CHARACTERIZATION OF FUNCTIONAL DOMAINS

OF *SACCHAROMYCES CEREVISIAE* SEPTINS.

BY

SATISH NAGARAJ

Master of Science

Bangalore University

Bangalore, India

1997

Submitted to the faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment
of requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2005

CHARACTERIZATION OF FUNCTIONAL DOMAINS

OF *SACCHAROMYCES CEREVISIAE* SEPTINS.

Dissertation Approved:

Dr. Mark. S Longtine
Dissertation advisor

Dr. Robert. L Matts

Dr. Marie. E Petracek

Dr. Jose. L Soulages

Dr. Rolf. A Prade

Dr. A. Gordon Emslie
Dean of the Graduate College

ACKNOWLEDGEMENTS

One person, who has influenced my graduate student experience, the most, is my advisor Dr. Mark Longtine. He was always there for me, guiding me in the right direction, challenging me in certain things and motivating me to perform the best. I am grateful for his support and friendship during my work. I thank Dr. Robert Matts, my committee member, for his continued support throughout my Ph D and also for guiding me through a part of my work done in his lab. I thank Dr. Marie Petracek and Dr. Jose Soulages, my committee members for their guidance and support during my work. I also want to thank Dr. Rolf Prade, my external committee member, for his stimulating discussions. I sincerely thank you all.

I would extend my acknowledgements to my colleagues, Ashok Rajendran and Manivannan Subramanian for their friendship, support and helpful criticisms during my work. My special thanks to Ashok, who helped me during my work and provided data to make my work a good manuscript. I also thank Lavanya Amirneni, my lab technician, who ensured that I do not run out media, plates and tubes. I thank Angie Thomure, Carrie Marston and Jamie Smith, all, who helped in generating various mutants for my work.

Nothing would have been possible without the constant encouragement, support and companionship of my wife Sumana Bhat. Inspite of her work, she ensured that I concentrate on my work and achieve my targets in a timely fashion. I thank her, for her

love and care. I would also thank my parents, Sudha Nagaraj and Nagaraj N R, my sister, brother-in-law and my sister-in-law, who always were keen on making my life easier and stress free.

Finally, I dedicate this thesis to my brother, Sunil Nagaraj, who always taught me to set goals and never stop until I reach them. He has been my role model and with his support, I have come to this important stage of my life. I thank him for being there for me always.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5FOA	5-fluoroorotic acid
AD	Activation domain
ATA	Aurintricarboxylic acid
$CaCl_2$	Calcium Chloride
CCD	Charge-coupled device
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
CRIB	Cdc42/Rac-interactive binding
DBD	DNA binding domain
DIC	Differential interference contrast
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence recovery after photobleaching
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GFP	Green fluorescent protein

GST	Glutathione S transferase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazine]ethanesulfonic acid
His	Histidine
HRP	Horseradish peroxidase
hr	Hour
kb	Kilo base
kDA	Kilo dalton
Leu	Leucine
MBP	Maltose Binding Protein
Mg^{++}	Magnesium ion
min	Minutes
mM	Milli molar
NaCl	Sodium Chloride
OD	Optical density
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PVDF	Polyvinylidene fluoride
RPM	Revolutions per minute
RRL	Rabbit reticulocyte lysate
SD media	Synthetic defined media

SDS	Sodium dodecyl sulfate
TBS	Tris buffered Saline
TNT	Transcription and translation
TPCK	L-(tosylamido-2-phenyl ethyl chloromethyl ketone)
Trp	Tryptophan
TRITC	Tetramethylrhodamine isothiocyanate
TWEEN 20	Polyoxyethylene sorbitan monolaurate
Ura	Uracil
UV	Ultraviolet
YCp	Yeast centromeric plasmid
YIp	Yeast integrative plasmid
YPD	Yeast extract, Peptone, Dextrose
μM	Micro molar
μl	Micro liter
μg	Micro gram
Δ	Deletion

CHAPTER I

INTRODUCTION

Understanding cell division

Cell growth and division are stringently regulated to ensure proper development of a healthy organism. In many cases, if a stage in eukaryotic cell division does not occur properly then a "checkpoint" mechanism will be activated that will prevent further progression through the cell cycle. By definition, a checkpoint delays cell cycle progression of a succeeding step if a preceding step is not completed or is delayed. Importantly, a checkpoint, is required only if some step is not properly completed; if the cell cycle is not perturbed the checkpoint protein and the checkpoint process are not required. Occasionally, checkpoint mechanisms that serve to regulate cell-cycle progression fail, often due to mutation in one of the many checkpoint proteins. Mutations in a checkpoint protein(s), allowing unregulated cell growth, are an important step in progression of many cancers. Understanding cell-cycle progression and cell division, and how these processes are regulated, is essential to our understanding of health and disease.

One complete cell cycle is the division of one cell and its components to give rise to two new cells. The cell cycle proceeds from G1 phase to S-phase (DNA synthesis) to G2 phase, then to M-phase (mitosis), in which the chromosomes are separated. Following the completion of mitosis the final stage of the cell cycle is cytokinesis, the

separation of the cytoplasm to form two separate daughter cells. A group of closely related proteins, called cyclins, appear and disappear in a cyclic pattern during the cell cycle (Sherr, 1996). In mammalian cells, there are about 16 cyclins, with four of them (Cyclin A, B, D and E) participating in cell-cycle progression (Tessema et al., 2004). Cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, associate with a particular cyclin(s) at a specific cell-cycle stages. These different CDK/cyclin complexes result in the phosphorylation of different substrates, thus promoting progression through the stages of the cell cycle. The first identified CDKs were Cdc28p and Cdc2p, from *Saccharomyces cerevisiae* and *Saccharomyces pombe*, respectively (Hartwell et al., 1973; Nurse and Thuriaux, 1980). Mammals have up to nine CDKs, of which four (CDK1, CDK2, CDK4 and CDK6) participate in cell-cycle progression (Elledge, 1996; Tessema et al., 2004). *S. cerevisiae* Cdc28p interacts with three partially redundant A-type cyclins (Cln1p, Cln2p, and Cln3p) during G1 to promote polarized growth and bud emergence. Next, Cdc28p complexed with two B-type cyclins (Clb5p and Clb6) promotes entry into S-phase, spindle-pole body duplication, and separation of the spindle-pole bodies in preparation for mitosis (Pringle et al., 1997). Next, Cdc28p associates with Clb1p and Clb2p, promoting the switch from apical to isotropic growth and entry into mitosis. Finally, inactivation of Cdc28/Clb complexes and activation of the mitotic exit network promotes the exit from mitosis and completion of cell division by cytokinesis (Yeong et al., 2002).

A number of proteins, including the Cak1p and Swe1p kinases, the Mih1p phosphatase, Far1p and Sic1p (inhibitors), tightly regulate these CDK/cyclin complexes in *S. cerevisiae* (Pringle et al., 1997). Cak1p is an essential protein required for the

activation of Cdc28p in G1 by phosphorylating the Cdc28p threonine residue at position 169 (Kaldis et al., 1998). Swe1p regulates the progression through the G2/M phase. Mih1p acts antagonistically to Swe1p. Under normal growth conditions, neither Swe1p nor Mih1p are needed. These proteins serve as "morphogenesis checkpoint" proteins, preventing cell division in the absence of a daughter cell, thus preventing the generation of binucleate cells. In cells lacking Sic1p, the Clb5p-6p/Cdc28p complex is activated prematurely leading to accelerated DNA replication (Pringle et al., 1997). In response to mating pheromones, Far1p inhibits the Cdc28p/Cln complexes, thereby causing a G1 cell-cycle arrest (Pringle et al., 1997).

The Cdc28p activator, Cak1p, is present in most phases of the cell-cycle with decreasing protein levels towards the stationary phase. Mostly they are present in the cytoplasm and are dispersed throughout the cell (Kaldis et al., 1998). The Cdc28p inhibitor, Sic1p is present both in the nucleus and cytoplasm at different stages of cell-cycle (Edgington and Futcher, 2001). However, Far1p, another Cdc28p inhibitor, is predominantly nuclear localized and is exported to the cytoplasm as a complex with Cdc24p to the sites of polarized growth in a shmoo in response to a mating pheromone (Blondel et al., 1999; Blondel et al., 2000). Septins are required for the localization of Swe1p and its negative regulators (Hsl1p and Hsl7p) (Longtine et al., 2000). In my thesis, I will first review the current status of our knowledge about septins and then present my research on septin structure and function.

Septins

Septins, a conserved family of proteins present in nearly all eukaryotes, were discovered in *Saccharomyces cerevisiae* during a screen for temperature-sensitive-lethal mutations designed to identify genes involved in cell-cycle control (Hartwell, 1971). At restrictive temperature, septin mutants stopped growth with multiple elongated buds, indicating defects in cell division and cell morphogenesis. Immunofluorescence studies indicated that septins co-localize to the cell cortex at the mother-bud neck for the entire budded phase of the cell cycle (Ford and Pringle, 1991; Haarer and Pringle, 1987; Jeong et al., 2001; Kim et al., 1991). At restrictive temperature, the septins are no longer localized to the neck. As the neck is the site of cytokinesis, together these results suggest that neck-localized septins are required for cytokinesis. Consistent with this idea, in higher eukaryotes septins localize to the cell cortex at the site of cytokinesis and disruption of septin localization results in defects in cytokinesis (Kinoshita and Noda, 2001).

The yeast genome encodes a total of seven septins. Five of these (Cdc3p, Cdc10p, Cdc11p, Cdc12p and Shs1p/Sep7p) are expressed during vegetative growth (Carroll et al., 1998; Ford and Pringle, 1991; Haarer and Pringle, 1987; Jeong et al., 2001; Kim et al., 1991; Mino et al., 1998). Deletion studies indicate that Cdc3p and Cdc12p are essential for vegetative growth under all conditions, while strains lacking Cdc10p, Cdc11p, or Shs1p are viable at 23˚C. Two other septins, Spr3p (Fares et al., 1996) and Spr28p (De Virgilio et al., 1996), are specifically expressed during sporulation, along with Cdc3p, Cdc10p, Cdc11p, and Shs1p. The vegetatively expressed septins co-purify in approximately stoichiometric ratios (Frazier et al., 1998). Septins are present in

other fungi (DiDomenico et al., 1994; Momany et al., 2001) , *Drosophila* (Adam et al., 2000), *C. elegans* (Nguyen et al., 2000), and mammals (reviewed in Kartmann and Roth 2001).

Electron microscopy (EM) of *S. cerevisiae* identified apparent filaments associated with the plasma membrane at the mother-bud neck and discovered that these apparent filaments are not detectable after incubation of septin mutant cells at restrictive temperature (Byers and Goetsch, 1976). These data suggest that septins might be components of the filaments at the mother-bud neck. Indeed, purified septin complexes from yeast and higher eukaryotes are able to form filaments *in vitro* (Field et al., 1996; Frazier et al., 1998; Hsu et al., 1998; Versele and Thorner, 2004), confirming that septins are filament-forming proteins.

The EM studies suggested these filaments form a helix or helices that spiral around the neck at the cell cortex (Byers and Goetsch, 1976). However, biochemical data (Field et al., 1996) and genetic data (Longtine et al., 1998) suggest that septins may be arranged as linear filaments that extend through the neck along the mother-bud axis, and the apparent helical filaments observed by EM are due to periodically distributed proteins that links adjacent septin filaments (Longtine et al., 1998). A recent EM study suggests that yeast septin organization is very complex, with septins forming both ring structures and mesh-like structures (Rodal et al., 2005). These mesh-like structures appear to be filaments that are oriented in multiple directions or to be parallel filaments that are very heavily crosslinked. Interestingly, these septin meshes appear to emanate from the septin ring structures with the approximate diameter of the mother-bud neck.

Although formation of long filaments *in vitro* is a conserved property of septins, the formation of long filaments appears dispensable for septin function in yeast, at least at low temperatures (Frazier et al., 1998). Consistent with the idea that long filament formation is not essential to septin functions, septins that are localized properly to the neck, but with clear defects in higher-order structure, function efficiently in a variety of septin-dependent processes (Frazier et al., 1998; Longtine et al., 1998).

Functions of septins

The initial identification of septins indicated that they have roles in cytokinesis and cell morphogenesis in yeast. A wide variety of data has emerged indicating that septins have multiple roles in yeast and other organisms, both in dividing and in non-dividing cells (Kartmann and Roth, 2001; Kinoshita, 2003). These roles are discussed briefly below.

1) Cell-cycle transition:

During bud formation in *S. cerevisiae*, there is an initial period of growth directed to the tip of the bud, resulting in apical growth. Cdc28p complexed with the G1 cyclins, Cln1p and Cln2p, promotes apical growth. Prior to mitosis, Cdc28p complexes with the G2 cyclins, Clb1p and Clb2p, and the pattern of growth switches to the entire bud periphery, resulting in isotropic growth. The shape of a yeast cell is dictated by the timing of this switch. If the Cdc28p/Clbp cyclin complexes are prematurely activated, the early induction of apical growth results in rounded cells. If the activity of

Cdc28p/Clbp complexes is delayed, then the resulting extended period of apical growth results in elongated buds (Lew and Reed, 1993).

In response to perturbation of the actin cytoskeleton, the activation of Cdc28p/Clbp complexes is delayed by phosphorylation of tyrosine 19 of Cdc28p by the Swe1p tyrosine kinase (Lew and Reed, 1995; Lew et al., 1997). Under normal conditions in wild-type cells, Swe1p becomes unstable during G2/M phase and is degraded in a Hsl1p-dependent pathway in G2/M allowing activation of Cdc28p/Clbp complexes (Sia et al., 1998). Surprisingly, Swe1p and its negative regulators, Hsl1p and Hsl7p, localize to the neck in a septin-dependent manner (Longtine et al., 2000). Hsl1p and Hsl7p are required for the neck localization of Swe1p and degradation of Swe1p (Shulewitz et al., 1999; Theesfeld et al., 2003). *In vitro*, activation of Hsl1p kinase activity and phosphorylation of Swe1p requires the binding of Hsl1p to Cdc11p and/or Cdc12p (Hanrahan and Snyder, 2003). The activation of Hsl1p upon septin binding is likely due to relieving an autoinhibition by Hsl1p (Hanrahan and Snyder, 2003). In the absence of neck-localized septins, Hsl1p and Hsl7p are unable to repress Swe1p activity, resulting in the phosphorylation of Cdc28p complexed with the Clbs. The consequent delay in the switch to isotropic growth results in elongated cells. It has been suggested that the interaction of septins with the Hsl/Swe1p complex is a mechanism by which the cell recognizes bud formation by monitoring a coincident shape-dependent change in septin organization (Lew, 2003; Theesfeld et al., 2003). These data suggest that septins localized at the neck and the monitoring of septin localization/organization by the Hslp/Swe1pcheckpoint proteins is a mechanism by which the cell determines whether or not a bud has been formed (Lew, 2003).

Recently, it was shown that an *SHS1/SEP7* deletion strain and a Cdc10p deletion strain were defective in the mitotic exit checkpoint. This checkpoint delays exit from mitosis if the spindle is not properly positioned at the mother-bud neck, and involves the localization to the bud cortex of Lte1p, the guanine nucleotide exchange factor (GEF) for the Tem1p small GTPase. In both shs1p/sep7Δ strains and in *cdc10*Δ strains, cells improperly exited mitosis when the spindle was not properly positioned (Castillon et al., 2003). These data suggest a role for septins in controlling mitotic exit, perhaps by the septins being involved in the localization and, perhaps, activation of mitotic exit checkpoint proteins in yeast cells.

