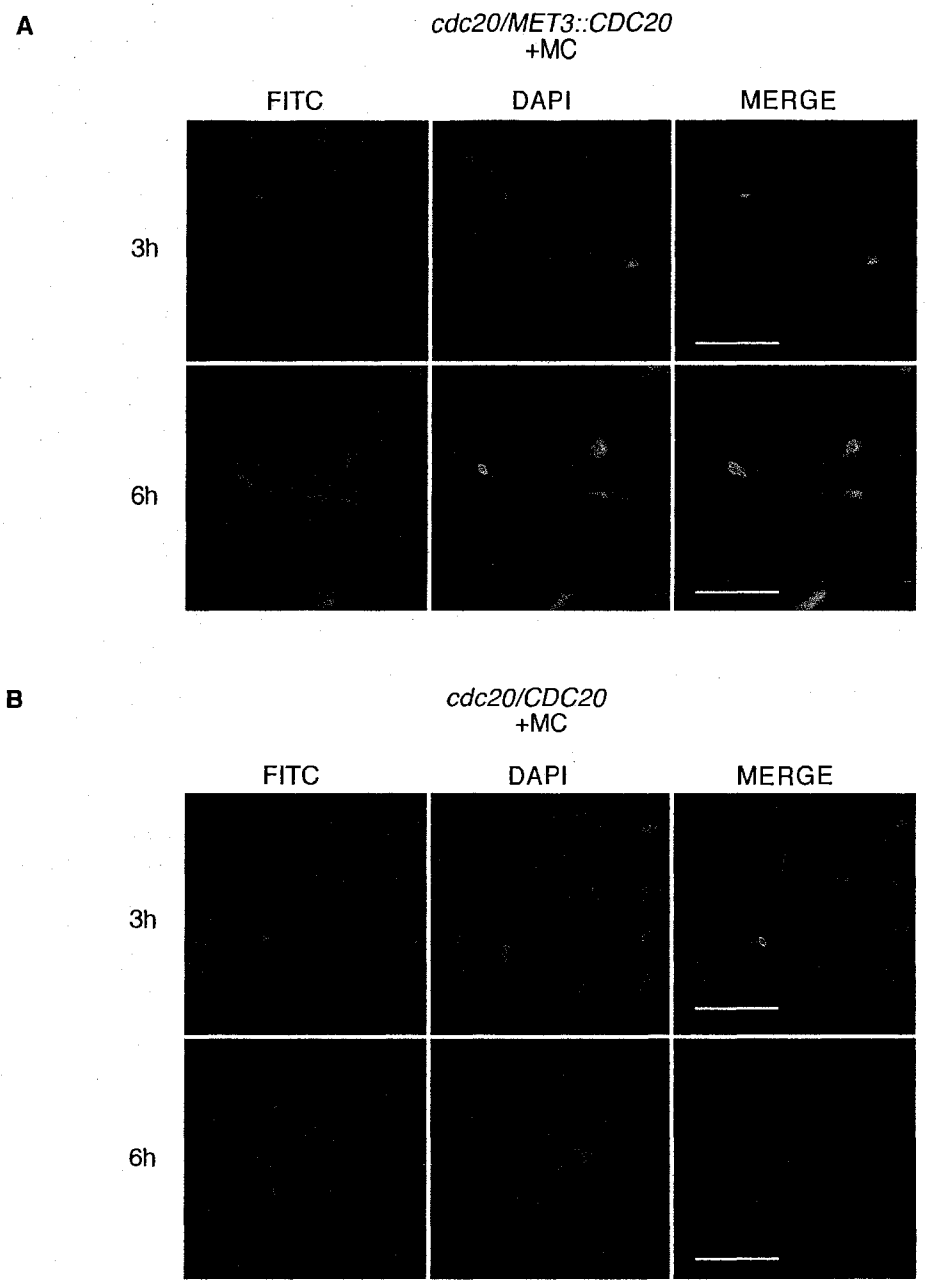


Figure 9: Cdc20p is important for the metaphase/anaphase transition and mitotic exit

(A) Strains HCCA109 (*cdc20/MET3::CDC20*) and (B) HCCA16 (*cdc20/CDC20*) were grown overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and fixed at 3 h or 6 h. Spindles were visualized with immunofluorescence, using anti-α tubulin antibody and FITC-conjugated secondary antibody. Bar: 10 μm

depletion of Cdc20p, suggesting that Cdc20p is important for both stages in mitosis. This is consistent with the function of Cdc20p in *S. cerevisiae*. The results also demonstrate that an early delay in mitosis precedes or is associated with polarized growth.

3.4 Cells lacking Cdc20p are defective in septation

In order to confirm that cells lacking Cdc20p were blocked in early mitosis, septa deposition was analyzed. Cells from strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were incubated overnight in inducing medium and diluted in repressing medium, collected at 3 and 6 h, fixed, and then stained with calcofluor white to visualize septa (Figure 10). At 3 h, only 5.6% (n=179) of cells from strain HCCA109 contained a septum. By 6 h, septa were present in 15.4% (n=344) of cells. In 2.6% of cells, 2 septa were present and located at the bud neck and within the elongated buds. Intriguingly, septa within the elongated filaments were not constricted, similar to septation patterns in true hyphae. In comparison, 19.9% (n=246) and 15.7% (n=319) of cells from strain HCCA16 contained 1 septum at 3 and 6 h, respectively, but these were in dividing yeast cells. The low proportion of cells containing septa in the absence of Cdc20p supports the concept that polarization is occurring during the mitotic block, but some cells were able to exit mitosis and form septa at later time points.

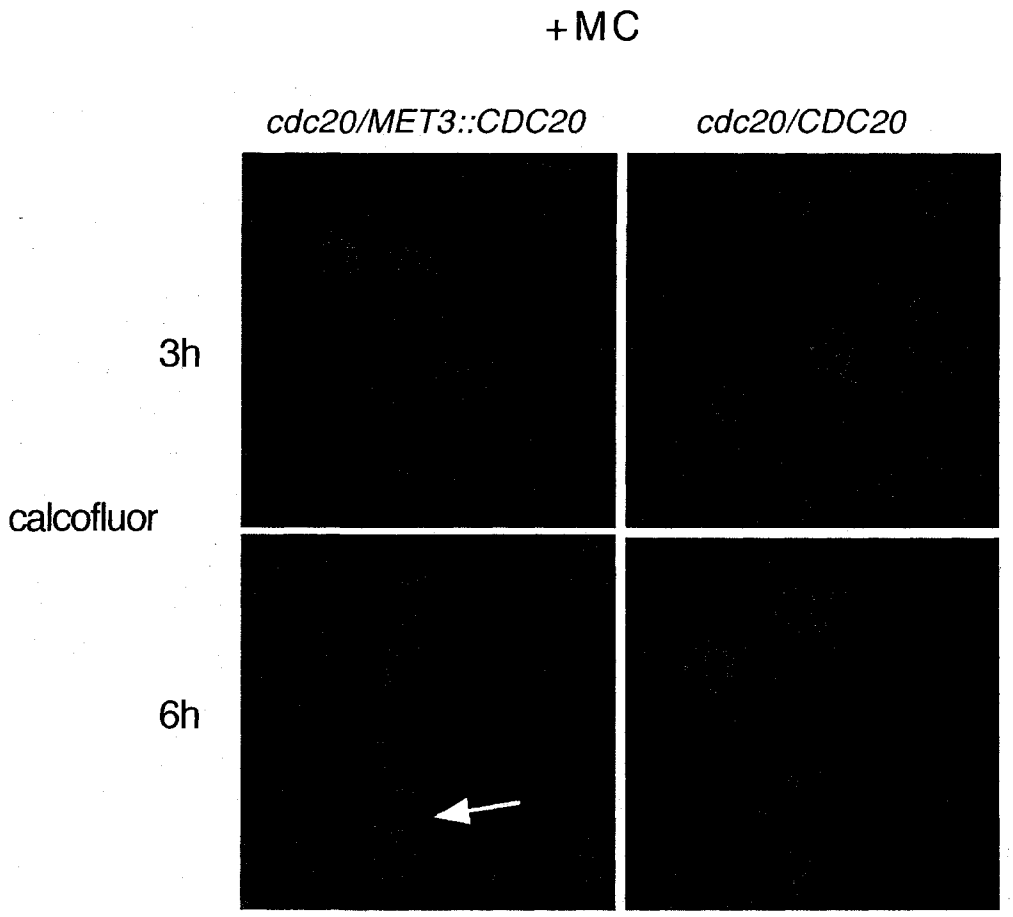


Figure 10: Septation is delayed in cells depleted of Cdc20p
Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were grown overnight in inducing medium lacking methionine and cysteine and diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine. Cells were fixed and stained with calcofluor white to visualize septa at 3 h and 6 h. Arrows indicate location of septa. Bar: 10 μm

3.5 Overexpression of *CDC20* partially suppresses the mitotic block in Cdc5p-depleted cells

Since depleting Cdc20p produced a similar phenotype as cells lacking Cdc5p, Cdc20p could be in the pathway downstream of Cdc5p function, leading to polarized growth. This was investigated by determining if overexpression of *CDC20* could suppress the Cdc5p-depleted phenotype. We first created a new *CDC5* conditional strain, HCCA118, in the BWP17 background, which had more auxotrophies than the previous *CDC5* conditional strain (Bachewich *et al.*, 2003). One copy of *CDC5* was deleted by the *URA3* blaster method, and the second copy was placed under the regulation of a *MET3* promoter. *CDC20* was then cloned after the regulatable *PCK1* promoter in plasmid pJA24, creating plasmid pHC4, which was transformed into strain HCCA118 to generate strain HCCA164. The *PCK1* promoter is activated in the presence of casamino acids and turned off by glucose (Leuker *et al.*, 1997). A control strain, HCCA180, was created by transforming strain HCCA118 with empty pJA24 plasmid. To test the effect of overexpressing Cdc20p alone in a wild-type background, strain HCCA223 was created, which consisted of plasmid pHC4 transformed into strain BWP17. PCR screenings confirmed proper construction of strains HCCA164 and HCCA223 (Figure 11).

Strains HCCA164 (*PCK1::CDC20, CDC20/CDC20, CDC5/MET3::CDC5*) and HCCA180 (*CDC20/CDC20, CDC5/CDC5,* pJA24) were inoculated overnight in inducing medium lacking methionine and cysteine but containing glucose to express *CDC5* and repress *CDC20*, respectively. Cells were then washed and diluted in fresh, Met3p-inducing medium that substituted glucose with casamino acids for 3 h to allow

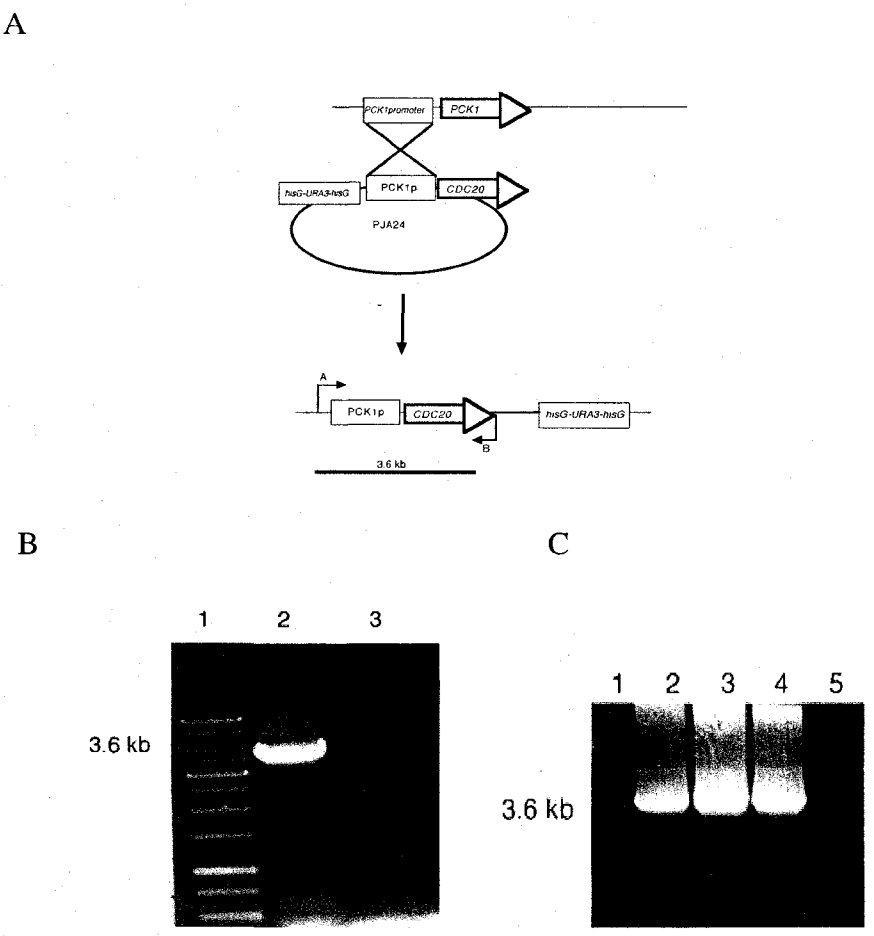


Figure 11: PCR screening for *PCK1*-regulated *CDC20* strains
(A) Map for PCR screening showing integration of *PCK1::CDC20* gene insert at the endogenous *PCK1* promoter site and resulting band sizes. (B) Lane 1: MWM; lane 2: *PCK1::CDC20, cdc5/MET3::CDC5*; lane 3: wild type. (C) Lane 1: MWM; lanes 2-4: *PCK1::CDC20, CDC5/CDC5*; lane 5: wild type

overexpression of *CDC20* prior to shutting off *CDC5* expression. Cells were then collected and transferred to fresh medium containing casamino acids to maintain overexpression of *CDC20*, and methionine and cysteine to repress *CDC5*. Cells were fixed and stained with DAPI and calcofluor white at 0, 3 and 5 h. At 3 h, the majority of cells from strain HCCA164 were elongated, but 38.3% (n=157) of cells showed rebudding from the mother cell (Table 6, Figure 12). A similar proportion of cells from control strain HCCA180 were elongated, but only 11.2% (n=196) were rebudding. A higher proportion of cells with rebudding was also seen in strain HCCA164 after 5 h, compared to the control strain HCCA180. Rebudding was not simply due to overexpression of *CDC20* alone, because when strain HCCA223 (*PCK1::CDC20*, *CDC20/CDC20*, *CDC5/CDC5*) was inoculated overnight in minimal medium containing glucose to shut off *PCK1*, then diluted in minimal medium containing casamino acids, cells appeared normal and remained as yeast with no abnormal rebudding from mother yeast cells (data not shown). When quantifying the number of nuclei, slightly more cells that overexpressed *CDC20* contained a single nucleus at 3 h as compared to the control (Table 7), but the values were similar between strains by 5 h. If the cells were previously in telophase, this could explain why the total number of nuclei did not increase upon overexpressing *CDC20*. However, we did not observe any major suppression of polarized growth upon overexpressing Cdc20p in Cdc5p-depleted cells. Despite this, the increase in rebudding suggests that Cdc20p may lie downstream of Cdc5p and could potentially mediate Cdc5p function.

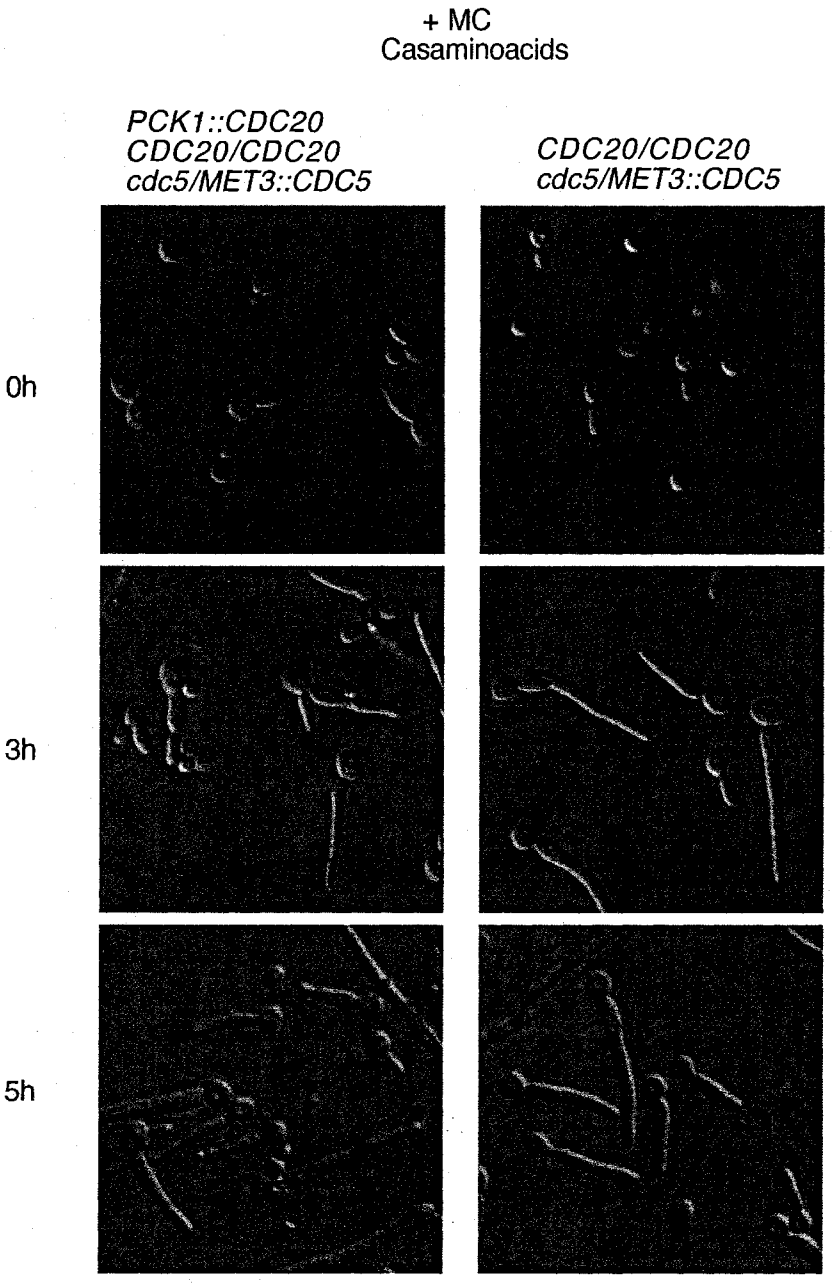


Figure 12: Overexpression of *CDC20* results in an increase in rebudding in Cdc5p-depleted cells

Strains HCCA164 (*PCK1::CDC20, CDC20/CDC20, cdc5/MET3::CDC5,*) and HCCA180 (*CDC20/CDC20, cdc5/MET3::CDC5,* pJA24) were inoculated overnight in inducing medium lacking methionine and cysteine, washed and diluted in the same medium containing casamino acids for 3 h. Cells were then inoculated in fresh medium containing 2.5 mM methionine and 0.5 mM cysteine to repress expression of *CDC5* and casamino acids to overexpress *CDC20*, collected and fixed at indicated time points. Bar: 10 μm

Table 6: Cell morphology[1] upon overexpressing *CDC20* in Cdc5p-depleted cells

Strain	hr	Cell morphology		
		Yeast	Elong.	Elong. Reb[2]
HCCA164 (*PCK1::CDC20, cdc5/MET3::CDC5*)	3 (n=157)	22	38.2	38.3
	5 (n=164)	5.0	71.8	23.2
HCCA180 (*cdc5/MET3::CDC5*, pJA24)	3 (n=196)	4.6	84.2	11.2
	5 (n=221)	5.8	91.9	2.3

[1] Values expressed in %. Cells from strains HCCA164 (*cdc5/MET3::CDC5, PCK1::CDC20*) and HCCA180 (*cdc5/MET3::CDC5,* pJA24) were inoculated overnight in inducing medium lacking methionine and cysteine to allow expression of *CDC5*, diluted in fresh inducing medium containing casamino acids for 3 h to overexpress *CDC20* prior to transferring into liquid repressing medium containing 2.5 mM methionine and 0.5 mM cysteine to repress expression of *CDC5*, and casamino acids to maintain overexpression of *CDC20*.

[2] Cells are elongated and show rebudding.

Table 7: Number of nuclei[1] in cells[2] overexpressing *CDC20* while repressing *CDC5*

Strain	hr	Number of nuclei			
		1	2	3	F[3]
HCCA164 (*PCK1::CDC20, cdc5/MET3::CDC5*)	3 (n=157)	33.1	63.1	3.8	-
	5 (n=164)	6.7	70.7	6.1	16.5
HCCA180 (*CDC20/CDC20, cdc5/MET3::CDC5*)	3 (n=196)	18.9	73	8.1	-
	5 (n=221)	10.4	77.8	1.4	10.4

[1] Values expressed in %.
[2] Cells from strains HCCA164 (*cdc5/MET3::CDC5, PCK1::CDC20*) and HCCA180 (*cdc5/MET3::CDC5,* pJA24) were inoculated overnight in inducing medium lacking methionine and cysteine, diluted in fresh inducing medium containing casamino acids for 3 h prior to transferring to liquid repressing medium containing 2.5 mM methionine and 0.5 mM cysteine to repress expression of *CDC5*, casamino acids to maintain expression of *CDC20*.
[3]Fragmentated chromosomes in cells.

3.6 Cdc20p is not required for serum-induced hyphal growth.

Since Cdc20p influenced polarized growth of yeast buds, we investigated whether it was necessary for serum-induced hyphal formation. Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were inoculated overnight in inducing medium lacking methionine and cysteine, then diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine for 2 h to repress *CDC20*. Cells were then transferred to fresh repressing medium with 10% fetal bovine serum for 3 h. After the 2 h mitotic block, prior to adding serum, 37.9% (n=269) of cells from strain HCCA109 were large doublets while 62.1% had short extensions approximately twice the length of diameter of the mother cell. After transferring to serum and incubating for 3 h, hyphal germ tubes formed in strain HCCA109. Interestingly, they emerged directly from the elongated buds or daughter cell of the large doublets formed from the previous 2 h block (Figure 13). The control strain responded to serum normally and formed hyphae of similar length to those in Cdc20p-depleted cells. Thus, Cdc20p does not influence hyphal growth. Intriguingly, if the cells were grown to an $O.D._{600nm}$ of > 5.0 prior to dilution into repressing medium to shut off *CDC20* and serum to induce germ tubes, cells from strain HCCA109 could not form hyphae. This is in contrast to that reported for stationary phase cells (OD_{600nm} >13), which are typically more responsive to hyphal induction signals (Kadosh *et al.*, 2005).

To determine if *CDC20* was important for nuclear division in hyphal cells as in yeast cells, hyphae were stained with DAPI and calcofluor. After 3 h in serum, 89.3% (n=56) of cells of strain HCCA109 contained 1 nucleus, while 10.7% contained 2 nuclei. None of the cells contained septa. In contrast, 92.3% (n=52) of cells of control strain

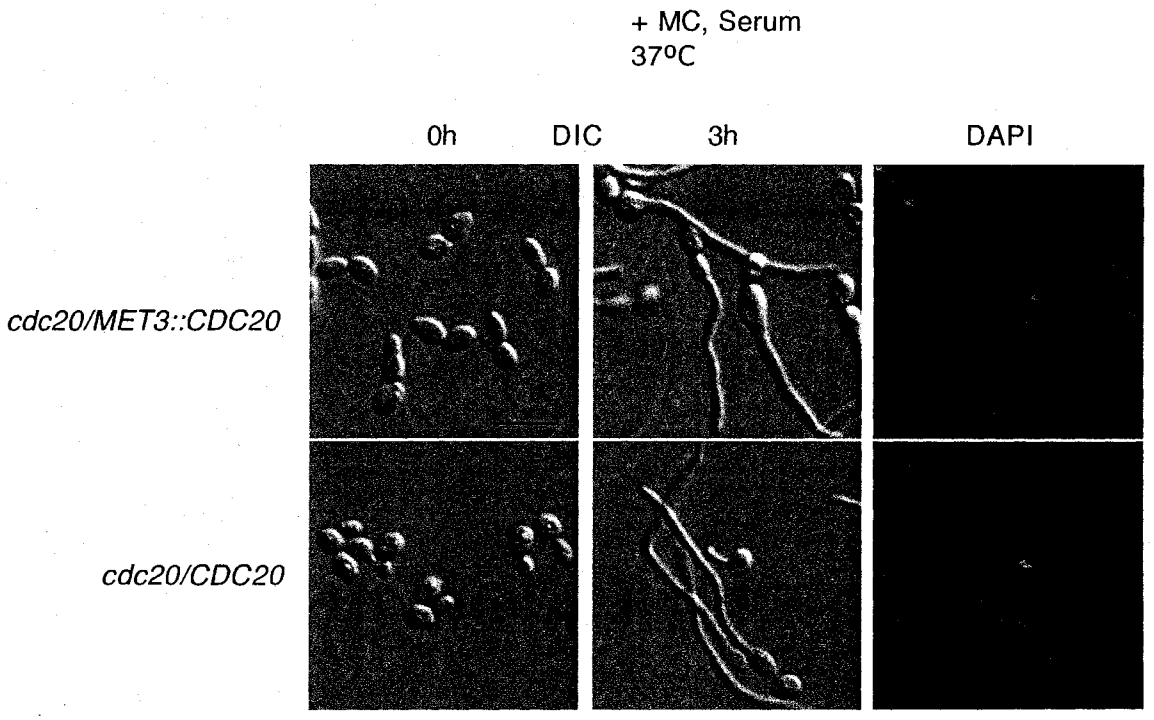


Figure 13: Depletion of Cdc20p under hyphal-inducing conditions does not affect hyphal growth

Strains HCCA109 (*cdc20/MET3::CDC20*) and HCCA16 (*cdc20/CDC20*) were grown overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine for 2 h at 30°C prior to transferring into repressing medium containing 10% fetal bovine serum at 37°C. Cells were fixed at 3 h. Bar: 10 μm

HCCA16 contained 2-3 nuclei as well as septa. The difference in number of nuclei could not be due to any difference in hyphal growth rate, since hyphae of the control strain (45.2 ± 2.2 μm (SEM; n=52)) and Cdc20p-depleted strains (41.2±1.2 μm (SEM; n=56)) were similar in length. This suggests that Cdc20p is also required for nuclear division in hyphal cells.

The results indicate that Cdc20p is not required for hyphal growth. They also demonstrate that germ tubes can form in yeast cells that were blocked in mitosis and preferentially from a pre-existing, as opposed to newly generated, polarized site. This data supports the notion that induction of hyphal growth is not restricted to G_1 phase of the yeast cell cycle (Hazan *et al.*, 2002).

3.7 Cells lacking the APC activator *CDH1* have diverse morphologies and do not resemble cells depleted of Cdc20p

Since Cdc20p is required for mitosis and the elongated bud growth response, we next investigated the function of another APC activator, Cdh1p. In *S. cerevisiae*, Cdh1p helps target the APC towards degrading Clb2p and Cdc5p, and is required for mitotic exit (Schwab *et al.*, 2001; Visintin *et al.*, 2008). A homologue of *CDH1* was annoted in the CGD website under orf19.2084. We first explored the function of *CDH1* by constructing a double knockout strain, HCCA45, by replacing one copy of *CDH1* with a *URA3* marker and the other copy with a *HIS1* marker. PCR screening and Southern analysis confirmed that the strain was properly constructed (Figure 14). The isogenic control strain HCCA100 was generated by transforming pRM100, which contains both *URA3* and *HIS1* markers, into strain BWP17.

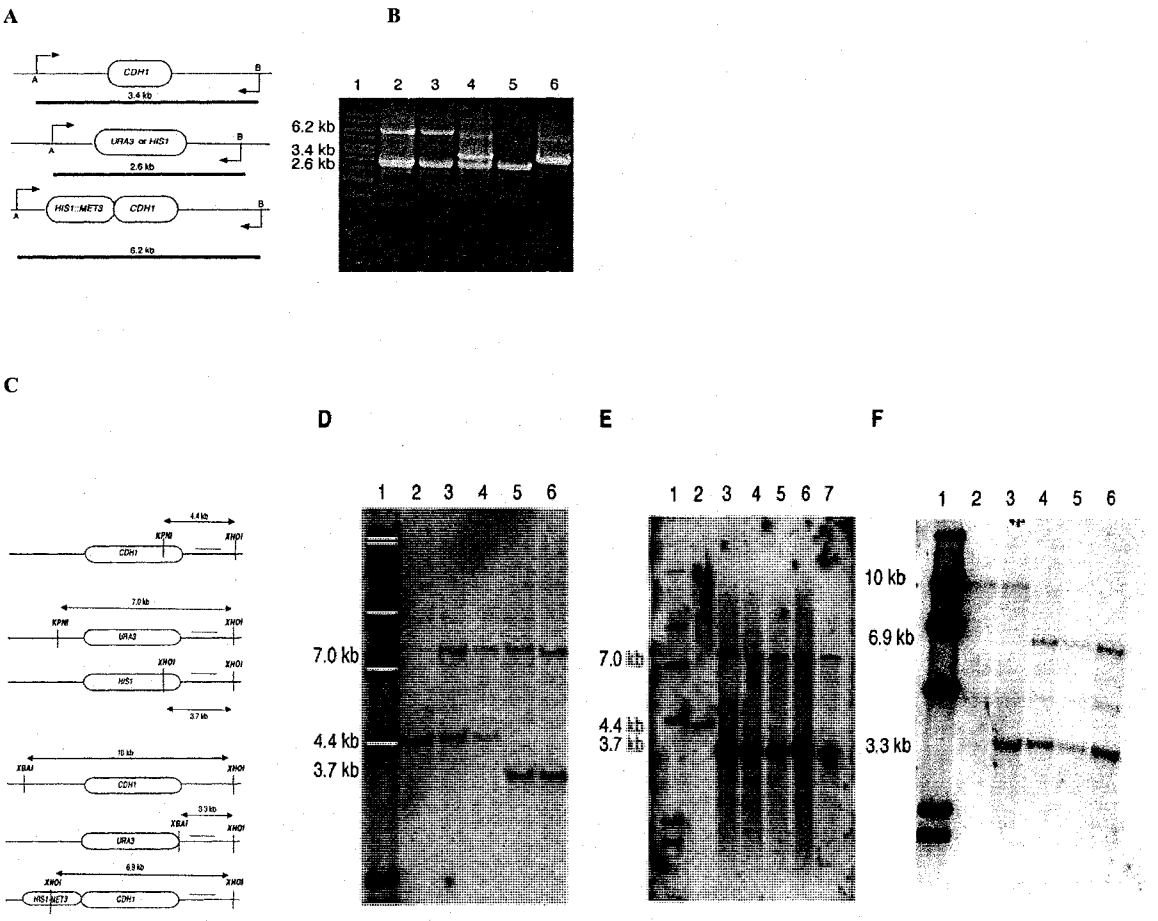


Figure 14: PCR screening and Southern analysis of *CDH1* deletion strains
(A) Expected band sizes for PCR screening of *CDH1*-regulated strains and *CDH1*-deleted strains. (B) Lane 1: MWM; Lane 2-3: *cdh1/MET3::CDH1*; lane 5: *cdh1/cdh1*, lane 6: wild-type. (C) Map showing of *Kpn*I/*Xho*I restriction digested gDNA for deletion strains and *Xba*I/*Xho*I digested gDNA for conditional strains. (D) Southern blot for *CDH1* deletion. Lane 1: MWM, Lane 2: wild-type; lanes 3- 4: *cdh1/CDH1*; lanes 5-6: *cdh1/cdh1*. (E) Southern blot of *CDH1*-deletion in *CDC5*-regulated background strains. Lane 1: MWM; lane 2: wild-type; lanes 3-7: *cdh1/cdh1, cdc5/MET3::CDC5*. (F) Southern blot of conditional *CDH1* strains. Lane 1: MWM; lane 2: wild-type; lane 3: *cdh1/CDH1*; lanes 4-5-6: *cdh1/MET3::CDH1*.

Strain HCCA45 was viable, indicating that *CDH1* is not essential. To determine the phenotype of cells lacking *CDH1*, strains HCCA45 (*cdh1/cdch1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in YPD liquid medium, diluted, streaked onto solid agar, and incubated at 30°C for 3 days. Colonies from strain HCCA45 had uneven edges indicating the presence of filaments, while strain HCCA100 grew smooth colonies (Figure 15A). The strains were also grown in liquid medium for comparison. Cells were grown overnight in YPD, diluted in fresh YPD, and incubated at 30°C for 7 h. At time 0, 70.6% (n=245) of cells from strain HCCA45 appeared as normal yeast, but the remaining cells were either abnormally enlarged yeast, pseudohyphal, or chained-cells. A small proportion of cells contained elongated buds that closely resembled Cdc20p and Cdc5p-depleted cells (Table 8, Figure 15B). In contrast, 96.3% (n=191) of cells from control strain HCCA100 remained in a normal yeast form, while the remaining cells were either elongated buds or enlarged cells. After incubating in fresh medium for 7 h, there was a more drastic change in cell morphology in strain HCCA45, where only 57.6% (n=283) of cells remained in the normal yeast form, 11.3% were abnormally enlarged yeast, and the remaining 31.1% were in a filamentous form. Of the filamenting cells, pseudohyphae, chained cells and elongated buds were observed, but the latter compromised only 11.7% of the total cells. In contrast, cells from strain HCCA100 grew normally, where 97.5% (n=202) remained in a normal yeast form, and only 2.5% appeared enlarged. Thus, absence of *CDH1* dramatically affected yeast cell morphology, but in a pleiotropic manner.

In order to confirm the *CDH1*-deletion phenotype, we constructed strain HCCA26, which contained a single copy of *CDH1* under control of the *MET3* promoter.