2) Bud-site selection:

Haploid yeast cells bud in an axial pattern, in which budding occurs adjacent to the immediately preceding site of cell division. Diploid cells bud in a bipolar pattern, in which budding is restricted to either of the two poles of the cell (Chant, 1999). Bud3p and Bud4p, which are required for axial budding, localize to the mother-bud neck and are delocalized in temperature-sensitive septin mutants at high temperatures (Chant et al., 1995; Sanders and Herskowitz, 1996). In contrast, septins localize properly when any of these genes are deleted. Bud8p and Bud9p are transmembrane proteins required for bipolar budding. Bud9p, which localizes solely to the neck, is dependent on septins for its localization (Schenkman et al., 2002). Bud8p localizes to both the bud tip and to the bud neck. Not surprisingly, the bud-tip localization of Bud8p is septin independent. However, as for the other proteins involved in bud-site selection, the bud-neck localization of Bud8p is dependent upon neck-localized septins (Harkins et al., 2001).

3) Chitin deposition:

Chitin, a cell wall polymer of β1-3 glucan is deposited in the cell wall at mother-side of the neck by the chitin synthase III complex. Following cell division, this chitin remains in the cell wall, yielding a chitin ring (called a "bud scar"), which can be visualized by staining with Calcofluor White. In cells with delocalized septins, chitin deposition is no longer restricted to the neck and it is deposited diffusely throughout the cell wall. Furthermore, two-hybrid and genetic data suggest that the septins directly interact with Bni4p, which in turn interacts with Chs4p an activator of Chs3p, the catalytic subunit of chitin synthase III (DeMarini et al., 1997). Together, these data suggest that septins direct localized deposition of chitin.

4) Cytokinesis:

Cytokinesis is the final stage of cell division, separating the cytoplasm of the cell after mitosis to yield the two resulting cells. In non-plant eukaryotic cells, an actomyosin ring is formed at the site of cytokinesis (the cleavage furrow in mammalian cells and the mother-bud neck in yeast). In yeast, as in other eukaryotes with an actomyosin contractile ring, the type II myosin (Myo1p) and actin are major components of the ring. Contraction of the ring using the ATPase activity of myosin promotes membrane contraction and, ultimately, cytokinesis (Bi et al., 1998; Lippincott and Li, 1998).

Septins are required for efficient localization, to the neck, of components of the actomyosin contractile ring (For e.g., Myo1p and actin) in yeast (Bi et al., 1998; Lippincott and Li, 1998) and in higher eukaryotes (Kinoshita et al., 2002; Kinoshita et al., 1997; Oegema et al., 2000). In contrast, septins localize apparently normally in the

absence of Myo1p, actin, and other components of the ring. Defects in cytokinesis upon disruption of septin function are also seen in *D. melanogaster* (Neufeld and Rubin, 1994). Similarly, defects in cytokinesis due to Nedd5p disruption was observed in mammalian cells (Kinoshita et al., 1997). Nedd5 and a large subset of the other mammalian septins, including Sept5p, Brain H5p, and MSF, are present at the cleavage furrow of dividing cells (Beites et al., 2001; Surka et al., 2002; Xie et al., 1999).

However MSF, unlike Nedd5p, did not colocalize with actin at the furrow but concentrated along tubulin at the central spindle. This localization difference among mammalian septins suggests different functions for different septins during cytokinesis in mammalian cells. Although septins are important for cytokinesis in higher eukaryotes, they may not be essential. C. elegans encodes only two septins, Unc59p and Unc61p. Both of these septins, localize to the leading edge of cleavage furrow. However, when mutated, C. elegans embryos are able to carry out cytokinesis during embryogenesis, but do display clear defects in post-embryonic cytokinesis (Nguyen et al., 2000).

Another protein, Hof1p/Cyk2p, suggested to be involved in linking primary septum synthesis machinery to the actomyosin contraction is also shown to be dependent on septins for its localization to the mother-bud neck (Vallen et al., 2000).

5) Mating:

Prior to mating, haploid *Mat* a and *Mat* α cells form a polarized growth projection, known as a shmoo, in response to pheromone from the opposite mating type. In shmoos, septins localize as a diffuse band at the base of these projections, corresponding to the region of chitin deposition (Ford and Pringle, 1991; Kim et al.,

1991; Longtine et al., 1998), suggesting a possible role of septins in mating. Consistent with this idea, *cdc12-6* cells are defective in shmoo morphogenesis (Giot and Konopka, 1997), and two-hybrid and genetic data suggest that Cdc12p interacts with Afr1p, a protein whose expression is induced by mating pheromone and localizes to the base of mating projections (Konopka, 1993; Konopka et al., 1995). Consistent with interaction of Afr1p and septins during mating, ectopic expression of high-levels of Afr1p in vegetative cells results in the neck localization of Afr1p and the disruption of normal septin localization at the neck (Konopka et al., 1995).

6) Sporulation:

Along with the sporulation-specific septins Spr3p and Spr28p, Cdc3p, Cdc10p, and Cdc11p (but not Cdc12p) are expressed during sporulation. The septins localize to the developing prospore wall (De Virgilio et al., 1996; Fares et al., 1996) suggesting that they may be involved in membrane extension during meiosis. Septin localization to the prospore wall requires the Glc7p protein phosphatase and a Glc7p-interacting protein, Gip1p (Tachikawa et al., 2001). After the growing prospore wall has captured the nuclei, septins are uniformly spread over the spore membrane, suggesting a possible role in formation of one or more layers of the spore wall. It seems likely that septins have a role in deposition of the chitosan layer of the spore wall, which involves chitin synthase III and Shc1p.

7) Secretion:

In *S. cerevisiae*, cells lacking Myo1p (and thus the actomyosin contractile ring) are nonetheless able to undergo cytokinesis, although it is inefficient. In contrast to Myo1p, septins play an essential role in cytokinesis, indicating that septins are involved in a Myo1p-independent cytokinesis mechanism. It seems likely that this mechanism involves targeted secretion and/or septum formation (Hales et al., 1999). Indeed, septins are required for the neck localization of the chitin synthase II complex, including the Chs2p catalytic subunit (Roh et al., 2002; Slater et al., 1985). Chs2p, the entire chitin synthase II complex and the chitin synthase III complex, are required for deposition of the primary septum during cytokinesis.

There are also several lines of evidence suggesting that septins are involved in targeted secretion in mammalian cells. First, septins are concentrated in the leading edge of the cellularization fronts during cellularization of *Drosophila* embryos (Fares et al., 1995; Neufeld and Rubin, 1994). As this is a region of rapid vesicle fusion, it is possible that septins are involved in vesicle targeting and/or secretion. Second, mammalian septins physically interact with the sec6/sec8 "exocyst" complex (Hsu et al., 1998), which is involved in the tethering of vesicles to the plasma membrane prior to vesicle fusion. Finally, syntaxin-1, a plasma-membrane localized t- (target) SNARE protein important for vesicle fusion directly binds the mammalian septin, Sept5p (Beites et al., 1999). Biochemical and genetic analysis suggests that, in these cells, septins may have a regulatory role in vesicle fusion (Beites et al., 2005; Beites et al., 1999). It has been suggested that septin filaments might have a role in tethering vesicles to the plasma membrane. Recent work from the Trimble lab indicates that septin interaction with the t-

snare syntaxin is mutually exclusive of the interaction of syntaxin with α-SNAP (Beites et al., 2005). α-SNAP serves to attract to the cis-snare complex an AAAtpase, which promotes separation of the cis-snare complex and in snare recycling.

Models for septin functions

1) Scaffold model:

A wide variety of data, mostly obtained from studies in *S. cerevisiae*, led to the development of the scaffold model for septin function. This model is based on previous work that showed the non-septin proteins involved in diverse processes, including bud-site selection (Chant et al., 1995; Harkins et al., 2001; Schenkman et al., 2002), chitin deposition (DeMarini et al., 1997; Vallen et al., 2000), and cell cycle regulation (Castillon et al., 2003; Hanrahan and Snyder, 2003; Longtine et al., 2000), are unable to localize to the neck in the absence of neck-localized septins. Conversely, septins localize efficiently to the mother-bud neck in the absence of these septin associated proteins. Unlike the actomyosin ring, septins do not undergo contraction during cytokinesis, also suggesting that septins function as a scaffold for proteins involved in cytokinesis (Bi et al., 1998). These results suggested that one role of septins is to serve as a "scaffold" that directs the localization of non-septin proteins to subcellular regions. In this model there are three possible roles for septins. First, the proper subcellular localization of septin-associated proteins is mediated by interactions with septins that can localize them to, or away from, their site of action. Second, septin interactions promote efficient interactions of colocalized, septin-associated proteins by increasing their local concentrations through

interactions with the septin scaffold. Third, based on the observation that an interaction with septins activates the kinase activity of Hsl1p *in vitro* (Hanrahan and Snyder, 2003), it appears as if septins also can regulate the biochemical activities of septin-associated proteins.

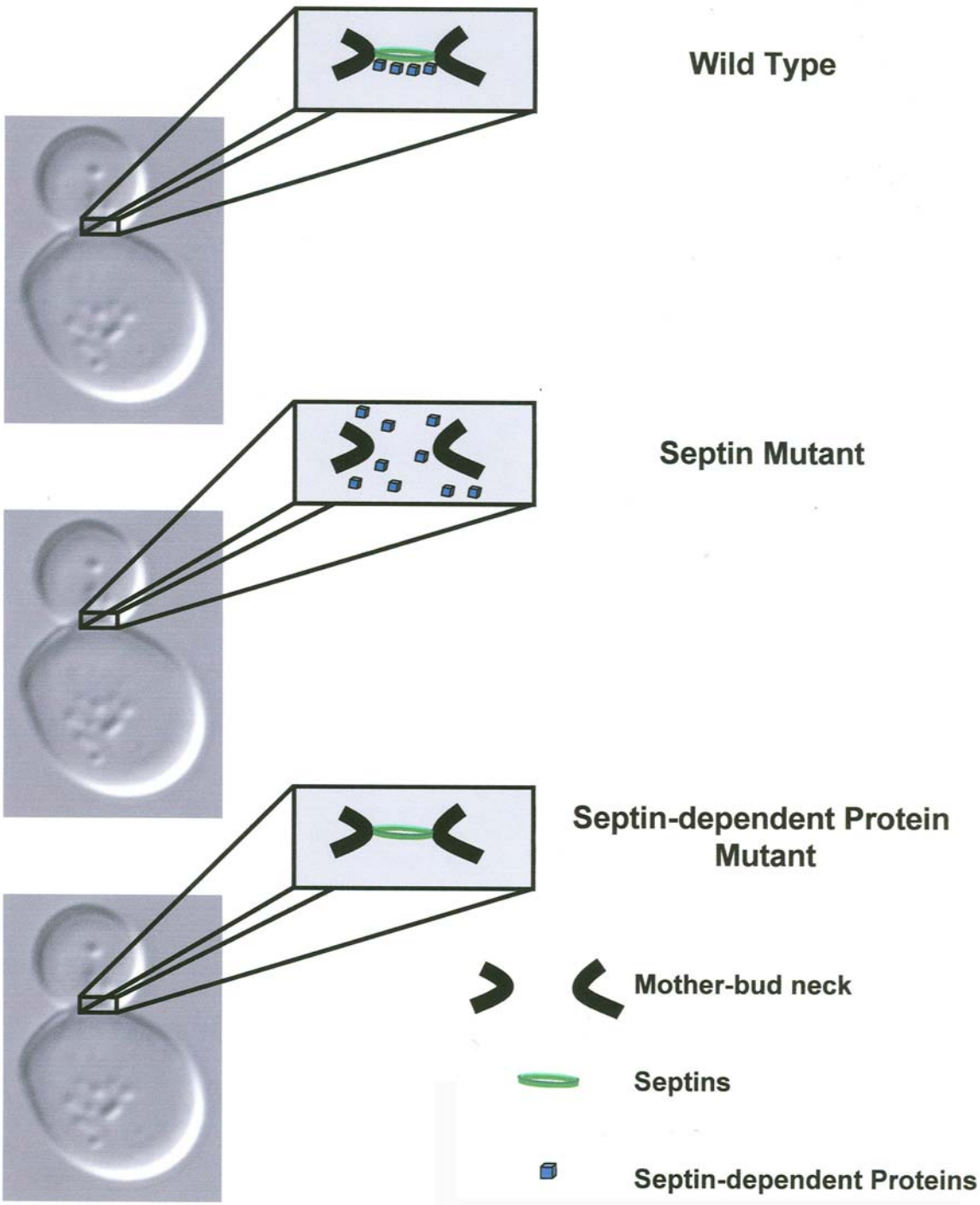

Figure 1. Scaffold model of septin function. Septin-dependent proteins localize to the mother-bud neck when septins are present, due to either direct or indirect interactions with the septin(s) protein. In the absence of the septins (septin mutant), the septin-associated proteins are unable to localize to the mother-bud neck. However, septin localization to the neck is not dependent on the localization of the septin-associated proteins (septin-dependent protein mutant).

2) Diffusion barrier model:

The generation of cellular asymmetry is required for polarized growth and the elaboration of different fates of daughter cells after cytokinesis. Thus, how cells generate asymmetry is an important question. One clue that septins may be involved in cellular asymmetry came several years ago from work done in the Vale and Snyder labs (Jensen et al., 2002; Takizawa et al., 2000). In yeast, the Ash2p mRNA was shown to be specifically transported into the bud where it is translated (Long et al., 1997; Takizawa et al., 1997). In a screen for other localized mRNAs in yeast, Takizawa et al. (2000) identified the *IST2* mRNA. *IST2* encodes a putative multipass transmembrane protein of unknown function. Ist2p localized specifically to the bud plasma membrane and was absent from the mother plasma membrane. However, upon shift of a septin mutant to restrictive temperature (and delocalization of septins from the neck), Ist2p now was localized in the plasma membranes of both the mother and bud. After the switch to isotropic growth, a number of membrane-associated proteins involved in polarized growth are specifically localized to the bud plasma membrane. Barrel et al. (2000) showed that upon septin delocalization, these proteins can diffuse into the mother cell. Together, these reports suggest that septins act as a physical barrier that restricts the movement of bud-localized, membrane-associated proteins into the mother cell (Figure 2).

Recent studies suggest a septin diffusion barrier also may function during cytokinesis. In *S. cerevisiae*, septin ring splitting occurs upon mitotic exit and just prior to cytokinesis. Work from the Barral lab (Dobbelaere and Barral, 2004) found that proteins involved in cytokinesis, including components of the secretion machinery and

proteins involved in actin polarization, localize within the region of the split septin rings. Upon septin delocalization, however, these proteins are no longer restricted to the region between the split septin rings and diffuse away. Thus, it seems likely that a role of the split septin ring during cytokinesis is to direct the restricted localization of proteins between the septin rings.

Septins also serve as a cortical barrier to the diffusion of membrane-associated proteins and lipids across the cleavage furrow of dividing mammalian cells (Schmidt and Nichols, 2004). Delocalization of the septins allows diffusion of proteins across this region. Thus, cortically localized septins are able to form a barrier of diffusion to membrane-associated molecules in yeast and in mammalian cells.

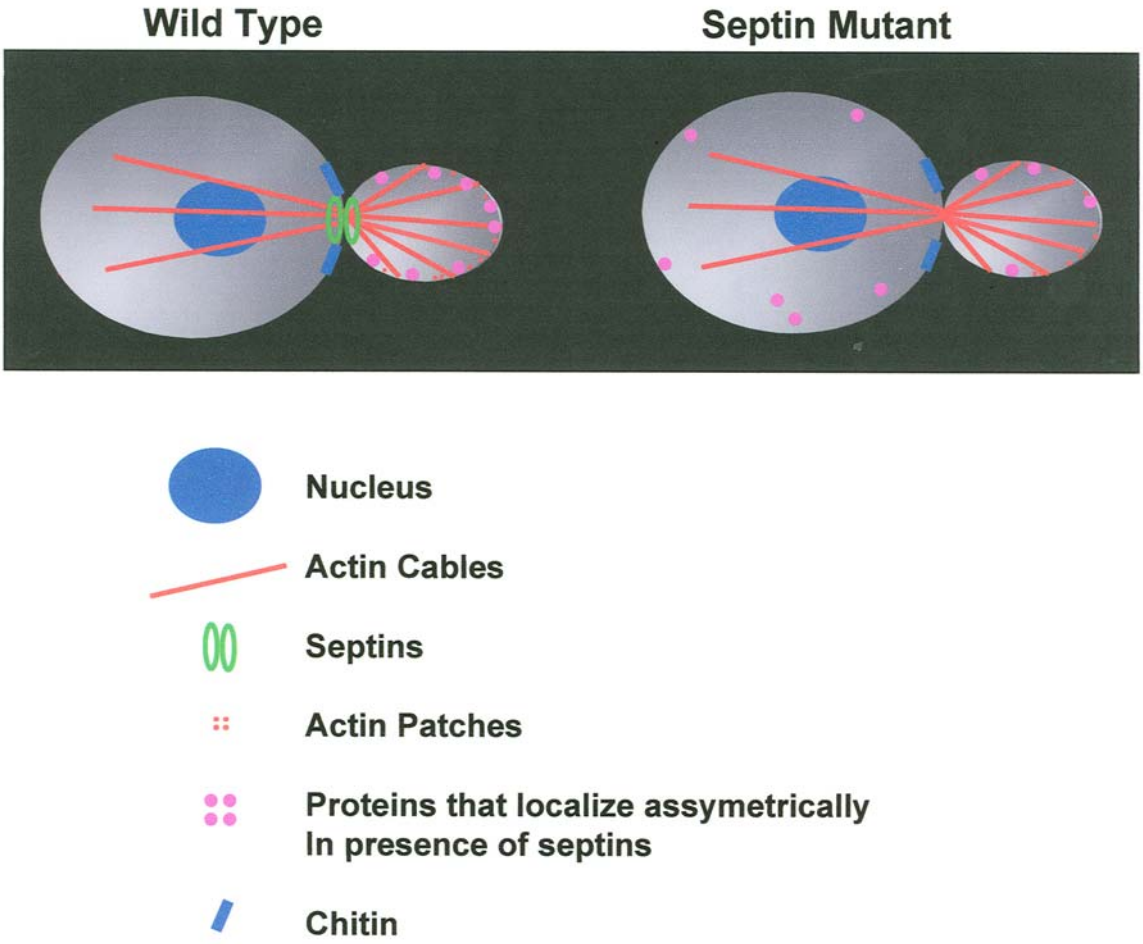

Figure 2. Diffusion barrier model of septin function. A subset of membrane-associated proteins localize asymmetrically to the bud in wild-type cells. These proteins are unable to move into the mother-cell because of the barrier formed by the cortically localized septins. In the absence of the septin barrier in a septin-mutant cell, these proteins are able to traverse the neck region, resulting in the loss of asymmetry.

Regulation of septin localization and organization

Many proteins collaborate to initiate bud formation in yeast. Initially, Cdc42p is activated by exchanging GTP for GDP by its guanine nucleotide exchange factor (GEF), Cdc24p (Johnson, 1999) (Figure 3, I). GTP-bound Cdc42p then interacts with its effectors Gic1p and/or Gic2p and/or one of the PAK-like kinases at the presumptive bud-site (Johnson, 1999) (Figure 3, II). Subsequently this complex interacts with Bni1p, a scaffold protein that is bound to the plasma membrane (Johnson, 1999) (Figure 3, III). Lastly, Bni1p interacts with profilin, Bud6p, Spa2p, and other actin-binding proteins (Johnson, 1999) (Figure 3, IV). It was suggested that this complex is the basis for localized assembly of septins (Johnson, 1999) (Figure 3, Stage One).

Initial septin localization to the cell cortex, formation of the septin ring and septin collar, splitting of the septin ring, and the disassembly of septins are likely to be regulated processes. Longtine and Bi (2003) recently reviewed our understanding of the regulation of septin localization and organization. The authors of this review propose that septins initially localize as a cortical cap (Figure 3, Stage One), then undergo transition to a dynamic ring structure (Figure 3, Stage Two) and, finally, mature into the stable septin hourglass structure (Figure 3, Stage Three) before splitting into two rings (Figure 3, Stage Four) just prior to cytokinesis.

Results from recent work we have done in collaboration with Erfei Bi, suggest a likelihood that a major pathway of septin localization as a cap to the incipient bud-site involves interaction between septins and Gic1p and Gic2p, two Cdc42p effectors. The Bi lab has shown that in Ts- *cdc42* mutant strains and in Ts- *gic* mutant strains, septins are unable to localize to the cell cortex. Furthermore, two-hybrid and co-

immunoprecipitation experiments indicate that septins interact with the Gic proteins. Consistent with this data, I found that Gic1p and Gic2p interact directly with septins. These results are further discussed in Chapter II, Appendix B. Based on these data, we suggest that the GTP-Cdc42p binds to the Gic proteins, promoting their interaction with the septins and the localization of septins as a cap to the cell cortex at the incipient bud site.

This hypothesis is refined by previous observations that suggest that cycles of GTP-binding and hydrolysis by Cdc42p are important in the initial localization of septins to the cell cortex. Gladfelter et al. (2002) found that GTP-hydrolysis defective mutants of Cdc42p displayed septin localization defects. Likewise, mutations in the Cdc42p GTPase activating proteins (GAPs) Bem3p, Rga1p, and Rga2p result in abnormal septin localization (Caviston et al., 2003). Together, these data suggest that hydrolysis of GTP by Cdc42 is important for proper regulation of septin localization. Perhaps the function of GTP hydrolysis is to result in the dissociation of Cdc42p from the Gicp proteins, allowing release of septins from the Gics and formation of the cortical septin ring in unbudded cells.

Two protein kinases, Cla4p and Gin4p are important for the initial localization of septins and their assembly into a ring structure (Figure 3, V & Stage Two). By doing Fluorescence Recovery After Photobleaching (FRAP) analysis, Caviston et al. (2003) show that the initial septin structure is dynamic and becomes immobile after converting to a stable ring. It is also shown that, during bud growth, Cla4p and Gin4p are necessary for the stable ring structure of septins that involves the phosphorylation of one of the septins, Shs1p/Sep7p (Dobbelaere et al., 2003) (Figure 3, V). Based on genetic studies

in isogenic strains, further classification of eight regulators of septin organization has been proposed wherein, proteins - Cdc42p, Rga1p, Rga2p, Bem3p and Cla4p are required for the initial assembly of the septin ring and proteins - Gin4p, Nap1p and Elm1p are required for the conversion of the ring to the hour-glass structure (Gladfelter et al., 2004) (Figure 3, VI & Stage Three).

The splitting of the septin hour-glass structure into two rings during cytokinesis is dependent on the Tem1p small GTPase and this splitting appears required for contraction of the actomyosin ring (Lippincott et al., 2001) (Figure 3, VII & Stage Four). Finally, it was also shown that the phosphorylation of one of the septins, Cdc3p, at two of its C-terminal serine residues, by Cdc28p might be important for the septin ring disassembly (Tang and Reed, 2002).

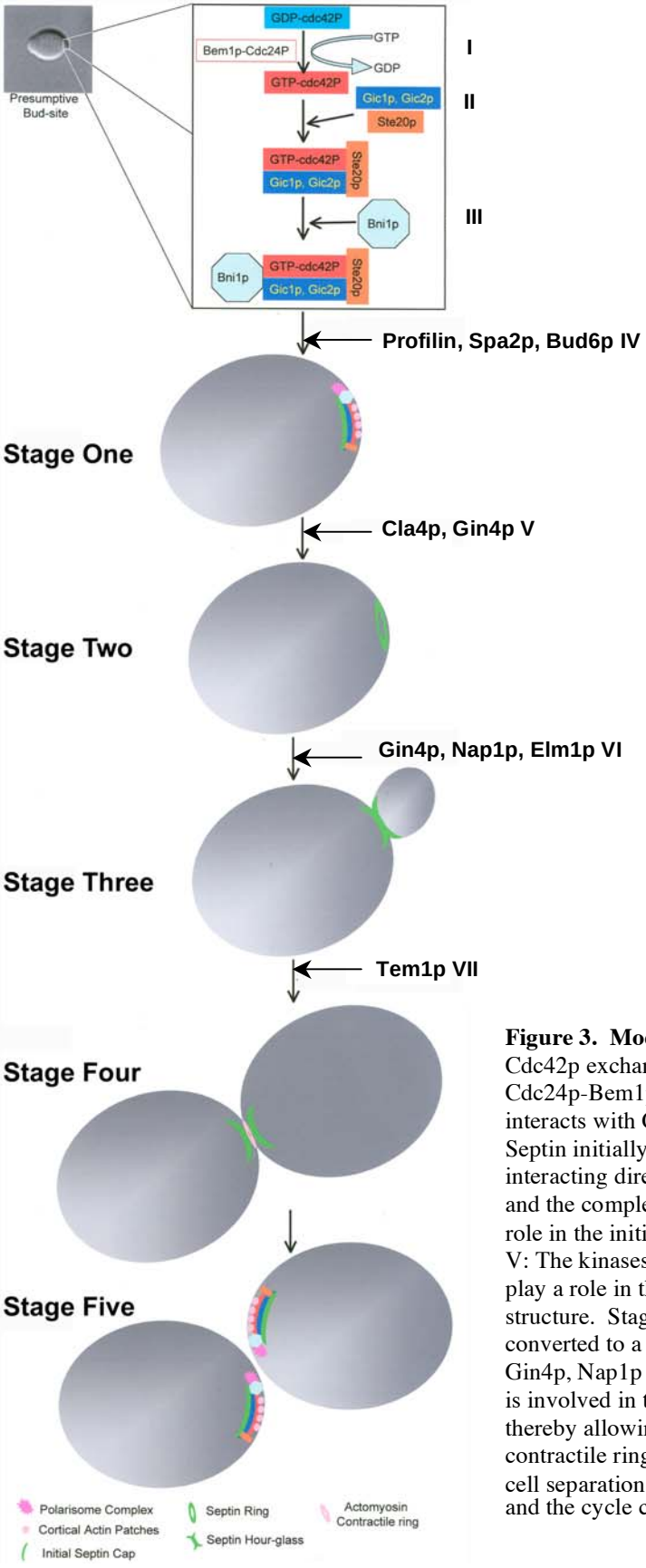

Figure 3. Model for Septin Regulation. I-IV: Cdc42p exchanges GTP for GDP in the presence of Cdc24p-Bem1p complex. Activated GTP-Cdc42p interacts with Gic1p and/or Gic2p. Stage One: Septin initially localize as a presumptive cap by interacting directly with Gic1p and Gic2p. Cdc42p, and the complex it is associated with, also plays a role in the initial localization of septins. Stage Two, V: The kinases, Cla4p and Gin4p, are suggested to play a role in the transition of the cap to a stable ring structure. Stage Three, VI: The ring is then converted to a stable hour-glass structure due to Gin4p, Nap1p and Elm1p. Stage Four, VII: Tem1p is involved in the splitting of the septin hour-glass thereby allowing the contraction of actomyosin contractile ring. Stage Five: Finally, cytokinesis and cell separation occurs giving rise to two new cells and the cycle continues.

Septin domain organization and septin-septin interactions

Septin proteins have an overall identity of $\geq$26% (Faty et al., 2002). They have greatest similarity in their central regions and less similarity at their N- and C-terminal regions (Figure 4). Molecular masses of septins range from 40–75 kDa with a chain length of 275–539 amino acids (Longtine et al., 1996) and they have conserved domains (Figure 5). All of the known septins, with the exception of a septin homologue from *Pyrenopeziza brassicae* (Singh et al., 2000), contain a P-loop (G1) nucleotide-binding domain near the N-terminal region and additional motifs (G3 and G4) that define the GTPase superfamily (Bourne et al., 1991). The P-loop consists of a glycine-rich sequence followed by a conserved lysine and a serine/threonine (Saraste et al., 1990). In the P-loop, the conserved glycines are necessary for proper folding of nucleotide-binding pocket, the conserved lysine bonds with β and γ phosphates, and the conserved serine (or threonine) helps coordinate the magnesium ion. Most septins also have positively charged residues just N-terminal to the P-loop which appear to be involved in the binding of septins to inositol-phosphate phospholipids (Zhang et al., 1999). Experiments on the interaction of phospholipid binding to the mammalian Sept4 septin, suggests that GTP binding (but not GDP binding) may be mutually exclusive with phospholipid binding. Most septins contain a predicted coiled-coil domain of 35–98 amino acids in their C–terminal region (Figure 4 and 5). This domain is suggested to be important for interactions of septins with other proteins (Casamayor and Snyder, 2003), or with other septins (Versele et al., 2004). By assaying the rate of GTP hydrolysis of a C-terminally truncated Cdc12p derivative, it was shown that the coiled-coil domain of Cdc12p appears to regulate Cdc12p GTPase activity (Versele and Thorner, 2004).

Septin complexes purified from *Drosophila* embryos consisted of septin subunits bound to GTP and GDP, with bound nucleotide in ~1:1 stoichiometry with the septin proteins (Field et al., 1996). GTP binding and hydrolytic properties similar to *Drosophila* septin complexes were seen in septin complexes purified from yeast (Vrabioiu et al., 2004). A single *Xenopus laevis* septin, expressed and purified from bacteria, hydrolyzed GTP. This hydrolysis appeared to promote septin polymerization *in vitro* (Mendoza et al., 2002). However, these results may not reflect *in vivo* septin function, since there is no evidence that a single septin forms homomeric complexes in vivo. Indeed, GTP binding and hydrolysis was not observed to be correlated with the efficiency of filament formation of human septins (Kinoshita et al., 2002; Sheffield et al., 2002). Thus, the role of GTP binding and hydrolysis in septin filament formation is yet unclear.

Mutations in P-loop motifs disrupt nucleotide binding and/or hydrolysis of many proteins (Bourne et al., 1991; Saraste et al., 1990). This disruption due to mutations has been directly demonstrated for the mammalian Nedd5 (Kinoshita et al., 1997), CDCrel-1 (Beites et al., 1999), and yeast Cdc10p and Cdc12p (Versele and Thorner, 2004). In the case of yeast septins, it has also been suggested that GTP binding is required for septin polymerization *in vitro* (Versele and Thorner, 2004). The P-loop motif (and thus nucleotide binding and hydrolysis) of ARTS is implicated to be important in mediation of TGF-β induced apoptosis: mutation in the P-loop region ceases to potentiate apoptosis induced by TGF-β (Larisch et al., 2000). An attempt to characterize the role of individual domains of yeast septins has yielded some data on septin interactions with other septins and non-septin proteins and their biological significance have been discussed (Casamayor and Snyder, 2003; Versele et al., 2004). My thesis is also an

attempt to characterize the role of nucleotide binding and/or hydrolysis in yeast septins. I have utilized various cell biological, biochemical and genetic techniques to achieve my goals. The rationale behind the approach, the methods applied and results obtained are described in detail in the subsequent chapters.