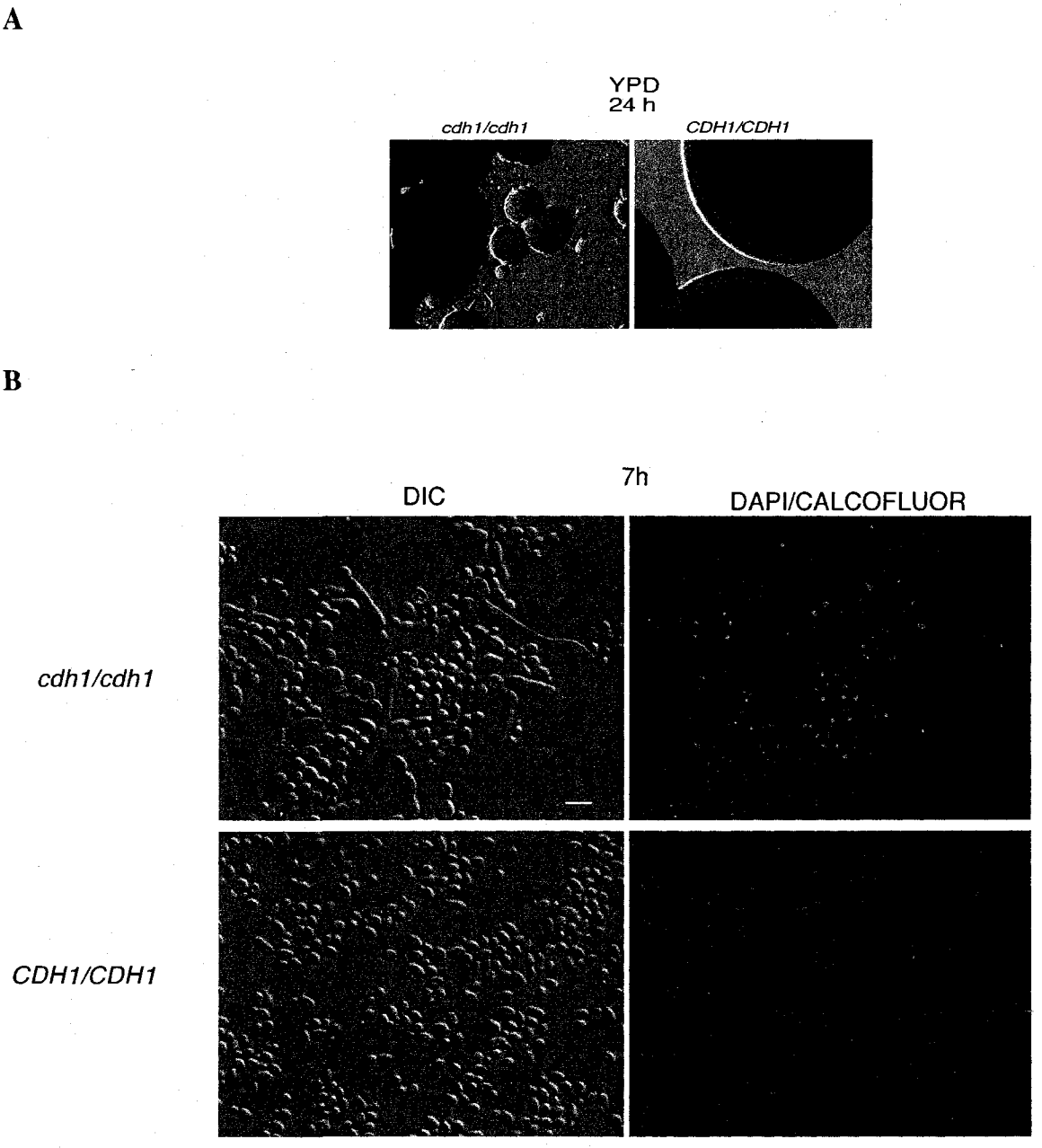
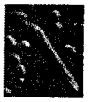


Figure 15: Deletion of *CDH1* results in pleiotropic changes in cell morphology and de-regulated nuclear division and septation
(A) Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in liquid YPD, diluted, streaked onto YPD solid medium and incubated for 72 h under yeast growth conditions. (B) Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in liquid YPD, diluted and collected after 7 h. Cells were stained with DAPI and calcofluor white. Bar: 10 μm.

Table 8: Proportion of *CDH1*-deleted cells[1] exhibiting different morphologies

	Yeast	Enlarged yeast	Chained/Pseudohyphal	Enlongated bud
				
cdh1/cdh1				
0 h (n=245)	70.6	8.6	11.4	9.4
7 h (n=283)	57.6	11.3	19.4	11.7
CDH1/CDH1				
0 h (n=191)	96.3	3.7	0	0
7 h (n=202)	96.5	3.5	0	0

[1] Values expressed in %. Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were inoculated in YPD overnight, diluted, collected and fixed at 7 h.

Southern analysis confirmed the proper construction of the strain (Figure 12). Cells from strains HCCA26 (*cdh1/MET3::CDH1*) and HCCA100 (*CDH1/CDH1*) were inoculated overnight in inducing medium, diluted in both inducing and repressing media, and collected at 0 and 8 h (Figure 16). The diversity and proportions of abnormal morphologies of strain HCCA26 under repressing conditions were very similar to those of the deletion strain, suggesting that the effects were due to the absence of *CDH1* (Table 8). Although some filamentation and abnormal cell morphologies were present in inducing medium, the proportion was significantly less than that under repressing conditions. The phenotype observed in inducing medium could be due to an effect from overexpression of *CDH1*. Thus, absence of APC factor *CDH1* alters cell morphology, but in a different manner from that seen with depletion of Cdc20p or Cdc5p.

The phenotype also differs significantly from that in *S. cerevisiae*, where deletion of *CDH1* resulted in small yeast cells, implying a role for Cdh1p in repressing Start (Jorgensen *et al.*, 2002). That deletion of *CDH1* resulted in some cell enlargement in *C. albicans* suggests that it may function in a different manner.

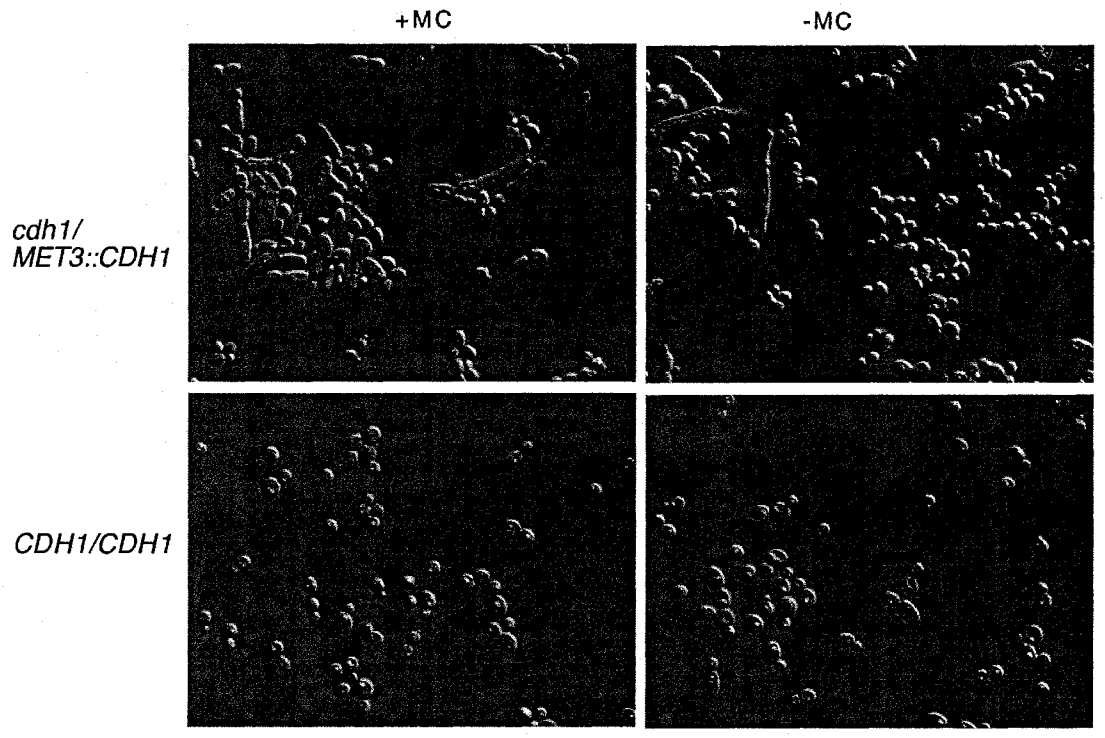


Figure 16: A conditional *CDH1* strain demonstrated a similar phenotype as the deletion strain under repressing conditions
Strains HCCA26 (*cdh1/MET3::CDH1*) and HCCA100 (*CDH1/CDH1*) were incubated in inducing medium overnight and diluted in repressing medium (+MC) and inducing medium (-MC). Cells were incubated for 8 h and fixed. Bar: 10 µm

3.8 Cells lacking Cdh1p show increased invasion of agar

Since cells lacking *CDH1* demonstrated some filamentation, we next tested the ability of the strains to invade agar. Agar invasion assays have been used to estimate the ability of *C. albicans* strains to exhibit invasive growth, involving hyphal and/or pseudohyphal formation independent of serum induction. Invasive growth in turn is an important attribute for virulence since it permits the organism to invade tissues. Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were spotted on solid YPD and incubated for 24 or 72 h. The plates were then thoroughly washed with distilled water to liberate any cells that had not invaded the agar (Figure 17). After 24 h, traces of control cells were present, indicating some degree of agar invasion. However, there were significantly more cells from strain HCCA45 remaining on the plate. After 72 h, this effect was less obvious, but more cells from strain HCCA45 were present. Thus, deletion of *CDH1* results in a more rapid and slightly enhanced invasion of agar.

3.9 Cdh1p is necessary to maintain proper nuclear division and septation

In *S. cerevisiae*, Cdh1p is necessary for degradation of mitotic cyclins and thus is important for cells to exit mitosis and undergo the M/G_1 transition (Schwab *et al.*, 1997; Schwab *et al.*, 2001). To determine whether defects in mitosis were associated with the *CDH1*-deleted cells, strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were inoculated in YPD medium overnight, diluted in fresh YPD for 7 h, and fixed and stained with DAPI and calcofluor. In strain HCCA45, nuclei appeared normal in cells that were in the yeast form (Figure 15B). Since the cells could clearly divide, this suggests that

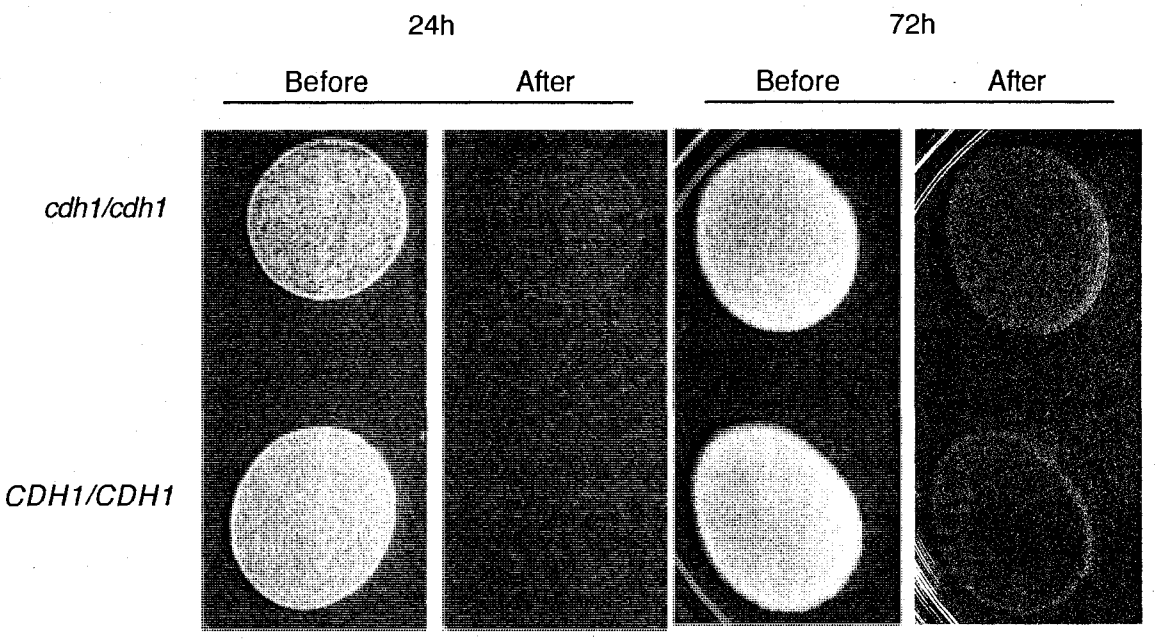


Figure 17: *CDH1*-deleted cells exhibit increased agar invasion
Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in liquid YPD, diluted, 50 µl was spotted on solid YPD medium and incubated for 24 h and for 72 h separately at 30°C after which the cells were thoroughly washed with water.

there were no dramatic defects in nuclear division. However, nuclear defects were prevalent in cells with abnormal morphology. For example, cell compartments defined by septa often contained clusters of 2-3 nuclei, while others contained no nuclei (Figure 15). In contrast, control cells demonstrated 1 nucleus per cell or cell compartment defined by septa. *CDH1*-deleted cells that grew as elongated buds contained 1-2 nuclei, but also had degraded chromatin, suggesting *CDH1* could contribute to normal nuclei division. In comparison, deletion of *CDH1* in *S. cerevisiae* did not result in any dramatic changes in nuclear division, but deficient chromosome segregation caused by spindle abnormalities in some cells was reported (Ross *et al.*, 2003). Calcofluor staining showed that septa could form in *C. albicans* cells lacking *CDH1*, but a de-regulation in the process may have contributed to the multi-nuclear phenotype in some cells. Intriguingly, septa in some filamentous cells were located in the tube and were not constricted, similar to septa in true hyphae (Sudbery *et al.*, 2004). In contrast, cells from control strain HCCA100 grew normally as budding yeast and did not demonstrate any nuclear or septation defects. Therefore, although most cells lacking *CDH1* could undergo nuclear division, a large proportion demonstrated de-regulation of the process and an uncoupling from septation, suggesting that *CDH1* may contribute to these processes.

3.10 Spindle patterns in *CDH1*-deleted cells suggest a delay in late mitosis

In order to determine the precise stage of mitosis in which Cdh1p was required, cells from strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were inoculated in YPD overnight, diluted in fresh YPD, collected after 7 h, fixed, and then processed for

immunolocalization of tubulin using anti-α tubulin antibody. At 7 h, both strains demonstrated a variety of tubulin patterns (Table 9, Figure 18), including single spots that represented spindle pole bodies and interphase cells, or short to long rod-like spindles which represented early (metaphase) or late (telophase) mitotic cells. Cells of strain HCCA45 also contained an abundance of cytoplasmic microtubules, particularly the elongated cells. However, 30.8% (n=260) of cells from strain HCCA45 contained long spindles, compared to 20% (n=240) for control strain HCCA100. The slightly higher proportion of cells in telophase in the *CDH1*-deleted cells suggests that there may be a delay in this cell cycle phase, and that Cdh1p may be important for mitotic exit. In agreement with this, deletion of *CDH1* in *S. cerevisiae* resulted in a slight increase in cells containing long anaphase spindles (20% compared to 14% in wild-type), in agreement with Cdh1p's role in mitotic exit (Visintin *et al.*, 1997).

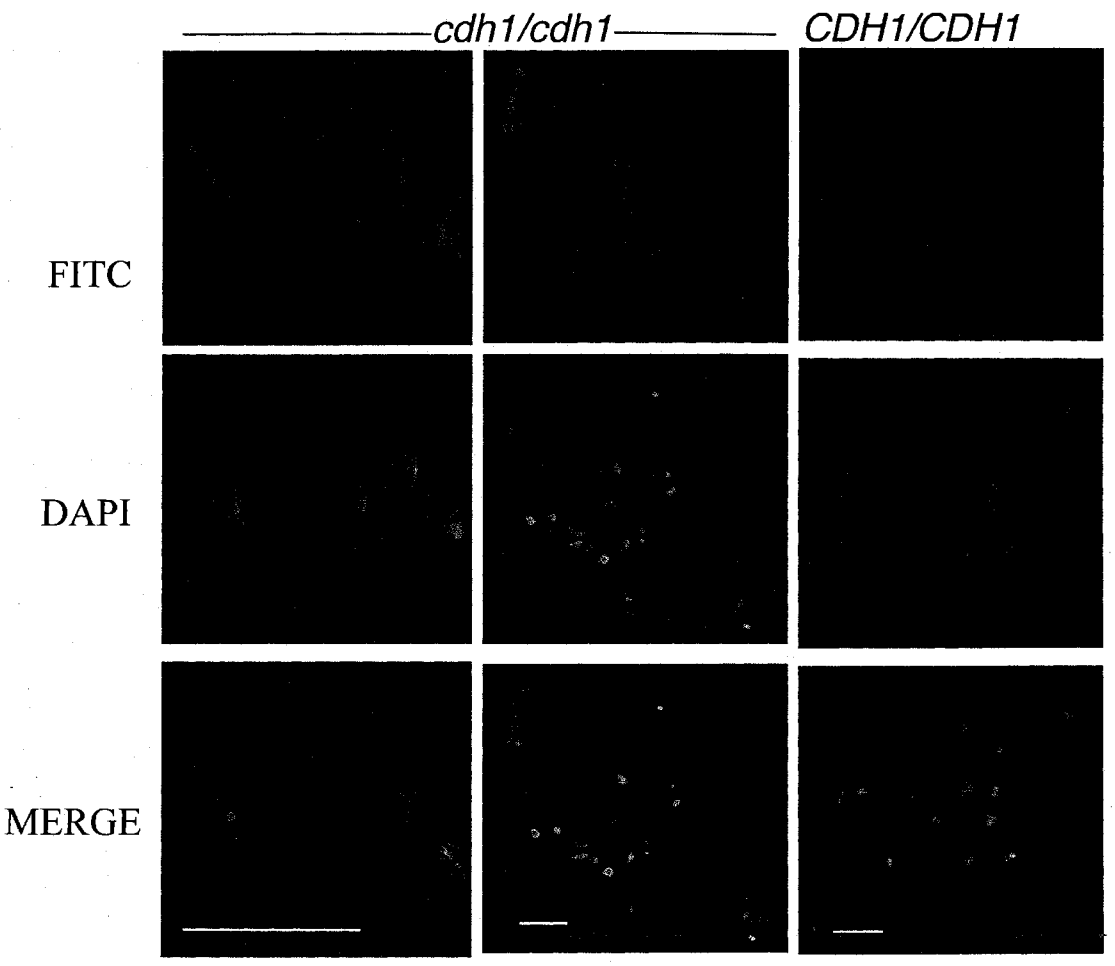


Figure 18: Spindle patterns in *CDH1*-deleted cells
Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in YPD and diluted in fresh medium. Cells were fixed at 7 h and spindles were visualized with immunofluorescence using anti-α tubulin antibody and FITC fluorochrome conjugate. Bar: 10 μm

Table 9: Spindle patterns in *CDH1*-deleted cells[1]

Strains	S/ early G_2[2]	G_2/ early M[3]	M[4]	Cytoplasmic[5]
HCCA45 (n=260) *(cdh1/cdh1)*	37.7	18.5	30.8	13
HCCA100 (n=240) *(CDH1/CDH1)*	62.1	17.9	20	--

[1] Values are expressed in %. Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were inoculated in YPD overnight, diluted, collected and fixed at 7 h and were processed for immunofluorescence of α-tubulin antibody.
[2] Cells containing single or double spots of tubulin indicating cells were in interphase.
[3] Cells containing short rod-like spindles indicating cells were in early mitosis.
[4] Cells containing long mitotic spindles indicating cells were in telophase.
[5] Cells containing cytoplasmic microtubules only.

3.11 Cdh1p is not necessary for Cdc5p-depleted polarized growth

Since a small proportion of cells lacking *CDH1* demonstrated the elongated bud phenotype, Cdh1p may contribute to the same process of cell polarization as seen in Cdc20p or Cdc5p-depleted cells. To test this possibility, a *CDC5*-regulated strain lacking both copies of *CDH1* was created. Southern analysis confirmed the genotype of strain HCCA143 (Figure 14F).

In order to compare the extent of polarization between the strains lacking or containing Cdh1p, strains HCCA143 (*cdh1/cdh1*, *cdc5/MET3::CDC5*), as well as control strains HCCA100 (*CDH1/CDH1, CDC5/CDC5*), HCCA126 (*CDH1/CDH1, cdc5/MET3::CDC5*) and HCCA45 (*cdh1/cdh1*, *CDC5/CDC5*) were inoculated overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5 mM of methionine and 0.5 mM of cysteine, and fixed at 0, 7 and 24 h. At time 0, the majority of cells in all strains were in a normal yeast form (Table 10). At 7 h in repressing medium, 80.2% (n=268) of cells from strain HCCA143 contained elongated buds, 10.1% remained in the yeast form, including enlarged yeast, and 9.7% were enlarged filamenting cells. In control strain HCCA126, 91% (n=178) of cells contained elongated buds, while 8.4% remained as normal yeast cells and 0.6% were enlarged yeast. The elongated bud phenotype observed in strain HCCA143 was due to the absence of Cdc5p, since strain HCCA45, which lacked only Cdh1p, contained 57.6% of cells in a yeast form, 11.3% in an enlarged yeast form, and 31.3% in a filamentous form, including elongated buds and pseudohyphae. Control strain HCCA100, which was not expected to filament due to the presence of Cdc5p, contained 94.4% (n=248) of normal yeast cells, 3.6% enlarged yeast, and 2% elongated cells. At 24 h, there was no significant difference

Table 10: Cell morphology[1] of *CDH1*-deleted cells in *CDC5*-regulated strains[2]

Strains	h	Yeast	Enl.[3]	Pseudo.[4]	Elong. Bud[5]
HCCA143	0 (n=234)	80.3	14.5	---	5.2
(cdh1/cdh1, cdc5/MET3::CDC5)	7 (n=268)	10.1	9.7	---	80.2
HCCA126	0 (n=202)	92.6	4.5	---	2.9
(CDH1,CDH1, cdc5/MET3::CDC5)	7 (n=178)	8.4	0.6	---	91
HCCA45	0 (n=376)	75.8	18.1	1.9	4.2
(cdh1/cdh1, CDC5/CDC5)	7 (n=238)	57.6	11.3	19.4	11.7
HCCA100	0 (n=232)	97	3	---	---
(CDH1/CDH1, CDC5/CDC5)	7 (n=248)	94.4	3.6	---	---

[1] Values are expressed in %.
[2] Cells were inoculated overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5 mM of methionine and 0.5 mM of cysteine, and fixed at 0 and 7 h.
[3] Enlarged yeast cells.
[4] Pseudohyphal cells.
[5] Abnormally enlarged elongated cells and/or elongated buds.

in the extent of filamentation in the presence or absence of *CDH1* (Figure 19). Thus, Cdh1p was not required for polarized growth produced by depletion of Cdc5p, suggesting that it is either not a downstream effector of Cdc5p function in the polarized growth response, or it plays only a partial role.

3.12 *CDH1* is important for serum-induced hyphal growth

Since Cdh1p influences cell polarization under yeast growth conditions and has a negative influence on agar invasion, we next investigated whether it was important for serum-induced hyphal growth. Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were grown overnight in rich YPD medium, and diluted in YPD medium containing 10% serum for 3 h at 37°C (Figure 20). In control strain HCCA100, 91% (n=110) of cells were able to form hyphae. In strain HCCA45, 70.5% (n=374) of the cells formed hyphae, but 29.5% of cells did not. The proportion of cells that were able to form hyphae is somewhat similar to the proportion of cells in a normal yeast form in the *CDH1* deletion strain prior to adding serum. At time 0, 70.6% of cells were in normal yeast form while 30% were of mixed morphologies including enlarged cells, pseudohyphal or elongated buds. Even when cells were incubated in YPD without serum at 30°C for 3 h, a similar proportion of cells were in the normal yeast form (64.1%, n=304), while the remaining 36% were enlarged, pseudohyphal or elongated. Thus, serum-induced hyphae do not form in all cells lacking *CDH1*, which could be due to previous defects in cell morphology. *CDH1* may therefore have some role in regulating the hyphal growth process.

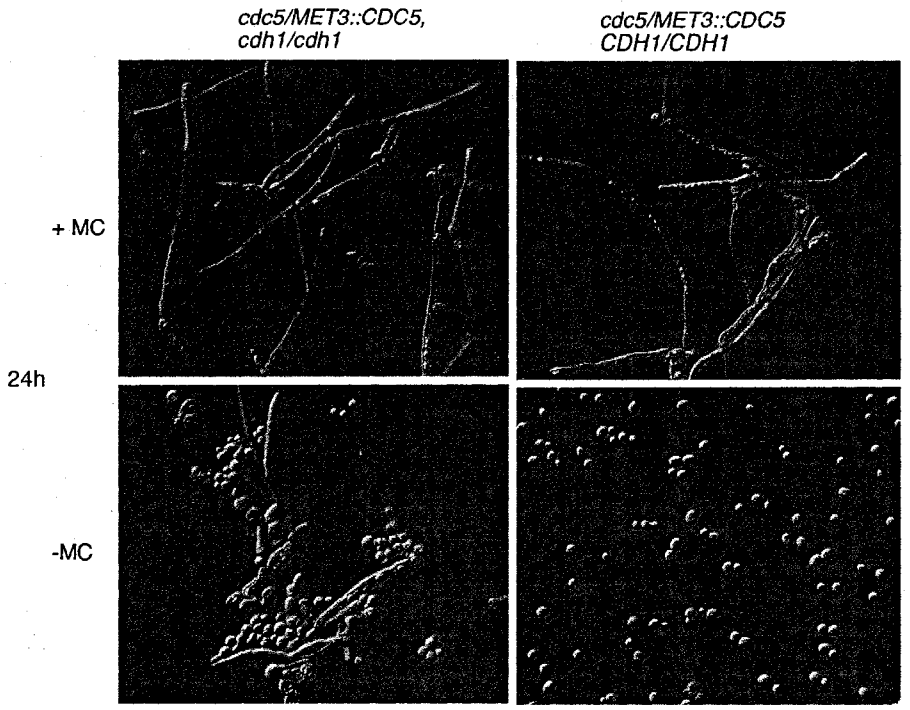


Figure 19: Absence of Cdh1p in Cdc5p-depleted cells does not affect cell polarization

Strains HCCA143 (*cdh1/cdh1, cdc5/MET3::CDC5*) and HCCA126 (*CDH1/CDH1, cdc5/MET3::CDC5*) were grown overnight in inducing medium at 30°C, diluted in repressing medium and fixed at 24 h. Bar: 10 μm

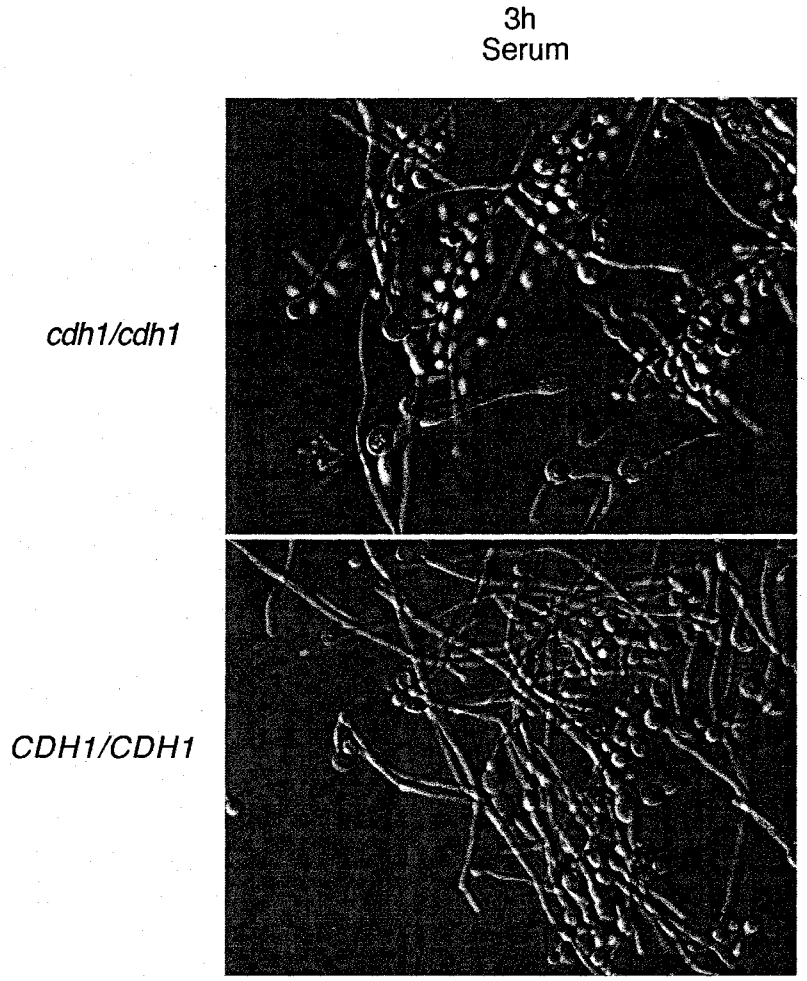
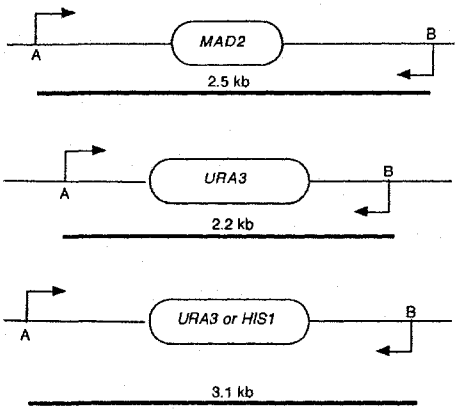


Figure 20: Hyphal formation can occur in some but not all of cells lacking Cdh1p Strains HCCA45 (*cdh1/cdh1*) and HCCA100 (*CDH1/CDH1*) were inoculated overnight in YPD medium, diluted in YPD containing 10% serum and incubated for 3 h at 37°C. Bar: 10 μm

3.13 Deletion of spindle assembly checkpoint factor *MAD2* partially impairs polarized growth in cells depleted of Cdc5p

Previous work demonstrated that the spindle checkpoint factor Mad2p was partially required for nocodazole-induced polarized growth in *C. albicans* (Bai *et al.*, 2002), while a different spindle checkpoint factor, Bub2p, contributed to Cdc5p-dependent polarized growth (Bachewich *et al.*, 2005). Polarization was not completely abolished in the absence of either Mad2p or Bub2p, suggesting that multiple checkpoint factors are involved. Since Cdc20p is a known target of Mad2p in *S. cerevisiae*, and our results suggest that Cdc20p may lie downstream of Cdc5p function in *C. albicans*, we next investigated whether Mad2p provided a link between Cdc5p and Cdc20p by determining if it was required for Cdc5p-depleted polarized growth. Both copies of *MAD2* were deleted from the conditional *CDC5* strain HCCA34, creating strains HCCA56 and HCCA57. As a control, both copies of *MAD2* were deleted from strain BWP17, which was wild-type for *CDC5*, creating strain HCCA110. Southern blotting was performed to confirm correct creation of the strains (Figure 21). A different amount of flanking DNA was removed in strains HCCA56 and HCCA57 compared to strain HCCA110, generating a different band size for deleted *MAD2* alleles. Non-specific bands were also present on the blots (Figure 21B), but were no longer detected when another probe was used for the Southern (Figure 21C). The second probe, however, was designed such that it was not possible to distinguish between the individual knockout alleles. Taken together, however, the Southern blots confirm correct construction of the strains.

A


MAD2
A
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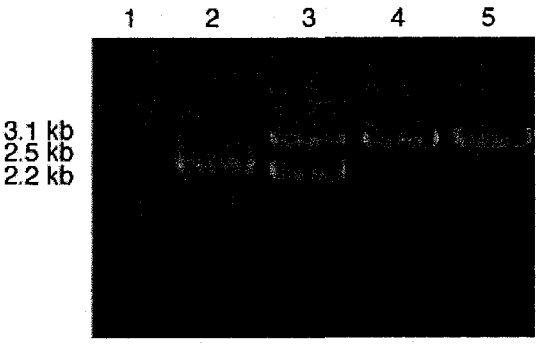
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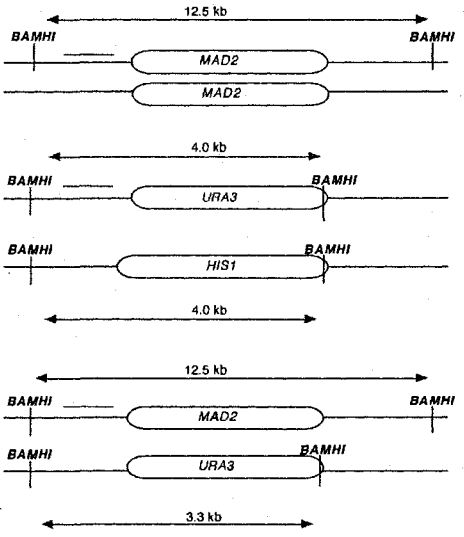
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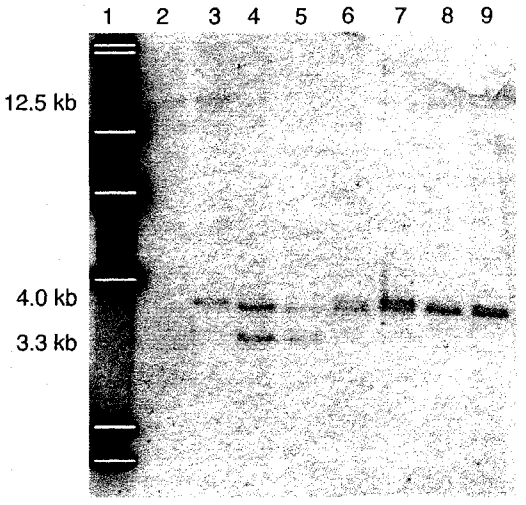
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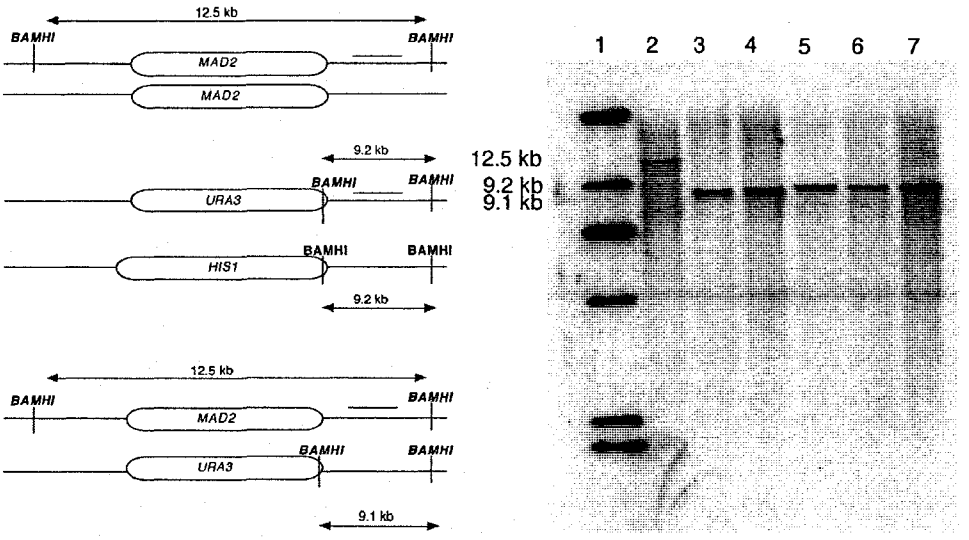


Figure 21: PCR and Southern screening of *mad2/mad2* strains

A) Map and PCR screen of *mad2/mad2* strains, showing different band sizes for alleles replaced with constructs containing 500 versus 80 nucleotides of homologous flanking DNA. Lane 1: MWM; lane 2: wild-type; lane 3: *mad2/MAD2*, *cdc5/MET3::CDC5*; lanes 4-5: *mad2/mad2*. B) Map and Southern blot using a 5' flanking probe. Lane 1: MWM; lane 2: wild-type; lane 3: *mad2/MAD2*; lanes 4-5: *mad2/mad2*, *cdc5/MET3::CDC5*; lanes 6-7-8-9: mad2/mad2. C) Map and Southern blot using different 3' flanking probe. Lane 1: MWM; lane 2: wild-type; lanes 3-4: *mad2/mad2, cdc5/MET3::CDC5*; lanes 5-6-7: *mad2/mad2*

We first investigated the growth rate of *MAD2*-deficient cells. Cells from strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*), HCCA110 (*mad2/mad2, CDC5/CDC5*), HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) and HCCA100 (*MAD2/MAD2, CDC5/CDC5*) were inoculated overnight in inducing medium, diluted in fresh inducing medium, and streaked onto solid inducing medium for 72 h at 30°C to compare colony sizes. Colonies from strains HCCA56 and HCCA110, which lacked *MAD2*, were much smaller compared to control strains HCCA126 and HCCA100 (Figure 22). Some mild filamentation was also observed. To determine the doubling rate of yeast cells, the strains were inoculated overnight in inducing medium, diluted in fresh inducing medium to maintain expression of *CDC5*, and collected every 2 h to measure the $O.D._{600nm}$. Since cells lacking *MAD2* had the tendency to clump together as they grew, sonification of live cells prior to measuring the optical density was performed. While control strains HCCA100 and HCCA126 grew normally, with a doubling time of approximately 1.5 h-2 h (Figure 23), strains HCCA56 and HCCA110 grew much slower, with a doubling time of approximately 6 h-8 h. This is in contrast to a previous study (Bai *et al.*, 2002), where deletion of *MAD2* in a different strain background did not affect cell growth in rich or minimal medium.