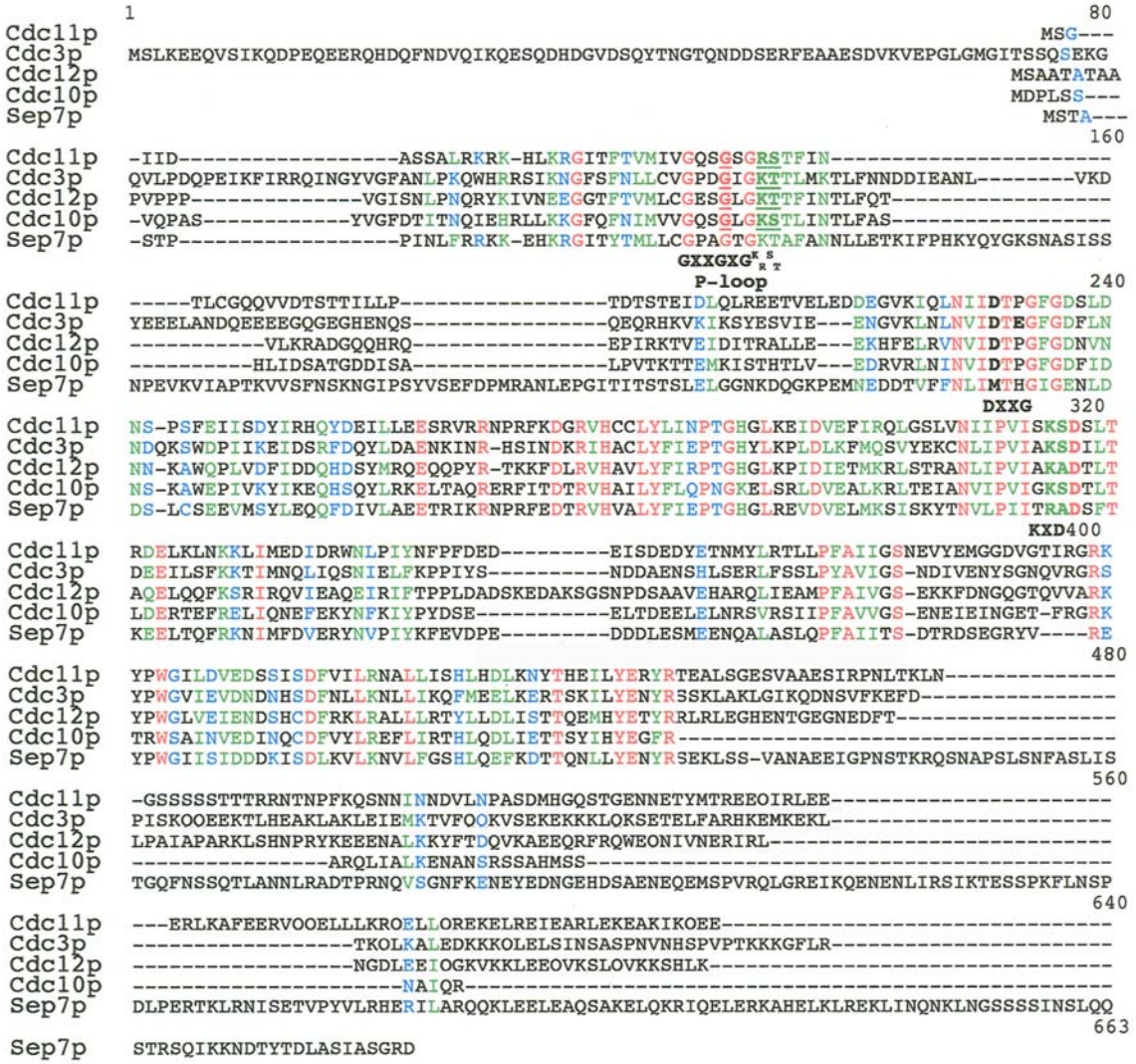

Figure 4. Clustal W alignment of *Saccharomyces cerevisiae* septins. Alignment was done using Clustal W (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page=/NPSA/npsa_clustalw.html). Indicated are the P-loop, G3 (DXXG) and G4 (KXD) nucleotide-binding domains. Residues mutated in the P-loop motif are underlined and highlighted.

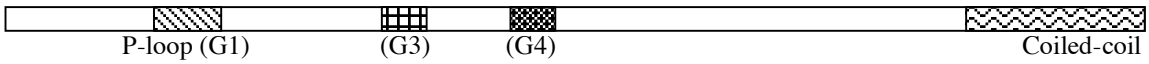

Figure 5. Schematic diagram showing septin domains. Septins have conserved domains. Shown here are the P-loop domain (G1) (hatched box), the GTP hydrolysis domain (G3) (gridded box), the nucleotide specificity domain (G4) (dotted box) and the coiled-coil domain (wavy box).

References

Adam, J.C., J.R. Pringle, and M. Peifer. 2000. Evidence for functional differentiation among *Drosophila* septins in cytokinesis and cellularization. *Molecular Biology of the Cell*. 11:3123-35.

Barral, Y., V. Mermall, M.S. Mooseker, and M. Snyder. 2000. Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. *Mol Cell*. 5:841-51.

Beites, C.L., K.A. Campbell, and W.S. Trimble. 2005. The septin Sept5/CDCrel-1 competes with alpha-SNAP for binding to the SNARE complex. *Biochem J*. 385:347-53.

Beites, C.L., X.R. Peng, and W.S. Trimble. 2001. Expression and analysis of properties of septin CDCrel-1 in exocytosis. *Methods Enzymol*. 329:499-510.

Beites, C.L., H. Xie, R. Bowser, and W.S. Trimble. 1999. The septin CDCrel-1 binds syntaxin and inhibits exocytosis. *Nat Neurosci*. 2:434-9.

Bi, E., P. Maddox, D.J. Lew, E.D. Salmon, J.N. McMillan, E. Yeh, and J.R. Pringle. 1998. Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *Journal Of Cell Biology*. 142:1301-12.

Blondel, M., P.M. Alepuz, L.S. Huang, S. Shaham, G. Ammerer, and M. Peter. 1999. Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes Dev*. 13:2284-300.

Blondel, M., J.M. Galan, Y. Chi, C. Lafourcade, C. Longaretti, R.J. Deshaies, and M. Peter. 2000. Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. *Embo J.* 19:6085-97.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature*. 349:117-27.

Byers, B., and L. Goetsch. 1976. A highly ordered ring of membrane-associated filaments in budding yeast. *Journal of Cell Biology*. 69:717-721.

Carroll, C.W., R. Altman, D. Schieltz, J.R. Yates, and D. Kellogg. 1998. The septins are required for the mitosis-specific activation of the Gin4 kinase. *Journal Of Cell Biology*. 143:709-17.

Casamayor, A., and M. Snyder. 2003. Molecular dissection of a yeast septin: distinct domains are required for septin Interaction, localization, and function. *Molecular and Cellular Biology*. 23:2762-2777.

Castillon, G.A., N.R. Adames, C.H. Rosello, H.S. Seidel, M.S. Longtine, J.A. Cooper, and R. Heil-Chapdelaine. 2003. Septins have a dual role in controlling mitotic exit in budding yeast. *Current Biology*. In press.

Caviston, J.P., M. Longtine, J.R. Pringle, and E. Bi. 2003. The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. *Molecular Biology of the Cell*. 14:4051-4066.

Chant, J. 1999. Cell polarity in yeast. *Annu Rev Cell Dev Biol.* 15:365-91.

Chant, J., M. Mischke, E. Mitchell, I. Herskowitz, and J.R. Pringle. 1995. Role of Bud3p in producing the axial budding pattern of yeast. *Journal Of Cell Biology*. 129:767-78.

De Virgilio, C., D.J. DeMarini, and J.R. Pringle. 1996. *SPR28*, a sixth member of the septin gene family in *Saccharomyces cerevisiae* that is expressed specifically in sporulating cells. *Microbiology*. 142:2897-905.

DeMarini, D.J., A.E. Adams, H. Fares, C. De Virgilio, G. Valle, J.S. Chuang, and J.R. Pringle. 1997. A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. *Journal of Cell Biology*. 139:75-93.

DiDomenico, B.J., N.H. Brown, J. Lupisella, J.R. Greene, M. Yanko, and Y. Koltin. 1994. Homologs of the yeast neck filament associated genes: isolation and sequence analysis of *Candida albicans CDC3* and *CDC10*. *Molecular And General Genetics*. 242:689-98.

Dobbelaere, J., and Y. Barral. 2004. Spatial coordination of cytokinetic events by compartmentalization of the cell cortex. *Science*. 305:393-6.

Dobbelaere , J., M.S. Gentry, R.L. Hallberg, and Y. Barral 2003. Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Developmental Cell*. 4:345-357.

Edgington, N.P., and B. Futcher. 2001. Relationship between the function and the location of G1 cyclins in S. cerevisiae. *J Cell Sci.* 114:4599-611.

Elledge, S.J. 1996. Cell cycle checkpoints: preventing an identity crisis. *Science*. 274:1664-72.

Fares, H., L. Goetsch, and J.R. Pringle. 1996. Identification of a developmentally regulated septin and involvement of the septins in spore formation in *Saccharomyces cerevisiae*. *Journal of Cell Biology.* 132:399-411.

Fares, H., M. Peifer, and J.R. Pringle. 1995. Localization and possible functions of *Drosophila* septins. *Molecular Biology of the Cell*. 6:1843-59.

Faty, M., M. Fink, and Y. Barral. 2002. Septins: a ring to part mother and daughter. *Current Genetics*. 41:123-31.

Field, C.M., O. Al-Awar, J. Rosenblatt, M.L. Wong, B. Alberts, and T.J. Mitchison. 1996. A purified *Drosophila* septin complex forms filaments and exhibits GTPase activity. *Journal of Cell Biology*. 133:605-16.

Ford, S.K., and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC11* gene product and the timing of events at the budding site. *Developmental Genetics*. 12:281-92.

Frazier, J.A., M.L. Wong, M.S. Longtine, J.R. Pringle, M. Mann, T.J. Mitchison, and C. Field. 1998. Polymerization of purified yeast septins: evidence that organized filament arrays may not be required for septin function. *Journal of Cell Biology*. 143:737-49.

Giot, L., and J.B. Konopka. 1997. Functional analysis of the interaction between Afr1p and the Cdc12p septin, two proteins involved in pheromone-induced morphogenesis. *Molecular Biology of the Cell*. 8:987-98.

Gladfelter, A.S., I. Bose, T.R. Zyla, E.S.G. Bardes, and D.J. Lew. 2002. Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *Journal of Cell Biology*. 156:315-326.

Gladfelter, A.S., T.R. Zyla, and D.J. Lew. 2004. Genetic interactions among regulators of septin organization. *Eukaryot Cell*. 3:847-54.

Haarer, B.K., and J.R. Pringle. 1987. Immunofluorescence localization of the *Saccharomyces cerevisiae CDC12* gene product to the vicinity of the 10-nm filaments in the mother-bud neck. *Molecular And Cellular Biology*. 7:3678-87.

Hales, K.G., E. Bi, J.Q. Wu, J.C. Adam, I.C. Yu, and J.R. Pringle. 1999. Cytokinesis: an emerging unified theory for eukaryotes? *Curr Opin Cell Biol.* 11:717-25.

Hanrahan, J., and M. Snyder. 2003. Cytoskeletal activation of a checkpoint kinase. *Mol Cell*. 12:663-73.

Harkins, H.A., N. Page, L.R. Schenkman, C. De Virgilio, S. Shaw, H. Bussey, and J.R. Pringle. 2001. Bud8p and Bud9p, proteins that may mark the sites for bipolar budding in yeast. *Molecular Biology of the Cell*. 12:2497-518.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast: IV. Genes controlling bud emergence and cytokinesis. *Experimental Cell Research*. 69:265-276.

Hartwell, L.H., R.K. Mortimer, J. Culotti, and M. Culotti. 1973. Genetic control of the cell division cycle in yeast. V. Genetic analysis of cdc mutants. *Genetics*. 74:267-286.

Hsu, S.C., C.D. Hazuka, R. Roth, D.L. Foletti, J. Heuser, and R.H. Scheller. 1998. Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. *Neuron*. 20:1111-22.

Jensen, S., M. Geymonat, A.L. Johnson, M. Segal, and L.H. Johnston. 2002. Spatial regulation of the guanine nucleotide exchange factor Lte1 in *Saccharomyces cerevisiae*. *Journal of Cell Science*. 115:4977-4991.

Jeong, J.W., D.H. Kim, S.Y. Choi, and H.B. Kim. 2001. Characterization of the *CDC10* product and the timing of events of the budding site of *Saccharomyces cerevisiae*. *Molecules to Cells*. 12:77-83.

Johnson, D.I. 1999. Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiology And Molecular Biology Reviews*. 63:54-105.

Kaldis, P., Z.W. Pitluk, I.A. Bany, D.A. Enke, M. Wagner, E. Winter, and M.J. Solomon. 1998. Localization and regulation of the cdk-activating kinase (Cak1p) from budding yeast. *J Cell Sci*. 111 (Pt 24):3585-96.

Kartmann, B., and D. Roth. 2001. Novel roles for mammalian septins: from vesicle trafficking to oncogenesis. *Journal of Cell Science*. 114:839-44.

Kim, H.B., B.K. Haarer, and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC3* gene product and the timing of events at the budding site. *Journal Of Cell Biology*. 112:535-44.

Kinoshita, M. 2003. Assembly of mammalian septins. *J Biochem (Tokyo)*. 134:491-6.

Kinoshita, M., C.M. Field, M.L. Coughlin, A.F. Straight, and T.J. Mitchison. 2002. Self- and actin-templated assembly of mammalian septins. *Dev Cell*. 3:791-802.

Kinoshita, M., S. Kumar, A. Mizoguchi, C. Ide, A. Kinoshita, T. Haraguchi, Y. Hiraoka, and M. Noda. 1997. Nedd5, a mammalian septin, is a novel cytoskeletal component interacting with actin-based structures. *Genes and Development*. 11:1535-47.

Kinoshita, M., and M. Noda. 2001. Roles of septins in the mammalian cytokinesis machinery. *Cell Structure and Function*. 26:667-70.

Konopka, J. 1993. *AFR1* acts in conjunction with the α-factor receptor to promote morphogenesis and adaptation. *Molecular and Cellular Biology*. 13:6876-88.

Konopka, J.B., C. DeMattei, and C. Davis. 1995. *AFR1* promotes polarized apical morphogenesis in *Saccharomyces cerevisiae*. *Molecular And Cellular Biology*. 15:723-30.

Larisch, S., Y. Yi, R. Lotan, H. Kerner, S. Eimerl, W. Tony Parks, Y. Gottfried, S. Birkey Reffey, M.P. de Caestecker, D. Danielpour, N. Book-Melamed, R. Timberg, C.S. Duckett, R.J. Lechleider, H. Steller, J. Orly, S.J. Kim, and A.B. Roberts. 2000. A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. *Nature Cell Biology*. 2:915-21.

Lew, D.J. 2003. The morphogenesis checkpoint: how yeast cells watch their figures. *Curr Opin Cell Biol*. 15:648-53.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *Journal Of Cell Biology*. 120:1305-20.

Lew, D.J., and S.I. Reed. 1995. A cell cycle checkpoint monitors cell morphogenesis in budding yeast. *Journal of Cell Biology*. 129:739-49.

Lew, D.J., T. Weinert, and J.R. Pringle. 1997. Cell cycle control in *S. cerevisiae*. *In* The molecular and cellular biology of the yeast *Saccharomyces*. Vol. 3. J.R. Pringle, J.R. Broach, and E.W. Jones, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 607-95.

Lippincott, J., and R. Li. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *Journal of Cell Biology*. 140:355-66.

Lippincott, J., K.B. Shannon, W. Shou, R.J. Deshaies, and R. Li. 2001. The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *J Cell Sci*. 114:1379-86.

Long, R.M., R.H. Singer, X. Meng, I. Gonzalez, K. Nasmyth, and R.P. Jansen. 1997. Mating type switching in yeast controlled by asymmetric localization of *ASH1* mRNA. *Science*. 277:383-7.

Longtine, M.S., and E. Bi. 2003. Regulation of septin organization and function in yeast. *Trends in Cell Biology*. 13:403-409.

Longtine, M.S., D.J. DeMarini, M.L. Valencik, O.S. Al-Awar, H. Fares, C. De Virgilio, and J.R. Pringle. 1996. The septins: roles in cytokinesis and other processes. *Current Opinion In Cell Biology*. 8:106-19.

Longtine, M.S., H. Fares, and J.R. Pringle. 1998. Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *Journal of Cell Biology*. 143:719-36.

Longtine, M.S., C.L. Theesfeld, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 20:4049-61.

Mendoza, M., A.A. Hyman, and M. Glotzer. 2002. GTP binding induces filament assembly of a recombinant septin. *Current Biology*. 12:1858-63.

Mino, A., K. Tanaka, T. Kamei, M. Umikawa, T. Fujiwara, and Y. Takai. 1998. Shs1p: a novel member of septin that interacts with Spa2p, involved in polarized growth in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications*. 251:732-6.