Despite the difference in yeast doubling rate, we next explored whether the absence of *MAD2* influenced Cdc5p-depleted polarized growth. Strains HCCA56 and HCCA126 were inoculated overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium to shut off *CDC5*, and incubated for 0, 4, 8 or 24 h, after which they were sonicated and fixed. At time 0, 100% (n=223) of cells in control strain HCCA126 were in a yeast form (Figure 24 and 25), while in strain HCCA56,

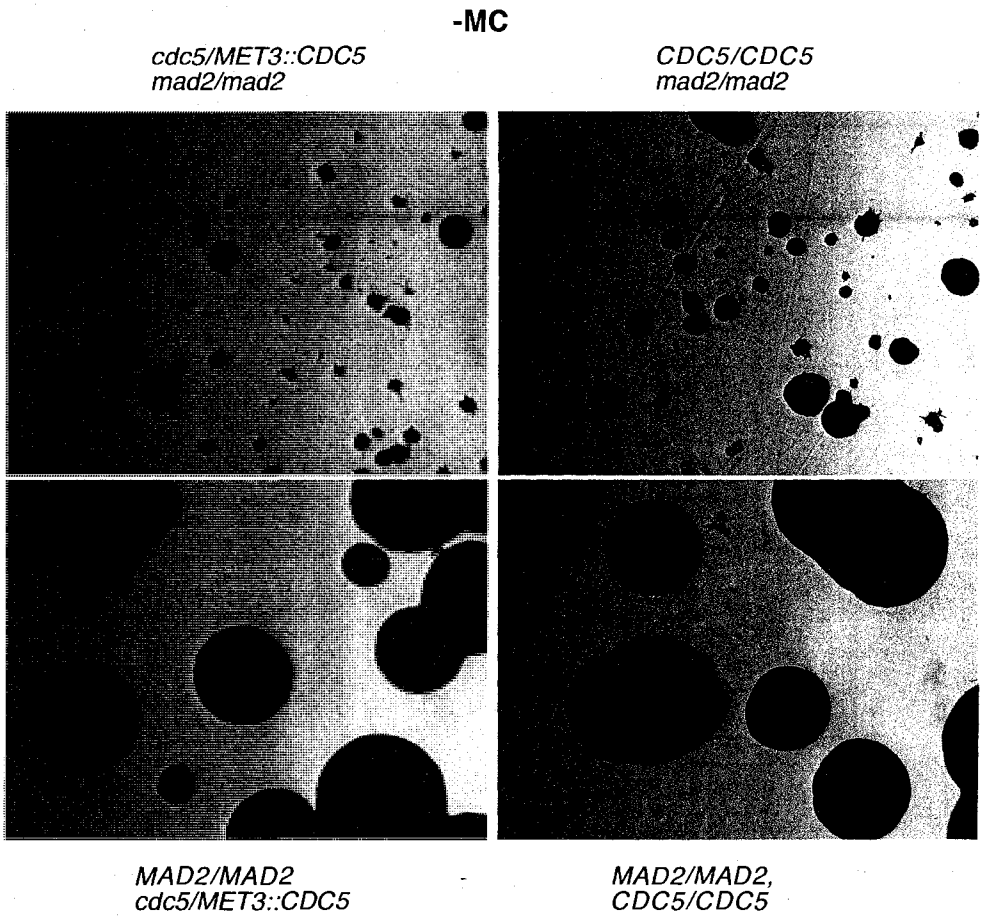


Figure 22: Cells lacking Mad2p produce smaller colonies and show signs of mild filamentation

Cells from strains HCCA56, HCCA110, HCCA126 and HCCA100 were inoculated overnight in inducing medium lacking methionine and cysteine, diluted and plated on solid inducing medium, and incubated for 72 h at 30°C under yeast growth conditions.

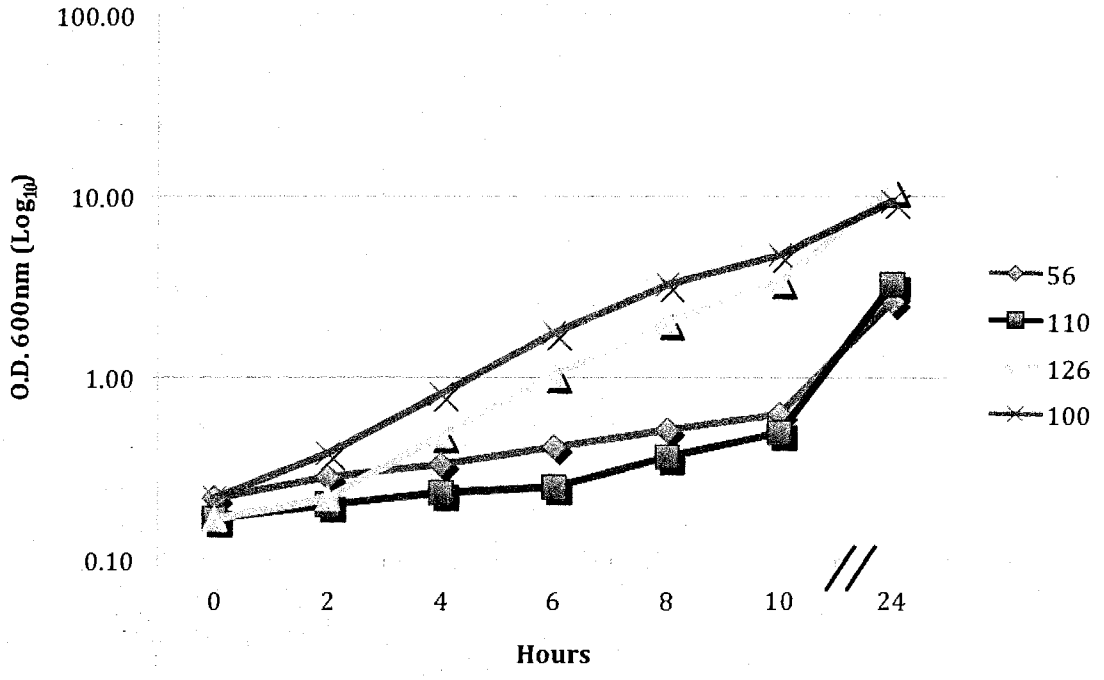


Figure 23: Growth curve for *MAD2* mutant strains and control strains
To measure cell growth rate, cells from strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*), HCCA110 (*mad2/mad2, CDC5/CDC5*), HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) and HCCA100 (*MAD2/MAD2, CDC5/CDC5*) were inoculated overnight in inducing liquid medium lacking methionine and cysteine, diluted in fresh inducing medium, and collected every 2 h. Graph is representative of 3 separate experiments.

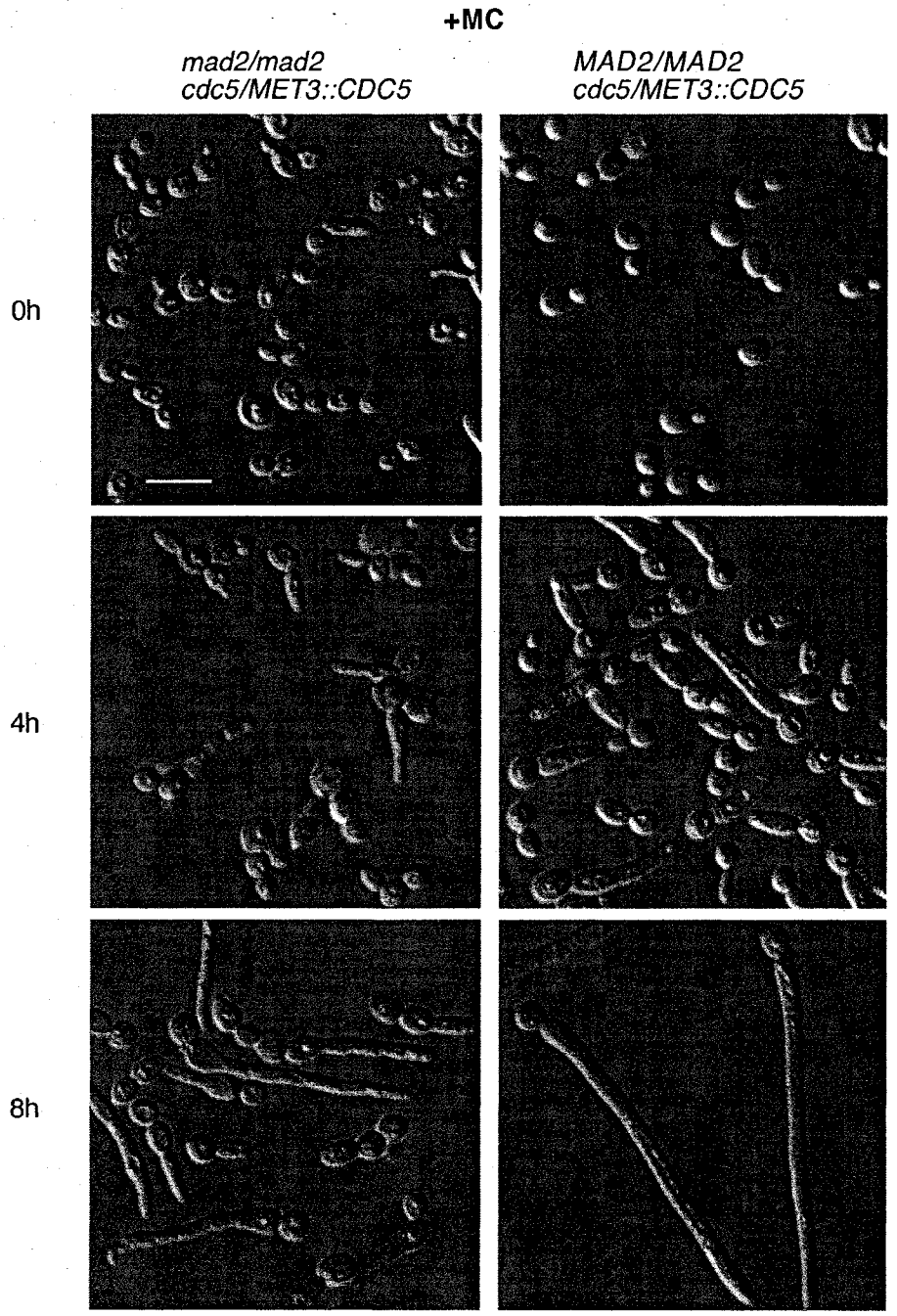


Figure 24: Deletion of the spindle checkpoint factor *MAD2* in Cdc5p-depleted cells compromises polarized growth

Cells of strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*) and HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) were grown overnight in inducing medium lacking methionine and cysteine at 30°C, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and fixed at 0, 4 and 8 h. Bar: 10 µm

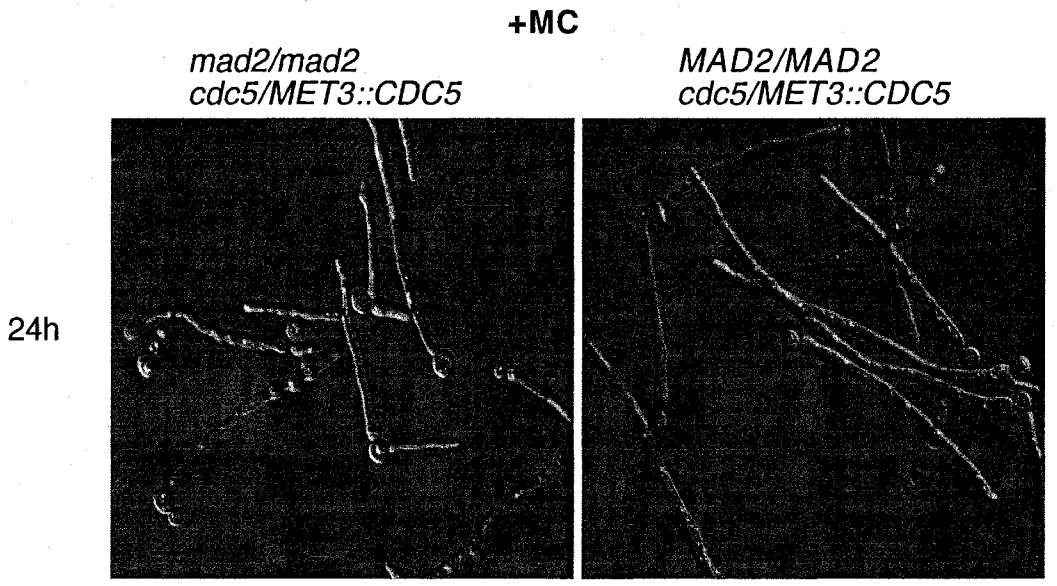


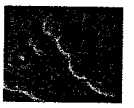
Figure 25: Depletion of Mad2p results in abnormal polarized growth
Cells of strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*) and HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) were grown overnight in inducing medium lacking methionine and cysteine at 30°C, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and fixed at 24 h. Bar: 10 μm

87.2% (n=321) of cells were yeast, 10% were chains of yeast composed of 3-4 cells, and 2.8% were elongated. Of the yeast cells, approximately 10% were irregular in morphology and enlarged, demonstrating that absence of *MAD2* under *CDC5*-inducing conditions had mild effects on morphology. After 4 h in repressing medium to shut off *CDC5*, 87.7% (n=219) of cells from control strain HCCA126 were elongated, while 12.3% remained in a yeast cell form. In contrast, only 25.6% (n=351) of cells from strain HCCA56 were elongated, most of which were shorter in length than those seen in the control strain HCCA126. The remaining proportions of cells were in a yeast form, where 43.6% were large budded, 28.2% were unbudded or small-budded, and 2.6% were chained cells (Figure 24). At 8 h, 100% (n=105) of cells from control strain HCCA126 were highly polarized. In contrast, 55.9% (n=247) of the cells from strain HCCA56 had short or long extensions, 25.9% were large budded, 13.4% were small or unbudded, and 4.9% were chained yeast cells. The filaments that did form were more irregular in shape compared to the control, often with jagged tips (Figure 24). The difference in filament morphology between the strains became more apparent at 24 h, where cells from strain HCCA56 were much more irregular in shape, and still shorter than those of the control (Figure 25). Although the slow growth rate likely contributed to the shorter length of filaments, it does not fully explain the difference in morphology, particularly the jagged tips and irregular-shaped filament walls that showed periodic isotropic expansion. Thus, Cdc5p-depleted cells lacking *MAD2* can still polarize, but the maintenance and regularity of the polarization is compromised, suggesting that Mad2p may partly lie downstream of Cdc5p function.

To determine whether absence of Mad2p partially suppressed the mitotic block in Cdc5p-depleted cells, strains HCCA56 and HCCA126 were inoculated overnight in inducing medium lacking methionine and cysteine, diluted in repressing medium, fixed at 0, 6 and 12h, and stained with DAPI and calcofluor to visualize nuclei and septa, respectively. At time 0, no defects in nuclear distribution were observed in control strain HCCA126, but approximately 13.4% (n=321) of the cells from strain HCCA56 contained compartments that either lacked nuclei, or contained 2 or more nuclei. At 6 h of repression (Table 11), when 97.7% (n=181) of cells of control strain HCCA126 were elongated and 2.3% were in the yeast form, 7.8% had a single nucleus, 44.2% has 2 nuclei and 42.3% contained fragmented chromatin. In strain HCCA56, however, where approximately 60% (n=262) of cells were in the elongated form, and the remaining cells were in a yeast form, 27.5% contained a single nucleus, 42.4% contained 2 nuclei, 6.5% contained 3 nuclei, and the remaining 23.6% showed fragmented chromatin. Although more cells had 1 nucleus in this strain, this was likely due to the fact that many were still in a yeast form, the majority of which were large-budded. Thus, absence of *MAD2* did not significantly suppress the block in nuclear division. However, the lower proportion of cells with fragmented chromatin suggests that nuclear integrity was improved. This effect could not be solely due to the slower growth rate of cells because even at a longer incubation time of 12 h, strain HCCA56 contained numerous intact nuclei compared to the control cells at 6 and 12 h (Figure 26).

In order to confirm that the results were due to absence of *MAD2*, a conditional strain was constructed that contained single copies of *CDC5* and *MAD2* under control of the *MET3* and *MAL2* promoters, respectively. However, under *MAD2*-repressing

Table 11: Number of nuclei and cell morphology in *CDC5*-repressed cells in the presence or absence of *MAD2*[1]

	Number of nuclei				Cell morphology		
	1	2	≥ 3	F[2]	Budding 	Elongated 	Chained 
HCCA56 (n=262) (*mad2/mad2 cdc5/MET3::CDC5*)	27.5	42.4	6.5	23.6	33.2	59.9	6.9
HCCA126 (n=181) (*MAD2/MAD2 cdc5/MET3::CDC5*)	7.8	44.2	5.5	42.5	2.2	97.8	--

[1]Values expressed in %. Strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*) and HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) were inoculated in inducing medium overnight, diluted in repressing medium, fixed at 6 h and stained with DAPI.
[2]Fragmentated chromosomes in cells.