Momany, M., J. Zhao, R. Lindsey, and P.J. Westfall. 2001. Characterization of the *Aspergillus nidulans* Septin (asp) Gene Family. *Genetics*. 157:969-77.

Neufeld, T.P., and G.M. Rubin. 1994. The *Drosophila peanut* gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. *Cell*. 77:371-9.

Nguyen, T.Q., H. Sawa, H. Okano, and J.G. White. 2000. The *C. elegans* septin genes, unc-59 and unc-61, are required for normal postembryonic cytokineses and morphogenesis but have no essential function in embryogenesis. *Journal of Cell Science*. 113:3825-37.

Nurse, P., and P. Thuriaux. 1980. Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. *Genetics*. 96:627-37.

Oegema, K., M.S. Savoian, T.J. Mitchison, and C.M. Field. 2000. Functional analysis of a human homologue of the *Drosophila* actin binding protein anillin suggests a role in cytokinesis. *Journal of Cell Biology*. 150:539-52.

Pringle, J.R., J.R. Broach, and E.W. Jones. 1997. The molecular and cellular biology of the yeast *Saccharomyces*. Vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Rodal, A.A., L. Kozubowski, B.L. Goode, D.G. Drubin, and J.H. Hartwig. 2005. Actin and septin ultrastructures at the budding yeast cell cortex. *Mol Biol Cell*. 16:372-84.

Roh, D.-H., B. Bowers, M. Schmidt, and E. Cabib. 2002. The septation apparatus, an autonomous system in budding yeast. *Molecular Biology of the Cell*. 13:2747-2759.

Sanders, S.L., and I. Herskowitz. 1996. The BUD4 protein of yeast, required for axial budding, is localized to the mother/bud neck in a cell cycle-dependent manner. *Journal of Cell Biology*. 134:413-27.

Saraste, M., P.R. Sibbald, and A. Wittinghofer. 1990. The P-loop: a common motif in ATP- and GTP-binding proteins. *Trends in Biochemical Science*. 15:430-434.

Schenkman, L.R., C. Caruso, N. Page, and J.R. Pringle. 2002. The role of cell cycle-regulated expression in the localization of spatial landmark proteins in yeast. *The Journal of Cell Biology*. 156:829-41.

Schmidt, K., and B.J. Nichols. 2004. A barrier to lateral diffusion in the cleavage furrow of dividing mammalian cells. *Curr Biol*. 14:1002-6.

Sheffield, P.J., C.J. Oliver, B.E. Kremer, S. Sheng, Z. Shao, and I.G. Macara. 2002. Borg/Septin interactions and the assembly of mammalian septin heterodimers, trimers and filaments. *J Biol Chem*. 21:21.

Sherr, C.J. 1996. Cancer cell cycles. *Science*. 274:1672-7.

Shulewitz, M.J., C.J. Inouye, and J. Thorner. 1999. Hsl7 localizes to a septin ring and serves as an adapter in a regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 19:7123-37.

Sia, R.A., E.S. Bardes, and D.J. Lew. 1998. Control of Swe1p degradation by the morphogenesis checkpoint. *Embo Journal*. 17:6678-88.

Singh, G., H. Sinha, and A.M. Ashby. 2000. Cloning and expression studies during vegetative and sexual development of Pbs1, a septin gene homologue from *Pyrenopeziza brassicae*. *Biochim Biophys Acta*. 1497:168-74.

Slater, M.L., B. Bowers, and E. Cabib. 1985. Formation of septum-like structures at locations remote from the budding sites in cytokinesis-defective mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology*. 162:1245-54.

Surka, M.C., C.W. Tsang, and W.S. Trimble. 2002. The mammalian septin MSF localizes with microtubules and is required for completion of cytokinesis. *Molecular Biology of the Cell*. 13:3532-45.

Tachikawa, H., A. Bloecher, K. Tatchell, and A.M. Neiman. 2001. A Gip1p–Glc7p phosphatase complex regulates septin organization and spore wall formation. *Journal of Cell Biology*. 155:797-808.

Takizawa, P.A., J.L. DeRisi, J.E. Wilhelm, and R.D. Vale. 2000. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. *Science*. 290:341-4.

Takizawa, P.A., A. Sil, J.R. Swedlow, I. Herskowitz, and R.D. Vale. 1997. Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. *Nature*. 389:90-3.

Tang, C.S., and S.I. Reed. 2002. Phosphorylation of the septin Cdc3 in G1 by the Cdc28 kinase is essential for efficient septin ring disassembly. *Cell Cycle*. 1:42-49.

Tessema, M., U. Lehmann, and H. Kreipe. 2004. Cell cycle and no end. *Virchows Arch*. 444:313-23.

Theesfeld, C.L., T.R. Zyla, E.G. Bardes, and D.J. Lew. 2003. A monitor for bud emergence in the yeast morphogenesis checkpoint. *Mol Biol Cell*. 14:3280-91.

Vallen, E.A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in cytokinesis in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*. 11:593-611.

Versele, M., B. Gullbrand, M.J. Shulewitz, V.J. Cid, S. Bahmanyar, R.E. Chen, P. Barth, T. Alber, and J. Thorner. 2004. Protein-protein interactions governing septin heteropentamer assembly and septin filament organization in Saccharomyces cerevisiae. *Mol Biol Cell*. 15:4568-83.

Versele, M., and J. Thorner. 2004. Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J Cell Biol*. 164:701-15.

Vrabioiu, A.M., S.A. Gerber, S.P. Gygi, C.M. Field, and T.J. Mitchison. 2004. The majority of the Saccharomyces cerevisiae septin complexes do not exchange guanine nucleotides. *J Biol Chem*. 279:3111-8.

Xie, H., M. Surka, J. Howard, and W.S. Trimble. 1999. Characterization of the mammalian septin H5: distinct patterns of cytoskeletal and membrane association from other septin proteins. *Cell Motility and Cytoskeleton*. 43:52-62.

Yeong, F.M., H.H. Lim, and U. Surana. 2002. MEN, destruction and separation: mechanistic links between mitotic exit and cytokinesis in budding yeast. *Bioessays*. 24:659-66.

Zhang, J., C. Kong, H. Xie, P.S. McPherson, S. Grinstein, and W.S. Trimble. 1999. Phosphatidylinositol polyphosphate binding to the mammalian septin H5 is modulated by GTP. *Current Biology*. 9:1458-67.

CHAPTER II

ROLE OF NUCLEOTIDE BINDING DOMAIN OF SEPTINS

Introduction

Septins are a conserved family of nucleotide-binding, filament-forming proteins. *S. cerevisiae* encodes five vegetatively expressed septins: Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Sep7p/Shs1p (Carroll et al., 1998; Ford and Pringle, 1991; Haarer and Pringle, 1987; Jeong et al., 2001; Kim et al., 1991; Mino et al., 1998) and two sporulation-specific septins-Spr3p and Spr28p (De Virgilio et al., 1996; Fares et al., 1996). Mutations in *CDC3*, *CDC10*, *CDC11*, or *CDC12* result in defects in cytokinesis, cell-cycle progression, chitin deposition, and other processes. The role of septins in cell-cycle processes is reviewed in Chapter I.

All yeast septins contain a P-loop (G1) nucleotide-binding domain, G3 and G4 domains that define the GTPase superfamily (Bourne et al., 1991) and yeast septins bind GTP (Vrabioiu et al., 2004). However, only two (Cdc10p and Cdc12p) of the four analyzed septins demonstrated GTPase activity (Versele and Thorner, 2004). However, the precise role of septin nucleotide binding and/or hydrolysis is not clear. Based on the role of nucleotide binding for many other proteins, it is likely that the status of nucleotide bound to the septins affects their conformation. Specifically, we propose that GTP-bound

septins have a different conformation than GDP-bound septins, and that this "nucleotide switch" alters protein-protein interactions. We propose that this switch affects one of two sets of septin interactions. First, based on the effect of GDP- versus GTP-binding to actin and tubulin (Belmont et al., 1999; Muller-Reichert et al., 1998), this nucleotide-dependent conformational change may affect septin-septin interactions, resulting in the assembly/disassembly of septin complexes and/or of septin filaments (Figure 1A). In this model, septin nucleotide-binding mutations would result in recessive, or possibly dominant, defects in septin-septin interactions. If septin-septin interactions are important for function, these mutants would be expected to have defects in septin localization and thus be defective in many septin-dependent processes.

Second, the role of septin nucleotide binding may be similar to that of small G proteins [reviewed in (Bourne et al., 1991; Sprang, 1997)], in which GTP-binding promotes interactions with downstream effectors, and this interaction is blocked upon hydrolysis of GTP to GDP (Figure 1B). In this model, we suggest that septin-septin interactions are independent of their GTP or GDP status. Rather, in this case we propose that septin-GTP promotes interactions with non-septin proteins that function in septin-dependent processes. Candidate interacting proteins whose interaction may be regulated by septin nucleotide binding include those that function in cytokinesis (Myo1p), in cell cycle progression (Hsl1p), in chitin deposition (Bni4p), and in bud-site selection (Bud3p, Bud4p). In this model, we would predict that nucleotide-binding mutant septins would have defects in some, but perhaps not all, septin-dependent processes (see Chapter I) with corresponding defects in interaction (and thus localization) of at least some proteins involved in the affected process.

To address these models, mutations were introduced in the GTP binding domains of the four septins (Cdc3p, Cdc10p, Cdc11p and Cdc12p) (Figure 2). By utilizing a UV cross-linking assay, we have verified that mutations in the septin P-loop domains disrupt GTP binding (Ashok Rajendran, personal communication). We assayed nucleotide-binding mutant septins for defects in viability, cytokinesis and cell morphogenesis, septin localization and for defects in septin-dependent processes. Also, we analyzed the defects in septin-septin interactions and septin complex formation using *in vitro* and *in vivo* assays. Our results do not provide support for the small G-protein model of septin nucleotide binding. Rather, our data strongly suggest that the role of septin nucleotide binding is to promote strong septin-septin interactions. Furthermore, our results suggest that efficient septin-septin interactions are a prerequisite for localization of septins as a cortical ring early in the cell cycle.

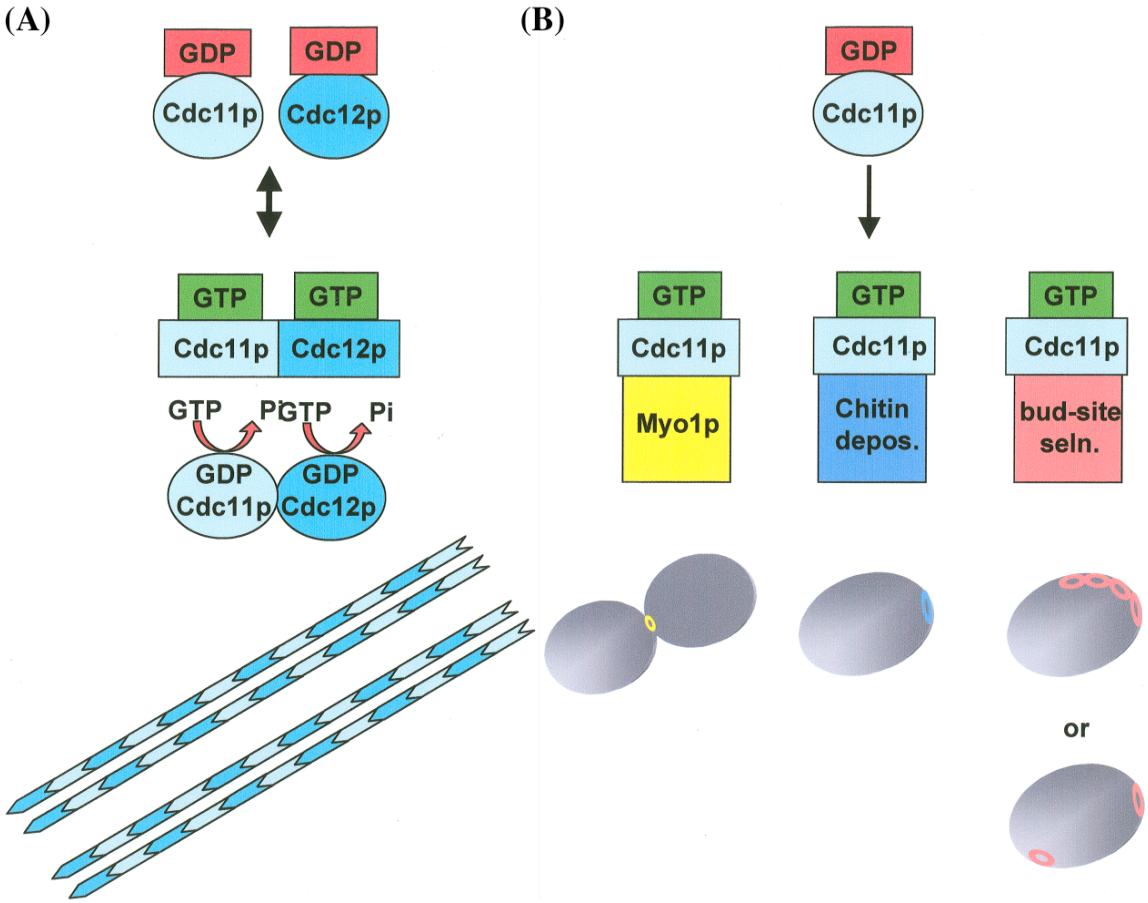

Figure 1. Models for the role of septin nucleotide binding and hydrolysis.
(A) The "filament formation" model predicts that the status of bound nucleotide and/or GTP hydrolysis regulates septin/septin interactions. (B) The "Small GTPase" model predicts that the status of bound nucleotide regulates septin/non-septin interactions.

	P-loop GXXGXGK(S/T)	G3 DXXG	G4 KXD
Cdc3p	126 GPDGIGKT 133	204 DTEG 207	287 KSD 289
cdc3p^{G129V}	126 GPDVIGKT 133		
cdc3p^{K132T}	126 GPDGIGTT 133		
cdc3p^{K132E}	126 GPDGIGET 133		
cdc3p^{T133N}	126 GPDGIGKN 133		
cdc3p^{G129V,K132E,T133N}	126 GPDVIGEN 133		
Cdc10p	39 GQSGLGKS 47	97 DTPG 100	180 KSD 182
cdc10p^{G42V}	39 GQSVLGKS 47		
cdc10p^{K45T}	39 GQSGLGTS 47		
cdc10p^{G42V,K45E,S46N}	39 GQSVLGEN 47		
Cdc11p	29 GQSGSGRS 36	89 DTPG 92	172 KSD 174
cdc11p^{G29D}	29 DQSGSGRS 36		
cdc11p^{G32E}	29 GQSESGRS 36		
cdc11p^{G32V}	29 GQSVSGRS 36		
cdc11p^{R35T}	29 GQSGSGTS 36		
cdc11p^{R35E}	29 GQSGSGES 36		
cdc11p^{S36N}	29 GQSGSGRN 36		
cdc11p^{G32V,R35E,S36N}	29 GQSVSGEN 36		
cdc11p^{D89A,G92A}		89 ATPA 92	
cdc11p^{K172A,D174A}			172 ASA 174
Cdc12p	41 GESGLGKT 48	98 DTPG 101	180 KAD 182
cdc12p^{G44V}	41 GESVLGKT 48		
cdc12p^{K47T}	41 GESGLGTT 48		
cdc12p^{G44V,K47E,T48N}	41 GESVLGEN 48		
cdc12p^{D98A,G101A}	41 GESGLGKT 48	98 ATPA 101	

Figure 2. Mutations in the P-loop, G3 and G4 domains in yeast septins.
Residues mutated in septins in the septin nucleotide binding motifs are discussed in the text. *cdc11-1* and *cdc11-7* alleles encode cdc11pG29D and the *cdc11-6* allele encodes cdc11pG32E. All other alleles were generated by *in vitro* mutagenesis. The expected effects of these mutations on nucleotide binding and hydrolysis are discussed in the text.

Materials and methods

Strains, growth conditions, and genetic methods

Yeast strains used (Table IA and B) are isogenic to YEF473 (Bi and Pringle, 1996), unless indicated. YPD, YM-1, synthetic defined complete (SDC) medium lacking appropriate amino acids for selection and sporulation medium were prepared as described (Guthrie and Fink, 1991; Lillie and Pringle, 1980). Cells were grown at 23˚C to mid-log phase except for sporulation assays, where cells were grown to saturation. For viability assays, five-fold serial dilutions were spotted on plates and incubated at the indicated temperature for three days. For sporulation assays, cells were washed once in water and transferred to sporulation medium with incubation continued at 23˚C for four days or seven days followed by counting of four-and three-spored asci. Cell cycle arrest at G1 with α-factor or at S-phase with hydroxyurea or at G2/M with nocodazole was done as described (Amon, 2002; Vallen et al., 2000). Yeast transformations and genetic manipulations were performed as described earlier (Gietz et al., 1992; Guthrie and Fink, 1991). *E.coli* DH5α (Life technologies, Rockville, MD) and standard media and methods (Ausubel et al., 1995) were used for all plasmid manipulations and *E.coli* BL21-Codonplus-RIL (Stratagene, La Jolla, CA) was used for protein expression.

Plasmid construction and DNA methods

Plasmids used in this study are listed in Table II and others are described below. *YCp111/CDC3*, *YCp111/CDC10*, *YCp111/CDC11* and *YCp111/CDC12* were made by cloning *Sal*I/*Eco*RI, *Sac*I/*Kpn*I, *Sal*I/*Pst*I and *Pst*I/*Xba*I fragments respectively, from the

pALTER vectors containing appropriate septins (provided by Mark Longtine). Mutations in the septin GTP binding and hydrolysis domains were made by PCR with primers listed in Table III and pALTER vector or YIp211 vector containing the appropriate septin as template. PCR products were used with *Xho*I-digested *YCP111/CDC3*, *Bam*HI-digested *YCP111/CDC10*, *Stu*I-digested *YCp111/CDC11* and *Mlu*I digested *YCP111/CDC12* to obtain YCp111 with septin nucleotide binding mutants by *in vivo* recombination in yeast (Petracek and Longtine, 2002). For integration into the yeast genome, an ~3.9kb *Sal*I/*Pst*I fragment containing wild-type or mutant *CDC11*alleles in YCp111 was cloned into YIp211 digested with *Sal*I/*Pst*I.. The resulting plasmids were then linearized with *Apa*I to target integration to the ura3-52 locus and transformed into yeast. For septin localization experiments, *YCp111/CDC3-GFP*^{S65T} was obtained by cloning an ~4.8kb *Sal*I/*EcoR*I fragment from *pRS316/CDC3-GFP*^{S65T} (Caviston et al., 2003) into YCp111 digested with *Sal*I and *EcoR*I. For proteins expressed in RRL/TnT system, full-length genes of *CDC3* and *CDC12* from pGEX4T derivatives, *CDC10* from pALTER and *CDC11* from pSL301 were cloned into *Bgl*II/*Sma*I or *Xba*I/*Sac*I digested pSP64T(S)XBSSE (Krieg and Melton, 1984). *TRP1*, *ARS1* genes were cloned into pSP64T(S)XBSSE to get pSP64TT1R1 by cloning the PCR product of ML639 and ML640 with YRp7 as template. Cdc11p nucleotide binding mutants were cloned into pSP64TT1R1 by *in vivo* recombination after amplifying the P-loop mutant region with primers listed in Table III and YCp111 derivatives as PCR template. To generate N-terminal 6XHis tags for affinity purification during protein-protein interaction assays, six histidine residues were added by cloning the PCR product of primers ML641 and ML 642 into pSP64TT1R1. Full-length, N-terminal or C-terminal regions of wild-type and

mutant septins were amplified by PCR and cloned into *Xho*I digested pSP64TT1R16XHis. To obtain a C-terminally 6XHis tagged *CDC10*, PCR amplified full-length *CDC10* was cloned into *Nde*I digested pSP64TT1R16XHis. To construct 2-hybrid plasmids, PCR products of ML856 and ML857 using pSP64T/cdc11p P-loop mutants as templates or PCR products of ML 582 and ML397 or ML582 and ML747 (in case of alanine scanning mutants) or ML397 and ML622 using YCp111 or YIp211 derivatives as templates were cloned by *in vivo* recombination into *Sma*I digested *pGBDU-C1/CDC11*, *Not*I/*Bsp*EI digested *pEG202/CDC11* and *Bsp*EI digested *pJG4-5/CDC11* respectively. PCR amplifications were done using Expand PCR kit (Roche, Indianapolis, IN) or Taq Polymerase (Promega, Madison, WI). Restriction enzymes were from Promega (Madison, WI), Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA). Nucleotide sequencing for verification was done by the core facility of Biochemistry department at Oklahoma State University.

Antibodies and western analyses

Anti-GFP was purchased from Covance (Denver, PA). Rabbit anti-Cdc11p against full-length Cdc11p, anti-Cdc10p against an N-terminal peptide, anti-Cdc28p against the C-terminus were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-Pma1p against an internal region and mouse anti-LexA against the DNA binding domain were also from Santa Cruz Biotechnology, Inc. Anti-Cdc3p and anti-Cdc12p were raised in rabbits, against synthetic peptides corresponding to the C-termini of Cdc3p (CNHSPVPTKKKGFLR) and Cdc12p (CEQVKSLQVKKSHLK), by Sigma-Genosys (St. Louis, MO) and purified over a column containing the peptide used as

antigen. Horseradishperoxidase (HRP) conjugated donkey anti-rabbit and sheep anti-mouse antibodies were from Amersham Biosciences (Arlington Heights, IL) and rabbit anti-goat HRP antibodies were Jackson ImmunoResearch Laboratories (West Grove, PA). Western analyses were done as described (Ausubel et al., 1995) using the Enhanced Chemiluminescence system (Amersham). Immunofluoresence experiments used Alexa 546-conjugated goat anti-rabbit IgG obtained from Molecular Probes (Eugene, OR).

Protein-protein interactions

^{35}S-labelled proteins were synthesized by rabbit reticulocyte lysate coupled *in vitro* transcription and translation (RRL/TnT) system, as described previously (Craig et al., 1992). RRL/TnT-produced proteins were allowed to interact 23˚C for one hour with gentle mixing. Proteins were isolated by immunoprecipitation using anti-Cdc11p or anti-Cdc12p antibodies and protein G beads (Pierce Biotechnology Inc., Rockford, IL) or, for 6XHis-tagged proteins, by affinity purification using Talon metal affinity resin (BD Biosciences, Palo Alto, CA). 5 µl of the remaining supernatant was collected as the unbound (UB) fraction, and the rest of the supernatant was discarded. The beads were washed four times, with 500 µl of IP150 (20mM PIPES pH 7.5, 150mM sodium chloride and 0.5% Tween 20), and the beads saved as the bound (B) fraction. The beads were resuspended in SDS-PAGE buffer, and the UB fraction was added to SDS-PAGE buffer followed by incubation at 95˚C for 5 min and 5 min of microcentrifugation at high speed. The resulting bound and unbound fractions were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and proteins detected by autoradiography. For co-immunoprecipitation (Co-IP) experiments, total protein was extracted using 50 O.D cells

as described earlier (McMillan et al., 1999), except the cells were lysed in buffer containing 25mM HEPES (pH 7.4), 150mM sodium chloride, 5mM magnesium chloride, 10mM β-mercaptoethanol, 1X protease inhibitors (Roche, Indianapolis, IN) and 0.1% Triton X100. For each Co-IP, 500 μg of total protein was mixed with 2 μg of anti-Cdc11p,anti-Cdc12p or anti-GFP antibodies for 1 hr followed and transferred to a tube containing 30 μl bed-volume Protein G beads. After gentle mixing at 23˚C or 4˚C, as indicated, the supernatant was collected as unbound fraction by spinning at 2500g on a microcentrifuge for one minute and the beads were collected as bound fraction after three 5 min washes with 1 ml each wash of binding buffer. The bound fraction and 20% of the unbound fractions were mixed with SDS-PAGE buffer, heated for 5 minutes and centrifuged for 5 min followed by separation on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Proteins were detected by western blot analyses using the appropriate antibodies.

Yeast two-hybrid assay

Two-hybrid interactions were done as described (De Virgilio et al., 1996), with each combination being assayed in 3-6 independent cultures, using the system developed by Brent and coworkers (Ausubel et al., 1995; Gyuris et al., 1993). Strain YML 140 (Table I) was transformed with pEG202 and derivatives which express the GAL4 DNA-binding domain (DBD) or the indicated DBD-fusion protein followed by selection on SDC-HIS medium. pJG4-5 and derivatives, which express the GAL4 transcriptional activation domain (AD) or the indicated AD-fusion protein, were transformed into strain YML 141 and transformants selected on SDC-TRP medium. Transformed YML 140 and

YML141 strains were mated on YPD plates and selected on minimal media+leucine to generate diploid strains carrying both a DBD- and an AD-plasmid. The diploid strains were inoculated in 3 ml minimal media containing leucine, 1% raffinose and 2% galactose and grown for 12 hr at 23˚C. Quantitative assays to determine β-galactosidase activity were done for 3-6 cultures for each plasmid combination, as described previously (De Virgilio et al., 1996).

Microscopy

Microscopy was done using Nikon Eclipse E800 (Tokyo, Japan). All assays were done in duplicates or triplicate, as indicated, and ≥200 cells were counted for each sample. Cell morphology, cell clumping, cytokinesis, and shmoo formation were observed by differential interference contrast (DIC) microscopy using cells that were fixed in formaldehyde for 1.5 hours at 23˚C and sonicated at setting 3 for 5 seconds in Sonic Dismembrator (Fisher scientific, Pittsburgh, PA). For cell clumping assay, more than 3 cells together after sonication were classified as a clump. Cells were prepared for immunofluorescence as described previously (Pringle et al., 1991; Pringle et al., 1989) with Hoechst in the mounting medium to visualize DNA and anti-Cdc11p as a 1:150 dilution as primary antibody and Alexa 546-conjugated anti-rabbit as a 1:2000 dilution as secondary antibody followed by observation under the FITC channel to visualize the Alexa 546 fluorophore and under the FITC channel to visualize GFP-tagged proteins. Budding patterns and chitin deposition were assayed by staining the cells with Calcofluor White (Sigma, St. Louis, MO), as described previously (Pringle, 1991). Haploid strains with ≥3 bud scars were classified as axial, if adjacent to each other as a chain, or non-

axial if one or more of the bud-scars were distinctly not contiguous with another of the bud scars. Chitin deposition in vegetative cells was classified as normal if chitin was present and clearly restricted to the mother-side of the neck. Other patterns of chitin localization were classified as abnormal, and sub-divided into predominantly on the daughter-side of the neck, on both sides of the neck, or diffusely localized categories. Diploid strains with 1, 2, 3 or 4 bud scars were counted to assay for bipolar budding pattern wherein cells with 1 bud scar was categorized as proximal, distal, or equatorial, and cells with 2, 3, or 4 bud scars were categorized as all proximal, all distal, all equatorial, proximal and distal or pole (either) and equatorial.

Table I. (A) **Strains used in this work**

Strain	Relevant genotype	Source
YML-95	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63*	Bi and Pringle, 1996
YML-97	*a/α ura3-52/" lys2-8011/" leu2-Δ1/" his3-Δ200/" trp1-Δ63/"*	Bi and Pringle, 1996
YML-136	*α leu2-3,-112 ura3-52 his3-Δ200 trp1-901 gal4Δ gal80Δ LYS2:GAL1:HIS3 GAL2:ADE2 met2:GAL7-lacZ*	James et al., 1996
YML-137	*a leu2-3,-112 ura3-52 his3-Δ200 trp1-901 gal4Δ gal80Δ LYS2:GAL1:HIS3 GAL2:ADE2 met2:GAL7-lacZ*	James et al., 1996
YML-140	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34*	Gyuris et al., 1993
YML-141	*α his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34*	Gyuris et al., 1993
YNT-81	*α cdc3ΔPCR-5::TRP1* with *YCp111/CDC3*	This Study
YNT-83	*α cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{K132E}*	This Study
YNT-85	*α cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{K132T}*	This Study
YNT-87	*α cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{T133N}*	This Study
YNT-89	*a cdc10Δ::HIS3* with *YCp111*	This Study
YNT-91	*a cdc10Δ::HIS3* with *YCp111/CDC10*	This Study
YNT-93	*a cdc10Δ::HIS3* with *YCp111/cdc10^{K45T}*	This Study
YNT-95	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111*	This Study
YNT-97	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/CDC11*	This Study
YNT-107	*a cdc12Δ::TRP1* with *YCp111/CDC12WT*	This Study
YNT-109	*a cdc12Δ::TRP1* with *YCp111/cdc12^{K47T}*	This Study
YNT-129	*a cdc10Δ::HIS3* with *YCp111/cdc10^{G42V,K45E,S46N}*	This Study
YNT-131	*a cdc12Δ::TRP1* with *YCp111/cdc12^{G44V,K47E,T48N}*	This Study
YNT-195	*a cdc11Δ::TRP1* with *YCp111/cdc11^{G32V,R35E,S36N}*	This Study
YNT-217	*α cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{G129V}*	This Study
YNT-225	*a cdc10Δ::HIS3* with *YCp111/cdc10^{G42V}*	This Study
YNT-233	*a cdc12Δ::TRP1* with *YCp111/cdc12^{G44V}*	This Study
M-493	*a cla4Δ-1::HIS3*	This Study
M-534	*a sep7Δ::HIS3*	This Study
M-1564	*a bni5Δ::HIS3-5*	This Study
M-2098	*a bni5Δ::HIS3 cdc11-6*	This Study
M-2297	*a cdc11-6*	This Study
M-2301	*a/α cdc11^{R35E}/cdc11^{R35E}*	This Study
M-2307	*a/α cdc11Δ::TRP1/" ura3-52:YIp211:cdc11^{S36N}/" lys2-8011/" leu2-Δ1/" his3-Δ200/"*	This Study
M-2313	*a/α cdc11Δ::TRP1/" ura3-52:YIp211:cdc11^{R35T}/" lys2-8011/" leu2-Δ1/" his3-Δ200/"*	This Study
M-2323	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{G32V} lys2-801 leu2-Δ his3-Δ200*	This Study
M-2325	*a/α cdc11^{G32V}/cdc11^{G32V}*	This Study
M-2332	*a cdc11Δ::TRP1 ura3-52:YIp211:CDC11WT lys2-801 leu2-Δ his3-Δ200*	This Study
M-2334	*a/α CDC11/CDC11*	This Study
M-2344	*a/α cdc11-6/cdc11-6*	This Study
M-2365	*a cdc11Δ::TRP1* with *YCp111/cdc11^{G29D}*	This Study
M-2437	*a cdc11Δ::TRP1 ura3-52:YIp211:CDC11* with *YCp111/CDC3-GFP*	This Study
M-2440	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{G32V} lys2-801 leu2-Δ his3-Δ200* with *YCp111/CDC3-GFP*	This Study
M-2443	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35T} lys2-801 leu2-Δ his3-Δ200* with *YCp111/CDC3-GFP*	This Study
M-2446	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35E} lys2-801 leu2-Δ his3-Δ200* with *YCp111/CDC3-GFP*	This Study
M-2449	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{S36N} lys2-801 leu2-Δ his3-Δ200* with *YCp111/CDC3-GFP*	This Study
M-2452	*a cdc11-6* with *YCp111/CDC3-GFP*	This Study
M-2517	*a cdc10Δ::HIS3* with *YCp111/cdc10PloopΔ*	This Study
M-2519	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCp111/cdc11 PloopΔ*	This Study
M-2722	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{G32V} bni5Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2723	*α cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{G32V} cla4Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2724	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{G32V} sep7Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2725	*a cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35T} bni5Δ::HIS3* with *YCp111/CDC3GFP*	This Study