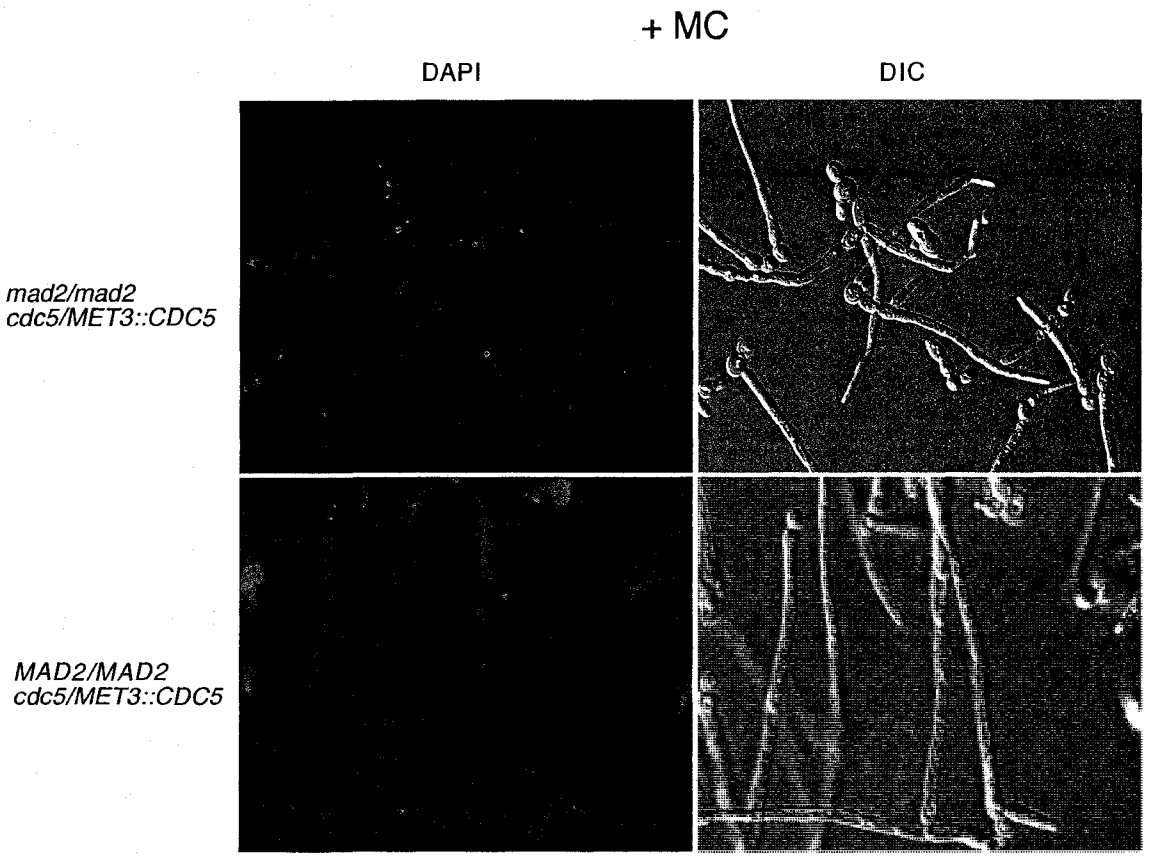


Figure 26: Absence of Mad2p when repressing *CDC5* results in improved integrity of chromatin

Cells of strains HCCA56 (*mad2/mad2, cdc5/MET3::CDC5*) and HCCA126 (*MAD2/MAD2, cdc5/MET3::CDC5*) were grown overnight in inducing medium at 30°C, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and fixed at stained with DAPI and calcofluor at 12 h. Bar: 10 μm

conditions, the strain did not show the slow growth phenotype as the deletion strains. Since the extent of *MAD2* repression in the conditional strain has not been determined via Northern blot, we can not yet make a conclusion based on this strain. Overall however, the results suggest that Mad2p may mediate at least part of Cdc5p function.

4. Discussion

We have characterized the APC activators Cdc20p and Cdh1p in *C. albicans*, and show that they are important for regulating similar stages of mitosis as their homologues in *S. cerevisiae*, but they dramatically influence morphogenesis in a different manner. While absence of *CDC20* results in elongated bud growth, deletion of *CDH1* results in a pleiotropic phenotype, including enlarged yeast, pseudohyphae and some elongated buds. In contrast, absence of *CDC20* or *CDH1* in *S. cerevisiae* results in large-budded or small cells, respectively. The results suggest that Cdc20p is a major component of the pathway that links mitotic arrest and spindle checkpoint activation to elongated growth of the bud, whereas Cdh1p is not essential for this process. Cdh1p, however, has a profoundly different influence on Start compared to its counterpart in *S. cerevisiae*. Overall, the results present the first picture of APC function and provide major insights into the mitotic regulatory circuit in *C. albicans*, which is similar but not identical to that in *S. cerevisiae*. The data also contribute to an emerging framework of the pathway underlying checkpoint-activated growth and morphogenesis in the pathogen.

4.1 Cdc20p is important for the metaphase-to-anaphase transition and mitotic exit

Since cells depleted of Cdc20p showed delays in metaphase and telophase, the results suggest that Cdc20p is required for both the metaphase-to-anaphase transition and mitotic exit. This is consistent with the function of Cdc20p in *S. cerevisiae*, although deletion of the gene results in a metaphase arrest only. However, an additional role in mitotic exit was implied by the fact that deletion of the downstream securin homologue

PDS1 partially suppressed the *CDC20* deletion phenotype, resulting in a cell cycle arrest at telophase (Lim *et al.*, 1998; Shirayama *et al.*, 1998). The fact that *C. albicans* cells lacking Cdc20p did not remain arrested in metaphase could be due to some leakiness of the *MET3* promoter, but this cannot account for the subsequent block in telophase. Although Cdc20p in *C. albicans* may function in regulating similar stages of mitotic progression as its counterpart in *S. cerevisiae*, the mechanism appears to be different. Cdc20p is required to target the APC towards degradation of the securin, Pds1p, and Pds1p/securin in turn is required to block seperase activity and thus seperation of sister chromatids at anaphase (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999). However, *C. albicans* lacks a homologue of Pds1p or any other known securin. In *S. cerevisiae*, Pds1p is essential for checkpoint-induced metaphase arrest, and cells lacking the gene are inviable (Giaever *et al.*, 2002). Although additional modes of inhibiting anaphase progression exist in other systems, including blocking seperase activity via CDK/cyclin B-dependent phosphorylation, securin homologues are present and function in these organisms (Stemmann *et al.*, 2001; Gorr *et al.*, 2005; Huang *et al.*, 2005; Stemmann *et al.*, 2005; Holland *et al.*, 2006). *C. albicans* may therefore utilize different mechanisms for a key step in mitotic progression compared to *S. cerevisiae*, suggesting a re-wiring of this critical process. How Cdc20p exerts its function in mitotic control, and the nature of the Cdc20p target(s) in *C. albicans*, are the focus of future experiments.

4.2 Cdc20p contributes to the regulation of elongated bud growth

Although Cdc20p may regulate a similar stage of mitosis as its counterpart in *S. cerevisiae*, it has a very different influence on morphogenesis in *C. albicans*. Depletion

of Cdc20p in *C. albicans* resulted in elongated bud growth, while *CDC20* mutants in *S. cerevisiae* arrested as large doublets (Hartwell *et al.*, 1973). Cdc20p does not appear to be a direct negative regulator of polarized growth, since a block in mitosis due to depletion of other factors or exposure to certain drugs induces a similar morphological response (Bai *et al.*, 2002; Bachewich *et al.*, 2003; Bachewich *et al.*, 2005; Bensen *et al.*, 2005), and absence of Cdc20p did not enhance or influence serum-induced hyphal growth in any way. Alternatively, the results support the possibility that a mitotic block underlies the polarized growth response, and suggest that Cdc20p lies in the pathway linking mitotic progression and checkpoint activation to polarized growth of the bud.

Since absence of *CDC20* resulted in the same phenotype as depletion of Cdc5p, the factors may lie in the same pathway. Overexpression of *CDC20* in Cdc5p-depleted cells partially suppressed the block in mitosis, as seen by rebudding of mother yeast cells, suggesting that Cdc20p may lie downstream of Cdc5p. Rebudding typically indicates that cells have re-entered a new cell cycle (Hoyt *et al.*, 1991). The fact that the total number of nuclei did not increase in cells overexpressing *CDC20* while repressing *CDC5* does not rule out the possibility that the cells were able to exit mitosis. In fact, cells can exit mitosis without separating chromatin. In addition, if cells were previously in telophase, with 2 masses of chromatin connected by a mitotic spindle, we would not expect to see a significant increase in the total number of nuclei if these cells exited mitosis. Analyzing spindles in these cells would help clarify this point. However, overexpression of *CDC20* did not suppress polarized growth of Cdc5p-depleted cells, suggesting that Cdc20p alternatively acts in a pathway parallel to that of Cdc5p. Since we do not yet know the extent of Cdc20p overexpression or stability during our time

course, and some rebudding did occur, the results are more consistent with the possibility that Cdc20p lies downstream and mediates at least part of Cdc5p function in *C. albicans*. Future work will involve determining the effects of depleting both Cdc5p and Cdc20p on polarized growth to help confirm the relationship between the two factors.

If Cdc5p lies upstream of Cdc20p, it could exert its function directly through phosphorylation. Although Cdc5p does not lie upstream of Cdc20p in the mitotic pathway in *S. cerevisiae*, Cdc20p was identified in a recent screen for Cdc5p targets based on combined chemical-genetic and proteomic tools (Snead *et al.*, 2007). However, the functional relevance of this hit has not yet been demonstrated. In higher organisms, a direct relationship between polo-like kinases and Cdc20p has also not been demonstrated, but in *Xenopus*, the Cdc5p homologue Plx1 influences APC activity through controlling a negative regulator, Emi1p, which directly binds to Cdc20p (Reimann *et al.*, 2001; Moshe *et al.*, 2004). Since *C. albicans* does not have a homologue of *EMI1*, any potential regulatory relationship between Cdc5p and Cdc20p would involve a different mechanism.

It is also possible that Cdc5p could influence Cdc20p activity indirectly via the SAC. Depletion of Cdc5p in *C. albicans* causes an early mitotic arrest and spindle defects, which could in turn activate the SAC. Given that the SAC factor Mad2p binds and inactivates Cdc20p in other systems, it is tempting to speculate that Mad2p provides a link between Cdc5p and Cdc20p in *C. albicans*. In support of this, deleting *MAD2* in Cdc5p-depleted cells partially affected polarized growth, resulting in irregular-shaped filamentation. The major growth difference due to absence of *MAD2* most likely accounted for the decrease in filament length compared to control cells, but it cannot

explain the major changes in morphology. The effect on growth rate was surprising, given that deletion of *MAD2* did not affect yeast doubling time in a different strain background (Bai *et al.*, 2002). However, there is a high level of genome instability and differences in sequence and behavior in lab strains of *C. albicans* (Selmecki *et al.*, 2005). In addition, after our work on *MAD2* was completed, a more recent annotation of the *C. albicans* genome identified a hypothetical, *C. albicans*-specific ORF lying in the 3' flanking region of *MAD2*, 50 nucleotides of which are missing in our *MAD2*-deleted strains. This may account for the growth rate difference, but cannot explain the changes in morphology that occur when Cdc5p is repressed in the absence of *MAD2*. In support of our results, the morphological phenotype was very similar to *C. albicans* cells exposed to nocodazole that also lacked *MAD2* (Bai *et al.*, 2002). To clarify the issue, future work will involve completing the re-introduction of a copy of *MAD2* into our deleted strains. Since polarization could still occur in the absence of Mad2p, albeit in an abnormal way, Mad2p could mediate part of Cdc5p function. In comparison, absence of Bub2p reduced but did not abolish polarized growth in Cdc5p-depleted cells (Bachewich *et al.*, 2005), suggesting that multiple checkpoint factors may contribute to the growth response. It would be informative to determine the extent of the polarization in Cdc5p-depleted cells upon deletion of both *MAD2* and *BUB2*.

In contrast to our results, deletion of *MAD2* in *S. cerevisiae* did not suppress the *CDC5* null phenotype, and Cdc5p was thought to alternatively lie downstream of Mad2p (Li *et al.*, 1991; Hu *et al.*, 2002). However, more recent evidence challenges this view, since the phenotype of specific mutants of *CDC5*, characterized by minor defects in spindle dynamics and cell growth, is suppressed by the addition of the microtubule-

depolymerizing agent benomyl in a Mad2p-dependent manner (Park *et al.*, 2008). In addition, Cdc5p phosphorylates the spindle checkpoint protein Mad3p, which is known to form a complex that targets Cdc20p during spindle activation (Rancati *et al.*, 2005). In other systems, additional lines of evidence suggest polo-like kinases can act upstream of Mad2p and/or other spindle checkpoint proteins. For example, depletion of human Plk1p causes a prometaphase arrest that was dependent on either Mad2p or BubR1p (van Vugt *et al.*, 2004), and in *Xenopus,* the polo-like kinase Plx1p phosphorylates kinetochores in response to low kinetochore tension, which is important for spindle checkpoint activation and increased levels of Mad2p and Cdc20p at the kinetochores (Ahonen *et al.*, 2005). While Plx1p and Cdc5p in *Xenopus* and *S. cerevisiae*, respectively, appear to function upstream of MAD proteins in a positive manner during checkpoint activation, Cdc5p in *C. albicans* appears to have a more negative influence, since absence of Cdc5p may lead to checkpoint activation (Bachewich *et al.*, 2003). Although the nature of the relationship between Mad2p and Cdc5p in *C. albicans* is unclear and possibly different than in other organisms, Mad2p appears to mediate at least part of Cdc5p function, and contribute to the polarized growth response.

4.3 Cdh1p is important, but not essential, for mitotic exit

The results show that Cdh1p, another activator of the APC, is important for the regulation of mitosis in *C. albicans*, but unlike Cdc20p, is not essential. The high proportion of *CDH1*-deleted cells containing telophase spindles suggests that Cdh1p may function during mitotic exit. Consistent with our results, *CDH1* mutant cells in *S. cerevisiae* were viable, but a high proportion contained separated chromatin (Schwab *et*

al., 1997) and long spindles, indicating a delay in mitotic exit (Visintin *et al.*, 1997). Since cells that are unable to exit mitosis have high levels of the B-type cyclin Clb2p, we have tagged Clb2p with the HA epitote (Appendix 1) in *CDH1*-deleted cells to gain further evidence for the role of Cdh1p in mitotic exit in *C. albicans*. In *S. cerevisiae*, Cdh1p acts with the cyclin-dependent kinase inhibitor Sic1p to control exit from mitosis via targeted degradation of Clb2p and inhibition of CDK/CLB2 activity. *C. albicans* contains a Sic1p homologue, called Sol1p (Atir-Lande *et al.*, 2005), which is also not essential, but its role in regulating mitosis was not determined. Construction of a *CDH1/SOL1* deletion strain would be informative to determine whether these factors play the crucial role in regulating mitotic exit as do their counterparts in *S. cerevisiae*. Sic1p and Cdh1p are in turn regulated by Cdc14p phosphatase in *S. cerevisiae*, of which *C. albicans* contains a homologue (Clemente-Blanco *et al.*, 2006). Since deletion of *CDC14* also results in a delay in mitotic exit, it is possible that Cdc14p may regulate Cdh1p in *C. albicans*. Therefore, *C. albicans* Cdh1p may lie in a similar position in the mitotic pathway as in *S. cerevisiae*.

4.4 Cdh1p is important for morphogenesis but dispensable for Cdc5p-depleted polarized growth

Cdh1p was also found to have a strong influence on cell morphogenesis, but in a different manner than Cdc20p. Deletion of *CDH1* resulted in pleiotropic effects, including a significant proportion of enlarged and abnormal-shaped yeast, normal yeast, and a small proportion of cells with elongated buds. In striking contrast, cells lacking *CDH1* in *S. cerevisiae* were small (Schwab *et al.*, 1997; Jorgensen *et al.*, 2002). The

deletion phenotype did not resemble cells depleted of Cdc20p or Cdc5p, and absence of *CDH1* did not dramatically influence Cdc5p-dependent polarized growth, indicating that Cdh1p is not necessary for the polarized growth response. The fact that a few *CDH1*-deleted cells grew as elongated buds suggests that Cdh1p may lie in the Cdc5p-dependent pathway, but at the same level as other redundant factors. Intriguingly, cells lacking the Sic1p homologue, Sol1p, grew transiently as elongated buds when in exponential phases, but resorted to a yeast growth mode at higher cell density, producing cells that more closely resembled those deleted of *CDH1* (Atir-Lande *et al.*, 2005). If a double *CDH1/SOL1* mutant in *C. albicans* showed synergistic effects on the elongated bud growth response, this would suggest that both factors are required for polarized growth and may redundantly lie downstream of Cdc5p function. In comparison, absence of both *CDH1* and *SIC1* in *S. cerevisiae* is synthetically lethal, and cells arrest in a large-budded state (Schwab *et al.*, 1997). Thus, Cdh1p dramatically influences cell morphogenesis in a manner different from that seen with Cdh1p in *S. cerevisiae*, and may contribute only partially to the Cdc5p-depleted phenotype. The mitotic defects in *CDH1*-deleted cells could precede and contribute to the more extreme phenotypes in these cells, but this would require confirmation with time-lapse imaging of synchronized cells with a GFP-tagged nuclear marker.

The role of Cdh1p in morphogenesis is complex, since it had a negative influence on elongated growth of yeast buds and during agar invasion but may have a positive role during hyphal growth. *CDH1*-deleted cells with abnormal morphology did not appear to be able to form normal hyphae upon exposure to serum, although this needs to be confirmed by time-lapse imaging of yeast cells prior to and during serum induction.