Table I. (B) **Strains used in this work**

Strain	Relevant genotype	Source
M-2726	α *cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35T} cla4Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2727	a *cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35T} sep7Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2772	a *cla4Δ-1::HIS3* with *YCp111/CDC3GFP*	This Study
M-2773	a *sep7Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2774	a *bni5Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2775	a *bni5Δ::HIS3 cdc11-6* with *YCp111/CDC3GFP*	This Study
M-2776	a *cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35E} cla4Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2777	a *cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35E} sep7Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2778	a *cdc11Δ::TRP1 ura3-52:YIp211:cdc11^{R35E} bni5Δ::HIS3* with *YCp111/CDC3GFP*	This Study
M-2840	a *cdc12ΔPCR-2::TRP1* with *YCp111/cdc12^{D98A,G101A}*	This Study
M-2855	a *cdc10Δ::HIS3* with *YCp111/CDC10* and *pRS316/GFP-CDC3*	This Study
M-2856	a *cdc10Δ::HIS3* with YCp111/cdc10^{G42V} and *pRS316/GFP-CDC3*	This Study
M-2857	a *cdc10Δ::HIS3* with YCp111/cdc10^{K45T} and *pRS316/GFP-CDC3*	This Study
M-2858	YNT-97 with *pRS316/GFP-CDC3*	This Study
M-2859	a *cdc11Δ::TRP1* with *YCp111/cdc11^{G29D}* and *pRS316/GFP-CDC3*	This Study
M-2860	a *cdc11Δ::TRP1* with *YCp111/cdc11^{G32V,R35E,S36N}* and *pRS316/GFP-CDC3*	This Study
M-2863	a *cdc12Δ::TRP1* with *YCp111/CDC12WT* and *pRS316/GFP*	This Study
M-2864	a *cdc12Δ::TRP1* with *YCp111/cdc12^{G44V}* and *pRS316/GFP*	This Study
M-2865	a *cdc12Δ::TRP1* with *YCp111/cdc12^{K47T}* and *pRS316/GFP*	This Study
M-2866	a *cdc12Δ::TRP1* with *YCp111/cdc12^{G44V,K47E,T48N}* and *pRS316/GFP*	This Study
M-2867	a *cdc12ΔPCR-2::TRP1* with *YCp111/cdc12^{D98A,G101A}* and *pRS316/GFP-CDC3*	This Study
M-2894	a *cdc10Δ::HIS3* with *YCp111* and *pRS316/GFP-CDC3*	This Study
M-2895	a *cdc10Δ::HIS3* with YCp111/cdc10^{G42V,K45E,S46N} and *pRS316/GFP-CDC3*	This Study
M-2897	YNT-95 with *pRS316/GFP-CDC3*	This Study
M-2908	α *cdc3ΔPCR-5::TRP1* with *YCp111/CDC3* and *pMS76*	This Study
M-2909	α *cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{G129V}* and *pMS76*	This Study
M-2910	α *cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{K132T}* and *pMS76*	This Study
M-2911	α *cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{K132E}* and *pMS76*	This Study
M-2912	α *cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{T133N}* and *pMS76*	This Study
M-2913	α *cdc3ΔPCR-5::TRP1* with *YCp111/cdc3^{G129V,K132E,T133N}* and *pMS76*	This Study
M-2915	a *ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCp111/cdc11^{D89A,G92A}*	This Study
M-2917	a *ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCp111/cdc11^{K172A,D174A}*	This Study
M-2934	M-2915 with *pRS316/GFP-CDC3*	This Study
M-2937	M-2917 with *pRS316/GFP-CDC3*	This Study
M-2962	a *cdc12-6* with *pRS316/GFP-CDC3*	This Study
2H-1	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202*	This Study
2H-25	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/CDC11*	This Study
2H-31	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202//cdc11^{G32V,R35E,S36N}*	This Study
2H-33	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{G32V}*	This Study
2H-41	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{R35T}*	This Study
2H-43	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{R35E}*	This Study
2H-45	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{S36N}*	This Study
2H-49	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{G32E}*	This Study
2H-71	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{D89A,G92A}*	This Study
2H-73	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11^{K172A,D174A}*	This Study
2H-101	a *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5*	This Study
2H-117	a *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC3*	This Study
2H-123	a *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC10*	This Study
2H-125	a *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC11*	This Study
2H-131	a *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC12*	This Study

Table II. **Plasmids used in this study**

Plasmid	Characteristics	Source
YCplac111	Low-copy *LEU2 CEN4/ARS1,*	Gietz and Sugino, 1988
YCplac111/CDC3	Low-copy *LEU2 CEN4/ARS1, CDC3*	This Study
YCplac111/cdc3^{G129V}	Low-copy *LEU2 CEN4/ARS1, cdc3^{G129V}*	This Study
YCplac111/cdc3^{K132E}	Low-copy *LEU2 CEN4/ARS1, cdc3^{K132E}*	This Study
YCplac111/cdc3^{K132T}	Low-copy *LEU2 CEN4/ARS1, cdc3^{K132T}*	This Study
YCplac111/cdc3^{T133N}	Low-copy *LEU2 CEN4/ARS1, cdc3^{T133N}*	This Study
YCplac111/cdc3^{G129V,K132E,T133N}	Low-copy *LEU2 CEN4/ARS1, cdc3^{G129V,K132E,T133N}*	This Study
YCplac111/CDC3-GFP^{S65T}	Low-copy *LEU2 CEN* CDC3-GFP^{S65T}	This Study
YCplac111/CDC10	Low-copy *LEU2 CEN4/ARS1, CDC10*	This Study
YCplac111/cdc10^{G42V}	Low-copy *LEU2 CEN4/ARS1, cdc10^{G42V}*	This Study
YCplac111/cdc10^{K45T}	Low-copy *LEU2 CEN4/ARS1, cdc10^{K45T}*	This Study
YCplac111/cdc10^{G42V,K45E,S46N}	Low-copy *LEU2 CEN4/ARS1, cdc10^{G42V,K45E,S46N}*	This Study
YCplac111/cdc10PloopD	Low-copy *LEU2 CEN4/ARS1, cdc10PloopD*	This Study
YCplac111/CDC11	Low-copy *LEU2 CEN4/ARS1, CDC11*	This Study
YCplac111/cdc11^{G29D}	Low-copy *LEU2 CEN4/ARS1, cdc11^{G29D}*	This Study
YCplac111/cdc11^{G32E}	Low-copy *LEU2 CEN4/ARS1, cdc11^{G32E}*	This Study
YCplac111/cdc11^{G32V}	Low-copy *LEU2 CEN4/ARS1, cdc11^{G32V}*	This Study
YCplac111/cdc11^{R35E}	Low-copy *LEU2 CEN4/ARS1, cdc11^{R35E}*	This Study
YCplac111/cdc11^{R35T}	Low-copy *LEU2 CEN4/ARS1. cdc11^{R35T}*	This Study
YCplac111/cdc11^{S36N}	Low-copy *LEU2 CEN4/ARS1, cdc11^{S36N}*	This Study
YCplac111/cdc11^{G32V,R35E,S36N}	Low-copy *LEU2 CEN4/ARS1, cdc11^{G32V,R35E,S36N}*	This Study
YCplac111/cdc11PloopD	Low-copy *LEU2 CEN4/ARS1, cdc11PloopD*	This Study
YCplac111/cdc11^{D89A,G92A}	Low-copy *LEU2 CEN4/ARS1, cdc11^{D89A,G92A}*	This Study
YCplac111/cdc11^{K172A,D174A}	Low-copy *LEU2 CEN4/ARS1, cdc11^{K172A,D174A}*	This Study
YCplac111/CDC12	Low-copy *LEU2 CEN4/ARS1, CDC12*	This Study
YCplac111/cdc12^{G44V}	Low-copy *LEU2 CEN4/ARS1, cdc12^{G44V}*	This Study
YCplac111/cdc12^{K47T}	Low-copy *LEU2 CEN4/ARS1, cdc12^{K47T}*	This Study
YCplac111/cdc12^{G44V,K47E,T48N}	Low-copy *LEU2 CEN4/ARS1, cdc12^{G44V,K47E,T48N}*	This Study
YCplac111/cdc12^{D98A,G101A}	Low-copy *LEU2 CEN4/ARS1, cdc12^{D98A,G101A}*	This Study
YIplac211	Integrative, *URA3*	Geitz and Sugino. 1988
YIplac211/CDC11	Integrative, *URA3*, *CDC11*	This Study
YIplac211/cdc11^{G32V}	Integrative, *URA3, cdc11^{G32V}*	This Study
YIplac211/cdc11^{R35E}	Integrative, *URA3, cdc11^{R35E}*	This Study
YIplac211/cdc11^{R35T}	Integrative, *URA3, cdc11^{R35T}*	This Study
YIplac211//cdc11^{S36N}	Integrative, *URA3, cdc11^{S36N}*	This Study
pRS316/CDC3-GFP^{S65T}	Low-copy *URA3, CEN,* CDC3-GFP^{S65T}	Caviston *et. al.* 2003
pEG202/	2*μ*, *HIS3*, *lexA* DNA Binding Domain (DBD)	Ausubel et al., 1995
pEG202/CDC11	2*μ*, *HIS3*, *lexA* DBD, *CDC11*	This Study
pEG202/cdc11^{G29D}	2*μ*, *HIS3*, *lexA* DBD, cdc11^{G29D}	This Study
pEG202/cdc11^{G32V}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{G32V}*	This Study
pEG202/cdc11^{G32E}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{G32E}*	This Study
pEG202/cdc11^{R35E}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{R35E}*	This Study
pEG202/cdc11^{R35T}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{R35T}*	This Study
pEG202/cdc11^{S36N}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{S36N}*	This Study
pEG202/cdc11^{G32V,R35E,S36N}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{G32V,R35E,S36N}*	This Study
pEG202//cdc11^{D89A,G92A}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{D89A,G92A}*	This Study
pEG202/cdc11^{K172A,D174A}	2*μ*, *HIS3*, *lexA* DBD, *cdc11^{K172A,D174A}*	This Study
pJG4-5PL	2*μ*, *TRP1*, transcriptional Activation Domain (AD)	DeMarini et al., 1997
pJG4-5PL/CDC3	2*μ*, *TRP1*, transcriptional AD, *CDC3*	De Virgilio et al., 1996
pJG4-5PL/CDC10	2*μ*, *TRP1*, transcriptional AD, *CDC10*	De Virgilio et al., 1996
pJG4-5PL/CDC11	2*μ*, *TRP1*, transcriptional AD, *CDC11*	De Virgilio et al., 1996
pJG4-5PL/CDC12	2*μ*, *TRP1*, transcriptional AD, *CDC12*	De Virgilio et al., 1996
pSP64T(S)XBBSE	Amp^r Vector for SP6 coupled transcription/translation.	Krieg & Melton 1984
pSP64T(S)XBBSE/CDC3	Amp^r Vector for SP6 coupled transcription/translation with *CDC3*	This Study
pSP64T(S)XBBSE/CDC10	Amp^r Vector for SP6 coupled transcription/translation with *CDC10*	This Study
pSP64T(S)XBBSE/CDC11	Amp^r Vector for SP6 coupled transcription/translation with *CDC11*	This Study
pSP64T(S)XBBSE/CDC12	Amp^r Vector for SP6 coupled transcription/translation with *CDC12*	This Study
pSP64T(S)XBBSE/cdc11^{G29D}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{G29D}*	This Study
pSP64T(S)XBBSE/cdc11^{G32E}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{G32E}*	This Study
pSP64T(S)XBBSE/cdc11^{G32V}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{G32V}*	This Study
pSP64T(S)XBBSE/cdc11^{R35E}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{R35E}*	This Study
pSP64T(S)XBBSE/cdc11^{R35}T	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{R35T}*	This Study
pSP64T(S)XBBSE/cdc11^{S36N}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{S36N}*	This Study
pSP64T(S)XBBSE/^{cdc11G32V,R35E,S36N}	Amp^r Vector for SP6 coupled transcription/translation with *cdc11^{G32V,R35E,S36N}*	This Study
pSP64TT1R16HisN/CDC11N$^{1-295}$	*pSP64T, TRP1 ARS1,* 6xHis, *URA3, CDC11N$^{1-295}$*	This Study
pSP64TT1R16His/cdc11G32EN$^{1-295}$	*pSP64T, TRP1 ARS1,* 6xHis, *URA3, cdc11G32EN$^{1-295}$*	This Study
pSP64TT1R16His/cdc11G32VN$^{1-295}$	*pSP64T, TRP1 ARS1,* 6xHis, *URA3, cdc11G32VN$^{1-295}$*	This Study
pSP64TT1R16His/cdc11R35EN$^{1-295}$	*pSP64T, TRP1 ARS1,* 6xHis, *URA3, cdc11R35EN$^{1-295}$*	This Study
pSP64TT1R16His/cdc11R35TN$^{1-295}$	*pSP64T, TRP1 ARS1,* 6xHis, *URA3, cdc11R35TN$^{1-295}$*	This Study
pMS76	Low-copy *HIS3, CEN,* CDC12-GFP	Schmidt et al., 2003
pRS316/CDC3-GFP^{S65T}	Low-copy *URA3, CEN,* CDC3-GFP^{S65T}	Caviston et al., 2003