Similar to our results with Cdh1p, cells lacking Cdc14p phosphatase were not able to respond to serum properly (Clemente-Blanco *et al.*, 2006), nor were cells lacking other important cell cycle regulators, such as Gin4p and Fkh2p (Bensen *et al.*, 2002; Wightman *et al.*, 2004). Cells lacking Cdc5p or Clb2p, on the other hand, can form true hyphae when incubated in the presence of serum (Bachewich *et al.*, 2003; Bensen *et al.*, 2005). If some cells lacking Cdh1p have higher Clb2p levels, due to the role of Cdh1p in mitotic exit, this may account for the inability to form hyphae properly, as cells overexpressing *CLB2* or *CLB4* are impaired in maintaining hyphal growth (Bensen *et al.*, 2005). Alternatively, pseudohyphal cells are not able to form hyphae efficiently when exposed to serum (Bensen *et al.*, 2002). This may explain why some *CDH1*-deleted cells, which were pseudohyphal, were unable to form true hyphae in presence of serum. However, other morphologies were also present and cells with morphological defects also had abnormal nuclear division, which in turn could have an influence on hyphal growth. Thus, Cdh1p is important in regulating polarized morphogenesis of different cell types, though the mechanisms are not yet clear. The results also suggest that different strategies are employed for polarized growth of yeast buds, true hyphae, and filaments formed during agar invasion.

4.5 Cdh1p does not regulate Start in *C. albicans* as it does in *S. cerevisiae*

The absence of a small cell phenotype in *C. albicans* cells lacking *CDH1* was in striking contrast to that seen in *S. cerevisiae* and suggests that Cdh1p is not a repressor of Start (Jorgensen *et al.*, 2002). Alternatively, the significant proportion of enlarged cells

resulting from deletion of *CDH1* in *C. albicans* suggests that Cdh1p may positively influence Start. Mutants in *S. cerevisiae* that exhibit large cell size include regulators of Start, such as *SWI4*, *SWI6* and *CLN3* (Jorgensen *et al.*, 2002). Intriguingly, deletion of *SWI4* in *C. albicans* results in a somewhat similar phenotype as deletion of *CDH1* (B. Hussein & C. Bachewich, unpublished results). It is not clear how Cdh1p could influence Start in a positive manner, but in *S. cerevisiae*, Cdh1p represses Start through acting upstream of the SBF (Swi4/Swi6) transcription factor complex, which is necessary for activation of G_1 cyclins and other factors at the G_1/S transition (Koch *et al.*, 1994; Jorgensen *et al.*, 2002). Thus, Cdh1p in *C. albicans* is clearly functioning in a different manner than its counterpart in *S. cerevisiae* during G_1 phase, and may play some novel role in regulating Start.

4.6 Linking mitotic progression to elongated bud growth

We have demonstrated that homologues of APC activators function during mitosis and strongly influence morphogenesis in a manner that suggests that APC activity is important for the checkpoint-associated elongated bud growth response in *C. albicans*. In contrast, activity of another ubiquitin ligase complex that functions during G_1 phase, Skp1-Cullin-1/Cdc53-F-box (SCF), influences morphogenesis in a different way. *C. albicans* cells lacking the SCF activator Cdc4p grow constitutively as true hyphae or pseudohyphae (Atir-Lande *et al.*, 2005), with active cell cycles. Thus, we have identified additional factors that contribute to the novel spindle checkpoint-activated polarized growth response in *C. albicans*, and provide a framework for the underlying mitotic pathway (Figure 27). However, it is not clear how arresting mitosis leads to polarized

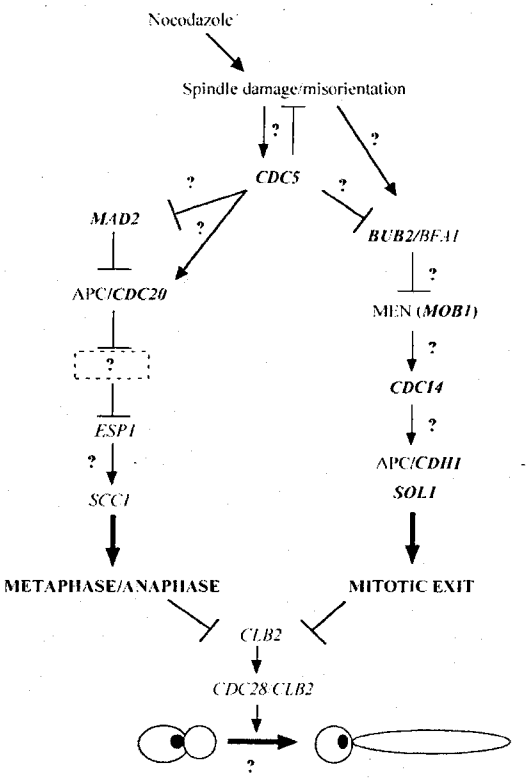


Figure 27: A model for the mitotic regulatory circuit involving spindle checkpoint factors in *C. albicans*, and the link with cell polarization

Our model of the mitotic regulatory circuit consists of two branches that regulate the metaphase-to-anaphase transition and mitotic exit, and is based on our current knowledge of factors that influence mitosis and morphogenesis in *C. albicans*. The proposed order of some genes is based on that in *S. cerevisiae* (See Figures 2 and 3). Factors in black exist in *C. albicans* but have not yet been investigated. Exposure to nocodazole causes spindle defects and leads to a metaphase arrest via activation of the spindle checkpoint factor Mad2p. Spindle defects caused by depletion of Cdc5p may also lead to activation of Mad2p, which in turn negatively regulates Cdc20p. Alternatively, Cdc5p has a direct negative effect on Mad2p or a positive role in regulating Cdc20p such that upon Cdc5p depletion, Cdc20p is also inactivated. During normal mitotic progression, Cdc20p is required to degrade the anaphase inhibitor securin, which would allow the separse Esp1p to cleave cohesin Scc1p and activate anaphase. *C. albicans* lacks a securin homologue, so it is unknown how the loss of Cdc20p activity leads to an arrest in anaphase. Spindle defects and /or loss of Cdc5p activity also lead to inhibition of the mitotic exit branch of the regulatory circuit, via activation of the checkpoint factor Bub2p. This in turn blocks activation of the mitotic exit network, Cdc14p phosphatase, and Cdh1p-APC activity, leading to defects in mitotic exit. The metaphase arrest and inability to exit mitosis due to depletion of Cdc5p, Cdc20p or exposure to nocodazole, may lead to accumulation of Clb2p, and maintained Cdc28p/Clb2p activity could drive polarized growth via unknown targets. Alternatively, targets of other factors that are maintained during mitotic arrest may be important for the response.

growth of the bud, or the nature of the mechanics of this type of polarization. Since Cdc20p is partly responsible for degradation of the B-type cyclin Clb2p (Shirayama *et al.*, 1998; Wasch *et al.*, 2002), and maintaining Clb2p in *C. albicans* cells through removal of the destruction box also results in elongated bud growth (Bensen *et al.*, 2005), it is possible that the ultimate result of arresting mitosis is maintaining the level of Clb2p, which leads to elongation of the bud through unknown means. If this is the case, the mechanics underlying polarized growth of the bud versus polarization of true hyphae is indeed quite different, since Clb2p and another B-type cyclin, Clb4b, have an inhibitory effect on hyphal extension (Bensen *et al.*, 2005). In addition, Bub2p, Mad2p and Cdc5p have no effect on true hyphal growth induced by serum (Bai *et al.*, 2002; Bachewich *et al.*, 2005), yet have strong effects on polarized growth of the yeast bud. In *S. cerevisiae*, transient apical growth of the bud is associated with Cdc28p/G_1 cyclin activity, while a switch to isometric growth occurs when Cdc28p associates with B-type cyclins like Clb2p (Rua *et al.*, 2001). Cell polarization during bud formation requires recruitment of Cdc42p and its associated guanine nucleotide exchange factor Cdc24p to help localize the actin cytoskeleton that is needed to direct bud growth (Pruyne *et al.*, 2000). Association of Cdc42p with the p21-activated kinase (PAK) Ste20p mediates apical growth, which is dependent on CLN/CDK1 phosphorylation of Ste20p, while association with the PAK Cla4p, due to Clb2p/CDK1-dependent phosphorylation of Cla4p, leads to isotropic growth (Pruyne *et al.*, 2000). However, this mechanism cannot explain polarized growth of the yeast bud in *C. albicans*, because maintained expression of *CLB2* leads to elongated bud growth, not isotropic expansion, and deletion of *CLA4* in *C. albicans* has very minor effects on yeast cell growth and morphology (Leberer *et al.*, 1997). If

maintained Cdc28p/Clb2p activity is responsible for the elongated bud growth response, then the nature of the targets is currently unknown. We also cannot rule out that other factors associated with mitotic arrest are maintained and responsible for triggering polarized growth. Future experiments will focus on determining how the polarization machinery in elongated buds compares with that in true hyphae, measuring Clb2p levels in Cdc20p-depleted and *CDH1*-deleted cells, and investigating how potential maintenance of Clb2p or other factors associated with mitotic arrest could be linked with activation of the polarization machinery in *C. albicans*.

The occurrence of checkpoint-activated growth in *C. albicans* is novel, and may have significance for pathogenesis. If the organism is in an environment within the host and is experiencing some insult to its cell cycle, the pathogen has a mechanism in place to help it escape that environment. Cells lacking *MAD2* are avirulent (Bai *et al.*, 2002), and elongated buds induced by mitotic arrest eventually express multiple virulence factors at later stages of growth (Bachewich *et al.*, 2005), supporting the importance of checkpoints and possibly this novel type of growth response during infection. It is therefore important to understand how checkpoint-activated growth, as well as the critical process of mitosis, is regulated in the pathogen. Our work has provided insights into the poorly understood mitotic regulatory circuit in *C. albicans*, and how it may be linked to polarized growth (Figure 27).

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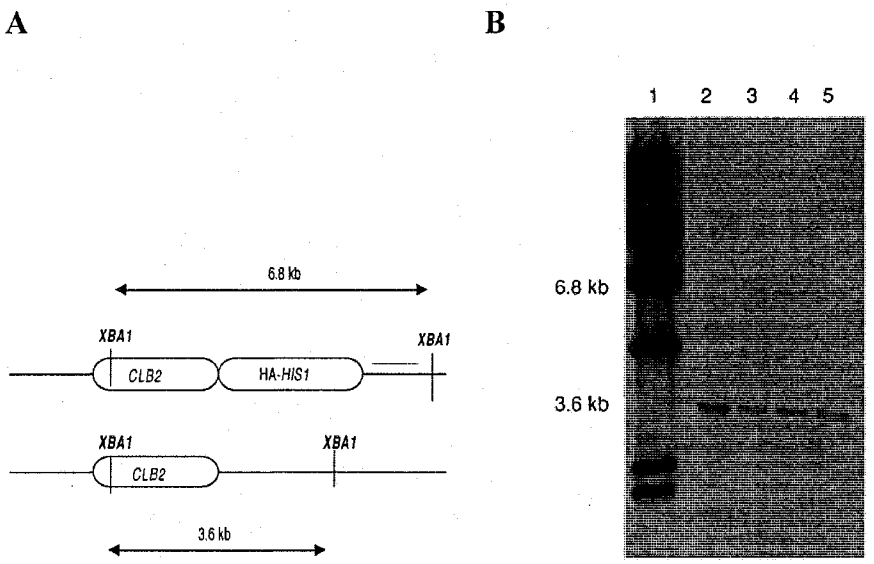
Appendix I

Tagging of Clb2p with HA tag in strains lacking *CDH1*

Since Cdh1p may be required for mitotic exit, the amount of mitotic cyclin Clb2p in cells lacking *CDH1* was determined. Clb2p was tagged with hemaglutinin (HA) tag using a 2-step PCR fusion method. Oligonucleotides HCGS32F and HCGS32R were used to amplify 540 bp flanking sequences of the 3' end preceding the stop codon of *CLB2*, while HCGS34F and HCGS34R were used to amplify 500 bp flanking sequence following the stop codon of *CBL2*. The fragment containing the *HIS1*-HA tag was amplified using oligonucleotides HCGS33F and HCGS33R and for plasmid pMG2091 (Bensen *et al.*, 2005), giving an expected fragment size of 3.2 kb. The 3 resulting fragments were combined and amplified using oligonucleotides HCGS32F and HCGS34R to make the deletion fragment with a total size of 4.3 kb. The PCR running conditions included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 41°C for 30 sec, 68°C for 3 min 15 sec, 15 cycles of the same with an additional 20 sec to the elongation time after each cycle, and a final 7 min elongation at 68°C. In order to transfer the *HIS1*-containing construct, we created a new *CDH1* deletion strain HCCA153 which was auxotrophic for *HIS1*. The *HIS1*-HA PCR construct was transformed into strain HCCA153 creating strains HCCA211, HCCA212 and HCCA213. The construct was also transformed into parental strain BWP17 to create strain HCCA228. Southern analysis confirmed the proper constructions of strains HCCA211, HCCA212 and HCCA213 (Figure 1). Oligonucleotides HCSP17 and HCSP18 were used to make a 1.1 kb probe for HA-tagged *CLB2*.



Appendix 1 Figure 1: Southern confirms *CLB2*-HA strain
(A) Southern map showing expected size bands. Strains were digested with *Xba*I restriction enzyme. (B) Lane 1: MWM; lane 2: wild-type; lanes 3-4-5 *cdh1/cdh1*, *CLB2*-HA.

Construction of *YKE2* deletion strains

(*yke2/yke2* and *yke2/yke2, cdc5/MET3::CDC5*)

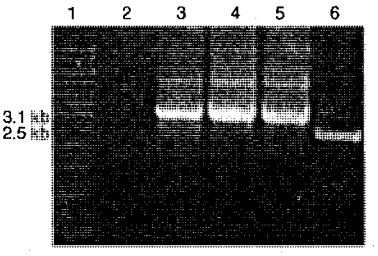
YKE2 is involved in folding of alpha-and beta-tubulin in *S. cerevisiae* (Geissler *et al.*, 1998), and is upregulated during Cdc5p-depleted polarized growth (Bachewich *et al.*, 2005) as well as during the yeast-to-hyphal transition in *C. albicans* (Nantel *et al.*, 2002). In order to determine if *YKE2* has a mechanical function in polarized growth in Cdc5p-depleted cells, both copies of *YKE2* were replaced with *URA3* or *HIS1* markers using 2-step PCR fusion method. Oligonucleotides HCGS26F and HCGS26R were used to amplify a 680 bp fragment identical to the 5' flank of *YKE2* and oligonucleotides HCGS28F and HCGS28R were used to amplify a 570 bp fragment identical to the 3' flank of the gene. PCR running conditions for the flanking fragments included 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 43°C for 30 sec and 68°C for 55 sec and a final 7 min run at 68°C. HCGS27F and HCGS27R were used to amplify a fragment containing the *URA3* or *HIS1* marker from plasmid pBS-*URA3* or pBS-*HIS1*, respectively. The PCR running condition included 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 40°C for 1 min and 68°C for 1 min 30 sec and a final 7 min run at 68°C. The 3 individual fragments were combined in a 1:3:1 (50:150:50 ng) ratio and amplified using oligonucleotides HCGS26F and HCGS28R to create a 2.5 kb deletion construct. The PCR running condition included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 43°C for 30 sec, 68°C for 2 min 30 sec, and 15 cycles of the same with an additional 20 sec extension/elongation after each cycle, followed by a final elongation of 7 min at 68°C. The deletion constructs were transformed sequentially into parental strain BWP17 and the *CDC5* conditional strain HCCA118 to create strains

HCCA184 (*yke2/yke2*) and HCCA193 (*yke2/yke2, cdc5/MET3::CDC5*), respectively. PCR screening and Southern analysis confirmed the proper construction of the strains (Figure 2). For PCR screening, oligonucleotides HC16F and HC16R were used with running conditions at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 37°C for 30 sec, 68°C for 3 min and 10 sec, and finally with a 7 min elongation at 68°C.

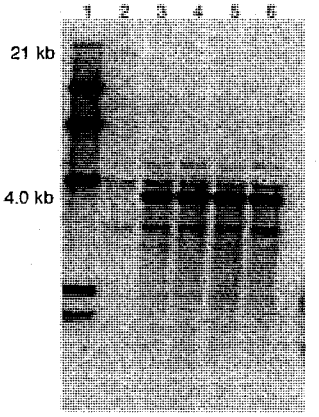
To determine the phenotype of cells lacking *YKE2*, strains HCCA184 (*yke2/yke2*) and HCCA100 (*YKE2/YKE2*) were grown overnight in YPD medium, diluted in fresh YPD medium, and incubated at 30°C for 0, 6 and 24 h (Figure 3). At 0 h, strains lacking *YKE2* exhibited pseudohyphal growth and yeast cells of which some were enlarged. At 7 h, cells grew as chained yeast cells, pseudohyphal cells, or as filament. At 24 h, most cells lacking *YKE2* grew in normal yeast form although some filamentous growth remained but was scarce. In contrast, cells from control strain HCCA100 grew mostly as normal yeast.

To test cells lacking Yke2p in Cdc5p-depleted cells, cells from strains HCCA194 (*yke2/yke2, cdc5/MET3::CDC5*) and control strain HCCA126 (*YKE2/YKE2, cdc5/MET3::CDC5*) were grown in liquid inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5 mM methionine and 0.5 mM cysteine and collected at 0, 6 and 24 h (Figure 4). At 0 h, most cells from strain HCCA194 were yeast cells with some enlarged yeasts, while control strain HCCA126 grew as normal yeast cells. At 6 h, most cells from strain HCCA126 and HCCA194 contained elongated buds. At 24 h, the filaments from cells lacking *YKE2* in Cdc5p-depleted cells were irregular in length and shape compared to filaments from the control strain HCCA126.

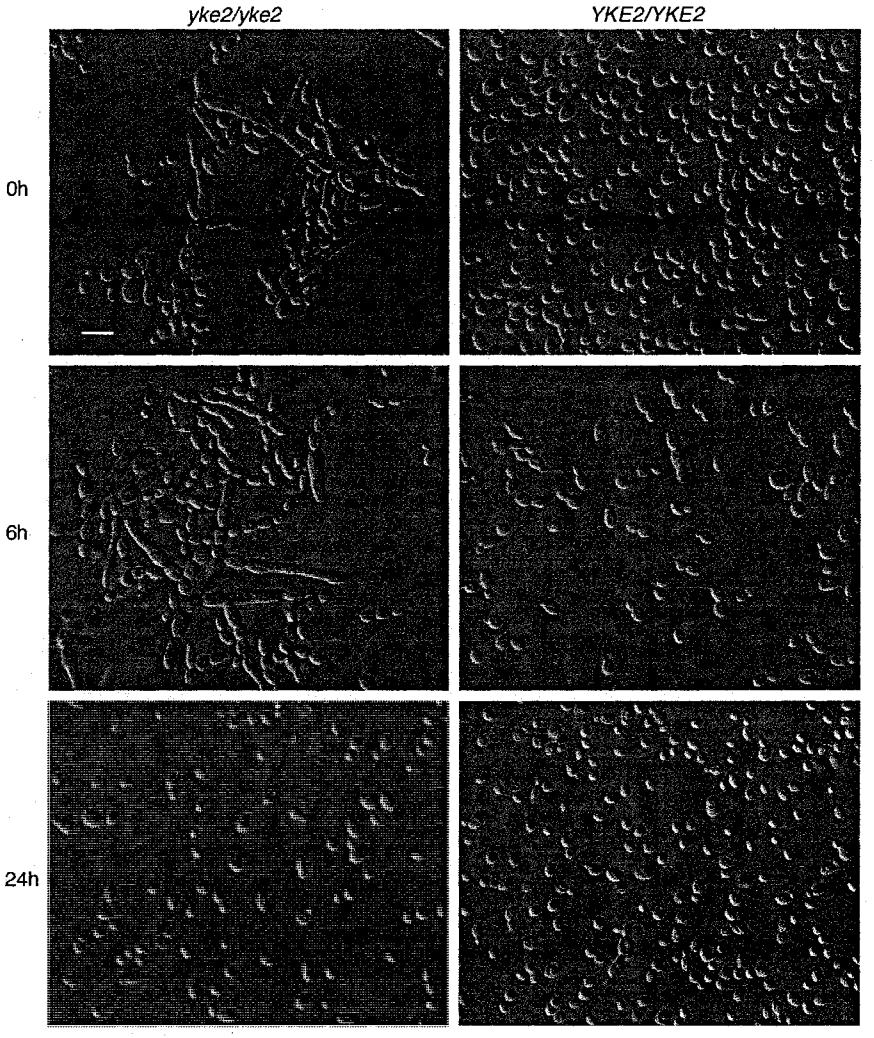
A



B

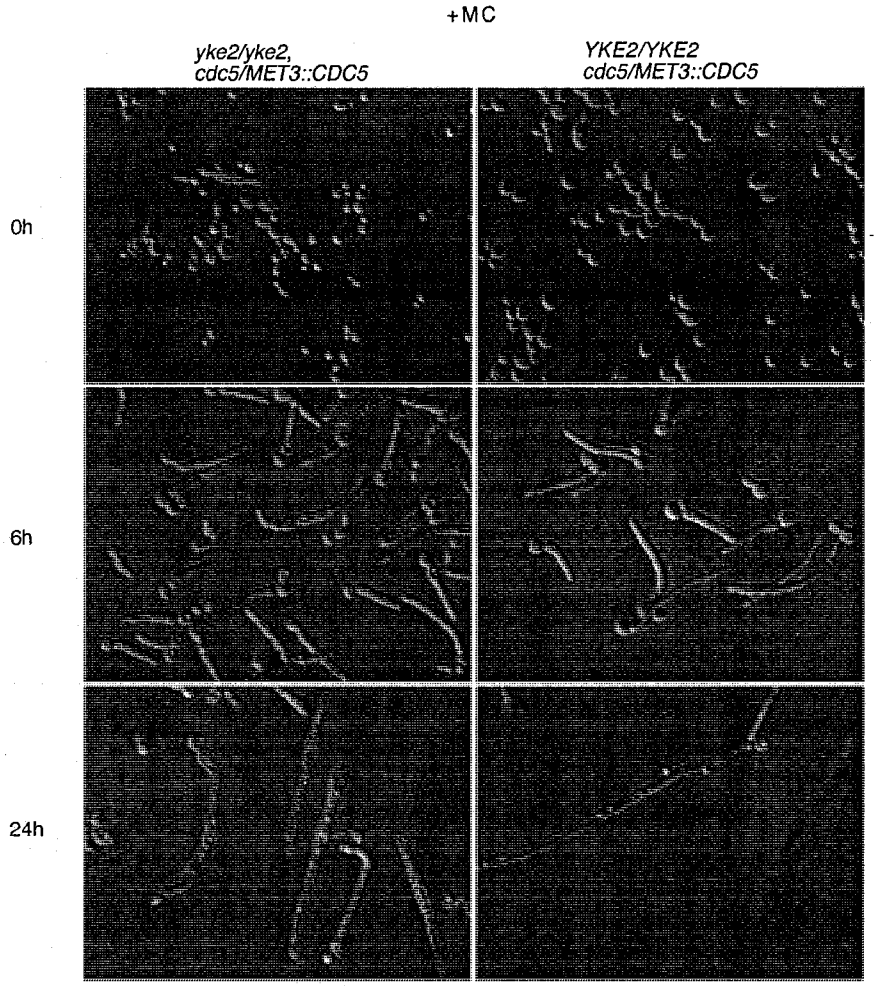


Appendix 1 Figure 2: PCR and Southern analysis of *YKE2* deletion strains
(A) PCR screen using oligonucleotides HC16F and HC16R where the expected size bands are 3.1 kb for *URA3* or *HIS1* and 2.5 kb for wild-type. Lane 1: MWM; lane 3: *yke2/yke2, cdc5/MET3::CDC5*; lanes: 4-5: *yke2/yke2, CDC5/CDC5*; lane 6: wild-type. (B) Southern blot for *YKE2* deletion with *XbaI* and *BamHI* cut gDNA. Expected size bands are 4 kb for *URA3* an *HIS1* replacement and 21 kb for wild-type. Lane 1: MWM; lane 2: wild-type; lanes 3-4-5-6: *yke2/yke2.*



Appendix 1 Figure 3: Deletion of *YKE2* resulted in multi-chained cells, pseudohyphal cells and elongated buds but grow normally as yeast after 24h of incubation

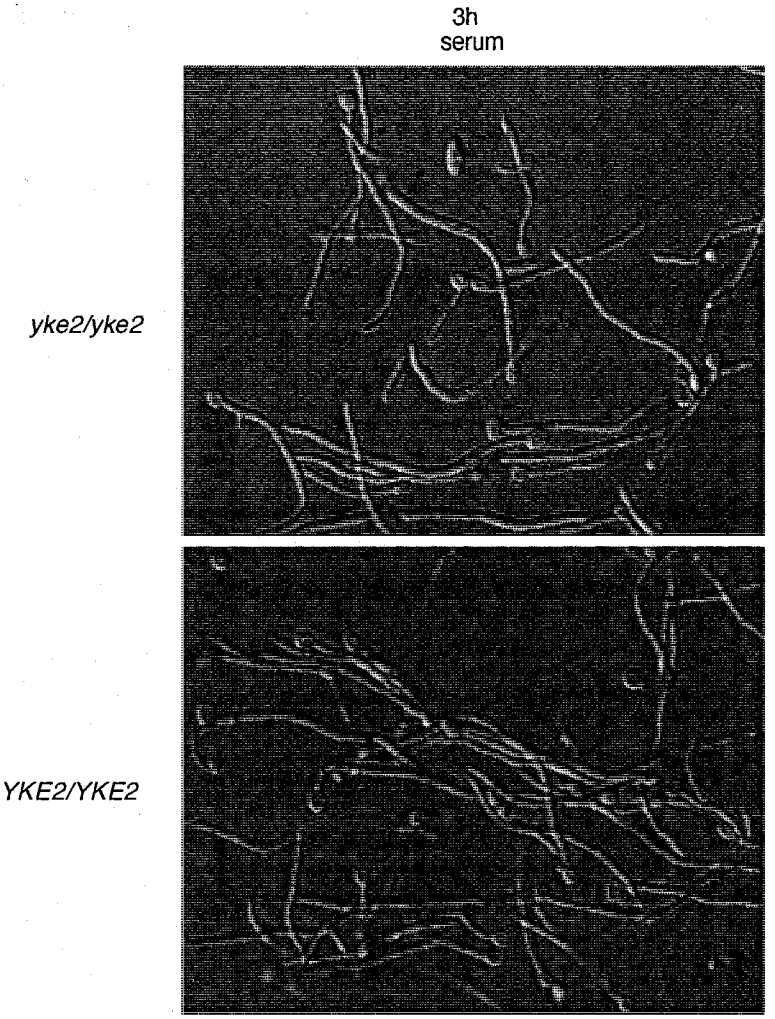
Strains HCCA184 (*yke2/yke2*) and HCCA100 (*YKE2/YKE2*) were grown overnight in liquid YPD, diluted to an $O.D._{600nm}$ of 0.2 and collected at indicated time points. Bar: 10 μm



Appendix 1 Figure 4: Cells lacking *YKE2* compromised Cdc5p-depleted polarized growth

Strains HCCA194 (*yke2/yke2, cdc5/MET3::CDC5*) and HCCA126 (*YKE2/YKE2, cdc5/MET3::CDC5*) were grown overnight in liquid inducing medium lacking methionine and cysteine, diluted in repressing medium containing 2.5mM methionine and 0.5 mM cysteine and collected at indicated time points. Bar: 10 μm

To determine if *YKE2* is important for serum-induced hyphal growth, cells from strain HCCA184 (*yke2/yke2*) and control strain HCCA100 (*YKE2/YKE2*) were grown overnight in rich YPD medium, diluted to an $O.D._{600nm}$ of 0.2 in YPD medium containing 10% serum for 3h at 37°C (Figure 5). Although *YKE2*-deleted cells exhibited mixed morphologies, most cells responded to serum and formed hyphae. Cells from control strain HCCA100 formed hyphae normally.



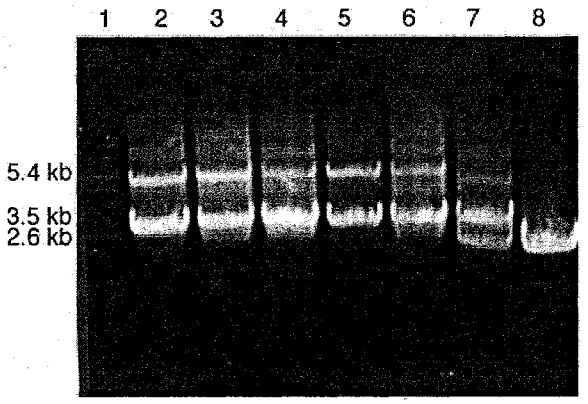
Appendix 1 Figure 5: *YKE2* is not necessary for serum-induced hyphal formation
Strains HCCA184 (*yke2/yke2*) and HCCA100 (*YKE2/YKE2*) were grown overnight in liquid YPD, diluted to an $O.D._{600nm}$ of 0.2 and incubated in liquid YPD containing 10% serum and collected at 3h. Bar: 10 μm

Construction of conditional *MOB1* strain (*mob1/MET3::MOB1*)

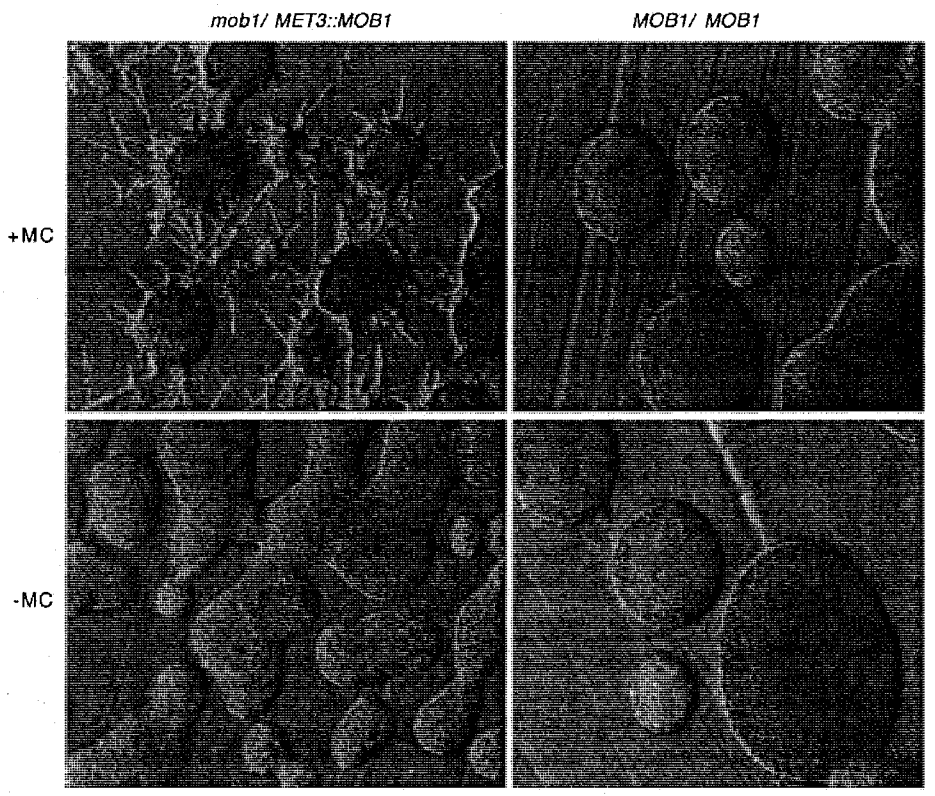
MOB1 is an essential gene in *S. cerevisiae* and is part of the mitotic exit network and also known to interact with Mps1p, a factor involved in the spindle checkpoint (Luca *et al.*, 1998; Luca *et al.*, 2001). *MOB1* was also highly up-regulated in Cdc5p-depleted cells. In order to study the roles of *MOB1*, we constructed a conditional *MOB1* strain. An attempt to delete both copies of *MOB1* was unsuccessful, suggesting it may be essential. We placed one copy of *MOB1* under the regulation of a *MET3* promoter while the other copy was replaced with a *URA3* marker by 2-step PCR fusion method. To create the PCR deletion construct, oligonucleotides HCGS29F and HCGS29R were used to amplify 610 bp of the 5' flanking sequence preceding Start of *MOB1*, while HCGS35F and HCGS35R were used to amplify 490 bp containing the start site of the *MOB1*. PCR running conditions included 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42-46°C for 30 sec, 68°C for 55 sec and finally a 7 min elongation at 68°C. A third fragment containing the *HIS1-MET3* promoter was amplified from plasmid pFA-*HIS-MET3* using oligonucleotides HCGS32F and HCGS33R, with running conditions consisting of 94°C for 4 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 3 min and finally with a 7 min elongation at 68°C. All 3 fragments were then combined in a final fusion PCR reaction in a 1:3:1 (50:150:50 ng) ratio of DNA. The final PCR, using oligonucleotides HCGS29F and HCGS35R, included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 46°C for 30 sec, 68°C for 4 min, and 15 cycles of the same set of temperature followed by additional 20 sec extension after each cycle and finally, a 7 min final elongation at 68°C. The final product was 3.9 kb. For replacing the other copy of *MOB1* with *URA3* marker, oligonucleotides HCGS29F and

HCGS29R were used to amplify 610 bp of the 5' flanking sequence of *MOB1*, while HCGS31F and HCGS31R were used to amplify 600 bp of the 3' flanking sequence. PCR running conditions included 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42-46°C for 30 sec, 68°C for 55 sec and finally a 7 min elongation at 68°C. A third fragment containing the *URA3* marker was amplified from plasmid pBS-*URA3* using oligonucleotides HCGS30F and HCGS30R, with running conditions consisting of 94°C for 4 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 1 min 30 sec and finally with a 7 min elongation at 68°C. All 3 fragments were then combined in a final fusion PCR reaction in a 1:3:1 (50:150:50 ng) ratio of DNA. The final PCR, using oligonucleotides HCGS29F and HCGS31R, included 94°C for 2 min followed by 10 cycles with 94°C for 10 sec, 46°C for 30 sec, 68°C for 2 min 35 sec, and 15 cycles of the same set of temperature followed by additional 20 sec extension after each cycle and finally, a 7 min final elongation at 68°C. The final product was 2.6 kb. PCR screening confirmed proper integrations of the markers and promoter (Figure 6). Using oligonucleotides HC17F and HC17R, the PCR running conditions included 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 5 min and 30 sec, and finally with a 7 min elongation at 68°C.

Strains HCCA218 (*mob1/MET3::MOB1*) and control strain HCCA100 (*MOB1/MOB1*) were grown on solid inducing medium lacking methionine and cysteine and repressing medium containing 2.5 mM methionine and 0.5 mM cysteine. Cells from strain HCCA218 grew smooth colonies indicating normal yeast growth while colonies exhibited filamentous growth on repressing medium (Figure 7) while cells from the control strain grew smooth colonies in both inducing and repressing media.



Appendix 1 Figure 6: PCR analysis confirms conditional *MOB1* strains.
The expected band size for wild-type is 2.6 kb, 3.1 kb for *URA3* replacement and 5.4 kb for *MET3::MOB1*. Lane 1: MWM; lanes 2-3-4-5-6: *mob1/MET3::MOB1*; lane 7: *mob1/MOB1*; lane 8: wild-type.



Appendix 1 Figure 7: Cells depleted of Mob1p exhibited filamentous growth
Strains HCCA218 (*mob1/MET3::MOB1)* and HCCA100 (*MOB1/MOB1*) were grown for 24h on inducing solid medium lacking methionine and cysteine and repressing solid medium containing 2.5 mM methionine and 0.5mM cysteine.

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