Table III. **Oligonucleotides used in this study**

Name	Sequence
ML 10[A]	5'-CAGTCCGGATCTGGTACCTCGACTTTTATA-3'
ML 299[D]	5'-TATATATAGAGAAAGAAGAAATAAGTGAGGAAGCCAAAAGGAATTCGAGCTCGTTTAAA-3'
ML 388[E]	5'-TAAAGTTTTCATTAAAGTTGTCTTACCAATAACATCAGGGCCGACACATAGGAG-3'
ML 389[E]	5'-AAATAAAGTTTTCATTAAAGTTGTCTCACCAATACCATCAGGGCCGACACATAG-3'
ML 390[E]	5'-ATTAAATAAAGTTTTCATTAAAGTATTCTTACCAATACCATCAGGGCCGACACA-3'
ML 391[E]	5'-TTCGTAAAAAGGAACCACCTGGGC-3'
ML 392[E, S1]	5'-TCGAAGTTCCTCCTCGTAATC-3'
ML 393[A]	5'-CAAAGTATTTATAAAAGTCGATCTACCAGATACGGACTGGCCCACGATCATCAC-3'
ML 396[S2]	5'-GACACCACGTATTGGCGACCCGATC-3'
ML 397[F, G, S2, S3]	5'-TTCGACCGTCTCCTCTCTCAA-3'
ML 429[A]	5'-TTGCTGACCGCACAAAGTATTTATAAAAGTCGACTCACCAGATCCGGACTGGCCCAC-3'
ML 430[A]	5'-AACTTGCTGACCGCACAAAGTATTTATAAAAGTATTTCTACCAGATCCGGACTGGCC-3'
ML 448[T, S4]	5'-GTATCAGTAATACTTAACTTTTTT-3'
ML 452[Q]	5'-TTGACGCATATAAGAATCG-3'
ML 473[E]	5'-CACCTGGGCGGCTAATTTAGTATT-3'
ML 474[E]	5'-TAAAGTTTTCATTAAAGTATTCTCACCAATAACATCAGGGCCGACACATAGGAG-3'
ML 477[H]	5'-AAAAAATCTAGATTAATGGATCCTCTCAGC-3'
ML 478[H]	5'-AAAAAAGAGCTCTGTTCATTCATTTTACCA-3'
ML 508[D]	5'-CACACATGAGATATTATATGAAAGATATAGAACCGAGGCATGAGGCGCGCCACTTCTAA-3'
ML 555[T]	5'-GGCAAATAACGTATTTATTAGAGTATTCTCACCCAAAACGGATTGGCCAACAACCATT-3'
ML 556[A, S2]	5'-ACCGCACAAAGTATTTATAAAAGTATTCTCACCAGAAACGGACTGGCCCACGATCAT-3'
ML 557[R]	5'-ACCGTGATGTTATGTGGTGAGAGCGTTTTGGGTGAGAATACATTTATTAATACCTTA-3'
ML 560[S4]	5'-AGGCAGGGCAGAAATATCATCACC-3'
ML 566[I]	5'-AGAGATCAATAACGCATTCCT-3'
ML 582[F]	5'-GACTGGCTGGAATTCCCGGGGATCCGTCGACCATGGCGGCCGCTCGAGATGTCCGGAATAATTGAC-3'
ML 586[S2]	5'-GAATGTAGTCGGAAATGATTTCGA-3'
ML 615[T]	5'-TAACGTATTTATTAGAGTACTTTTACCCAAAACGGATTGGCCAACAACCATTAT-3'
ML 616[R]	5'-GTGATGTTATGTGGTGAGAGCGTTTTGGGTAAAACTACATTTATTAATACCTTA-3'
ML 621[I]	5'-TTTTTTTTAGATCTATGTCCGGAATAATT-3'
ML 622[G]	5'-GTGCCAGATTATGCCTCTCCCGAATTCGGCCGACTCGAGATGTCCGGAATAATTGACGC-3'
ML 639[J]	5'-AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGGTCGAAAAAAGAAAAGG-3'
ML 640[J]	5'-TATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTACCCACATGTTAAAATAGT-3'
ML 641[L]	5'-AACGCTCAACTTTGGCAGATCTCGACTCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACCTCGAG-3'
ML 642[L]	5'-ATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCTCACTCGAGGTGATGGTGATGGTG-3'
ML 649[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACATGAGCACTGCTTCAACAC-3'
ML 650[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATCAATCTCTACCCGATGCA-3'
ML 662[M]	5'-GGATCCCCGGGTAATGCATCACCATCACCATCACCTCGAGTTCAATTCAATTCATCATT-3'
ML 663[M]	5'-AGGAAACAGCTATGACCATGATTACGAATTCTCACTCGAGGGGTAATAACTGATATAAT-3'
ML 668[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACATGTCCGGAATAATTGACG-3'
ML 669[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATGCCTCGGTTCTATATCTT-3'
ML 671[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATCATTCTTCCTGTTTGATT-3'
ML 672[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACATGAGTGCTGCCACTGCGA-3'
ML 673[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATCTTAATCTTCTGTATGTT-3'
ML 674[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACCTTGAGGGTCACGAGAACA-3'
ML 675[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATCATTTTAAATGGGATTTT-3'
ML 677[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACATGAGTTTAAAGGAGGAAC-3'
ML 678[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCACTAACGTAAAAATCCCTTC-3'
ML 679[N]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACATGGATCCTCTCAGCTCAG-3'
ML 680[N]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCATCAACGTTGAATGGCGTTG-3'
ML 687[N]	5'-TTTCAGTTCATCTCTTGTCAAGGAATC-3'
ML 691[C]	5'-GATGAAGGTGTCAAGATTCAACTTAATATCATCGCTACTCCGGCATTCGGTGATTCTCTC-3'
ML 695[N]	5'-TTAATCAACCCAACTGGCCACGGTTTA-3'
ML 696[C]	5-TTTATTCAGTTTCAGTTCATCTCTTGTCAAGGAAGCTGATGCGCTGATCACAGGGAT-3'
ML 729[B]	5'-AACTTGCTGACCGCACAAAGTATTTATAAAAGTCACGATCATCACAGTGAAGGT-3'
ML 747[F,N]	5'-AATTGGCAAGTTCCATCTGTC-3'
ML 748[N]	5'-ATAAATACTTTGTGCGGTCAGCAA-3'
ML 749[N]	5'-CAACTGTCTGATGAATTCCACATC-3'
ML 750[N]	5'-CTCGGTTCTATATCTTTCATATAA-3'
ML 836[O]	5'-GAATTTTTGATTCGAACTCATCTC-3'
ML 837[O]	5'-AATAACATAAGATATATAATCGCCACCATTCTTATGAGATTCAGTGATGGTGATGGTGATGACGTTGAATGGCGTTGCT-3'
ML 846[K]	5'-CGCTCAACTTTGGCAGATCTCGACTCTAGAGGATCCCCGAATGAGCACTGCTTCAACAC-3'
ML 847[K]	5'-CCCACATAGTAACATTGTGTATGTGAT-3'
ML 899[P]	5'-TCTAGAGGATCCCCGGGTAATGCATCACCATCACCATCACGCTCAAGATCGTAATCTTT-3'
ML 900[P]	5'-TCACACAGGAAACAGCTATGACCATGATTACGAATTCTCAACCCATGAAGTAACCACAGTAGT-3'
ML 947[Q, R]	5'-GTGATGTTATGTGGTGAGGTCGGATTGGGTAAAACTACA-3'
ML 949[Q]	5'-AGGGCAGATGGTCAGCAACACCGC-3'
ML 950[Q, R]	5'-TTTGTTGTTATTCACGTTATCTCCAAAAGCTGGGGTCGCGATGACATTAACGCG-3'

A. Primers containing altered nucleotides (double underline) used for mutating residues in nucleotide binding domain of Cdc11p. B. Primer used to delete the P-loop region of Cdc11p. C. Primers containing altered nucleotides (single underline) used for mutating residues by alanine scanning. D. Primers used to delete the coiled-coil domain of Cdc11p. E. Primers used for generating nucleotide binding mutants of Cdc3p F. Primers used to clone Cdc11p mutants into pEG202. G. Primers used to clone Cdc11p mutants into pJG4-5. H. Primers used to clone Cdc10p into pSP64T(S)XBSSE. I. Primers used to clone Cdc11p mutants into pSP64T(S)XBSSE. J. Primers used to clone *Trp1*, *ARS1* into pSP64T(S)XBSSE. K. Primers used to clone Sep7p into pSP64TT1R1. L. Primers used to clone 6xHis tag into pSP64TT1R1. M. Primers used to clone *URA3* gene into pSP64TT1R16xHis. N. Primers used to clone Cdc3p, Cdc10p, Cdc11p, and Cdc12p (wild-type/mutants). O. Primers used to clone 6xHis tag at the C-terminal of pSP64TT1R1/CDC10. P. Primers used to clone C-terminal end of *NFI1* from genomic DNA into pSP64TT1R16xHis. Q. Primers used to clone Cdc12p mutants into pSP64TT1R16xHis/CDC12. R. Primer containing altered nucleotides (double underline) used for mutating residues in Cdc12p. S1. Primer used for sequencing to check Cdc3p nucleotide mutants. S2. Primer used for sequencing to check pEG202/cdc11, pJG4-5/cdc11 & pSP64T/cdc11 nucleotide mutants. S3. Primer used for sequencing to check YIp211/cdc11 nucleotide mutants. S4. Primer used for sequencing to check Cdc10p nucleotide mutants. T. Primers used for generating nucleotide binding mutants of Cdc10p.

Results

Mutations in the septin nucleotide-binding domains result in temperature sensitive viability, defective morphology, and cytokinesis

All *S. cerevisiae* septins, and almost all septins from all organisms, contain three conserved domains involved in nucleotide binding and hydrolysis. The P-loop domain (also called the Walker A or G1 motif) consists of the consensus sequence GxxGxGK(S/T) that envelopes the nucleotide. In the P-loop, the first and third conserved glycines are required to allow the torsional angles necessary for proper folding, the conserved lysine interacts with the beta and gamma phosphates, and the conserved serine/threonine is involved in the coordination of Mg^{++}. Mutation of these conserved residues in a variety of nucleotide binding proteins, including septins, disrupts nucleotide binding [(Beites et al., 1999; Bourne et al., 1991; Kinoshita et al., 1997; Saraste et al., 1990; Versele and Thorner, 2004) and references therein]. The G3 domain (DxxG) is involved in Mg^{++} coordination and disruption of this domain typically does not affect nucleotide-binding affinity but does reduce the rate of nucleotide hydrolysis. The G4 domain (KxD) interacts with the guanine ring and confers specificity for GTP. Mutation of the G4 domain can allow quite high affinity binding to non-native nucleotides (e.g., XTP) but results in a reduction in the binding affinity for GTP (Hoffenberg et al., 1995; Hwang and Miller, 1987; Schmidt et al., 1996; Weijland et al., 1994; Yu et al., 1997; Zhong et al., 1995).

To evaluate the role of Cdc11p nucleotide binding and hydrolysis, we generated seven alleles of *CDC11* (Figure 2). cdc11pG32V is predicted to not allow proper folding

of the P-loop, and thus have dramatic defects in nucleotide binding. Indeed, this mutation disrupts nucleotide binding of a variety of GTP-binding proteins, including mammalian septins [(Beites et al., 1999; Bourne et al., 1991; Kinoshita et al., 1997; Saraste et al., 1990) and references therein]. cdc11pR35T and cdc11pR35E are expected to reduce GTP binding by decreasing an interaction with the β and γ phosphates of GTP. cdc11pR35T, which introduces a neutral amino acid, is expected to display a lower reduction in nucleotide binding than cdc11pR35E, which introduces a negatively charged glutamate. cdc11pS36N is expected to result in reduced binding to Mg^{++} and thus in a defect in nucleotide binding. Finally, we generated a triple mutant allele, *cdc11*^{G32V,R35E,S36N}. It is expected that cdc11pG32V,R35E,S36N is unable to bind nucleotide at significant levels.

These *cdc11* P-loop mutant alleles were integrated into the genome of *cdc11*Δ strains, except for *cdc11*^{G32V,R35E,S36N}, which was introduced into a *cdc11*Δ strain on a low copy number plasmid. *CDC11*, *cdc11*Δ, and *cdc11* P-loop mutant strains were then grown at 23˚C and assayed for viability at 23, 30, 32, 34, and 37˚C (Figure 3A, B). As shown previously (Frazier et al., 1998), *cdc11*Δ cells are inviable at 30˚C and above [(Frazier et al., 1998) (Figure. 3B)] with reduced viability at 23˚C (Figure 3B). At 23˚C, all P-loop mutant strains, including *cdc11*^{G32V,R35E,S36N}, appeared fully functional, displaying growth rates and viabilities indistinguishable from a wild-type strain (Figure 3A, B). At elevated temperatures, most *cdc11* P-loop-mutant strains showed reduced viability, with a correlation between the minimal restrictive temperature and the expected defect in nucleotide binding. Both *cdc11*^{G32V} (Figure 3A) and *cdc11*^{G32V,R35E,S36N} (Figure 3B) strains, predicted to have strong defects in nucleotide binding, were inviable at 30˚C

and above. *cdc11*^{R35E} strains were inviable at 37˚C while *cdc11*^{R35T} strains were viable at all temperatures (Figure 3A), consistent with the prediction that cdc11pR35E would have a larger defect in nucleotide binding than cdc11pR35T. *cdc11*^{S36N} showing reduced viability only at 37˚C (Figure 3A). Identical minimal restrictive temperatures were obtained for P-loop mutant haploid strains of both mating types and for diploid mutants (data not shown), indicating no cell-type specific effects of nucleotide binding on viability. When crossed to a wild-type strain, all *cdc11* P-loop mutations were fully recessive for viability at 37˚C (data not shown).

Previous work identified three recessive ts- alleles of *CDC11*, *cdc11-1*, *cdc11-6*, and *cdc11-7* (Hartwell, 1971). At restrictive temperature, these mutations result in defects in septin localization, cytokinesis and morphogenesis (Adams and Pringle, 1984). Sequencing of these mutant genes indicates that these alleles each contain a single point mutation resulting in an amino-acid substitution in the Cdc11p P-loop (Mark Longtine, personal communication). Both *cdc11-1* and *cdc11-7* (GGC→G<u>A</u>C) result in *cdc11*^{G29D} while *cdc11-6* (GGA→G<u>A</u>A) results in *cdc11*^{G32E}. Indeed, these two alleles confer temperature-sensitive viability in a *cdc11*Δ strain (Figure 3A, B) and, as with the targeted P-loop mutations we generated, they both fully rescued the reduced viability of a *cdc11*Δ strain at 23˚C (Figure 3A, B). Both the *cdc11*Δ strains expressing YCp111/*cdc11*^{G32E} (data not shown) and a strain carrying a chromosomal *cdc11G32E* allele were inviable at 32˚C (Figure 3A) while a *cdc11*Δ strain carrying YCp111/*cdc11*^{G29D} was inviable at 30˚C (Figure 3B). To further investigate the idea that the Ts- inviability of the cdc11p P-loop mutant strains is due to defects in GTP binding, we assayed effects of mutation of the Cdc11p G4 domain on viability. When introduced into a *cdc11*Δ strain on a low-copy

number plasmid, *cdc11*$^{K172A, D174A}$ supported full viability at 23˚C and was inviable at 32˚C. Together, the effects of mutations on the Cdc11p P-loop and G4 domain suggest that GTP binding is not essential for Cdc11p function in viability at 23˚C, but is essential at elevated temperatures.

To investigate the role of GTP binding in other vegetatively expressed septins, mutations were introduced into the P-loop regions of Cdc12p, Cdc3p, and Cdc10p (Figure 2) and the effects of these mutations on viability assayed (Figure 3C, D, and E). These results were similar, but not identical to, the results obtained with Cdc11p. At 23˚C *cdc10*Δ strains show a small (~several fold) reduction in viability and subtle defects in septin localization and cell morphogenesis [(Frazier et al., 1998) and our unpublished observations]. Like a *cdc10*Δ strain, a cdc10pG42V, K45E, S36N strain showed reduced viability at 23˚C and was inviable at 30˚C (Figure 3E). However, western blot analysis indicated that the cdc10p42V, K45E, S36N protein is highly unstable and present in undetectable levels at 23˚C (Figure 4), explaining its lack of function. Both cdc10pG44V and cdc10pK45T strains were fully viable at 23˚C and 30˚C, suggesting these proteins retain significant function. However, in contrast to the analogous mutations in Cdc11p, cdc10pR35T appeared less functional than cdc10pG32V. The reason for the apparent differences in effects of analogous mutations in septin P-loops is not clear, but it is likely this difference reflects quantitative differences in the extent of the GTP binding defect.

Unlike Cdc10p and Cdc11p, both Cdc3p and Cdc12p are required for viability at 23˚C (Frazier et al., 1998). All *cdc3* and *cdc12* P-loop mutant strains, including *cdc3*$^{G129V, K132E, T133N}$ and *cdc12*$^{G44V, K27E, T48N}$, were fully viable at 23˚C, indicating these proteins function efficiently at 23˚C (Figure 3C, D). At 37˚C, the cdc12pK47T strain was fully

viable, the *cdc12*^{G44V} strain displayed reduced viability, and the *cdc12*^{G44V,K47E,S48N} strain was inviable (Figure 3C). All *cdc3* single-mutant P-loop mutant strains were fully viable at 23˚C and 37˚C. In contrast, a *cdc3*$^{G129V,\ K132E,\ T133N}$ strain was fully viable at 23˚C but was inviable at 32˚C. These data suggest that, as for Cdc10p and Cdc11p, GTP binding by Cdc3p and Cdc12p is required for their function in viability at high temperature but not at low temperature.

Next, we investigated the role of GTP hydrolysis in septin function. Mutations were introduced into the G3 domains of Cdc11p and Cdc12p, resulting in cdc11pD89A, G92A and cdc12pD98A, G101A. These mutations are predicted to permit efficient GTP binding but to reduce or eliminate GTP hydrolysis (McGrath et al., 1984; Versele and Thorner, 2004). The cdc11pD89A, G92A strain was fully viable at 23˚C and 37˚C while the cdc12pD98A, G101A strain was fully viable at 23˚C with a modest reduction in viability at 37˚C (Figure 3B, C). We conclude that GTP hydrolysis by Cdc11p or Cdc12p is unlikely to be essential for their function in viability at 23˚ or 37˚C.

Together these data suggest that GTP binding by Cdc11p, Cdc3p, and Cdc12p may not be required for their function in viability at low temperatures and that GTP binding is required for septin function in viability at elevated temperatures. In addition, these data suggest that GTP hydrolysis may have little role in septin function in viability.

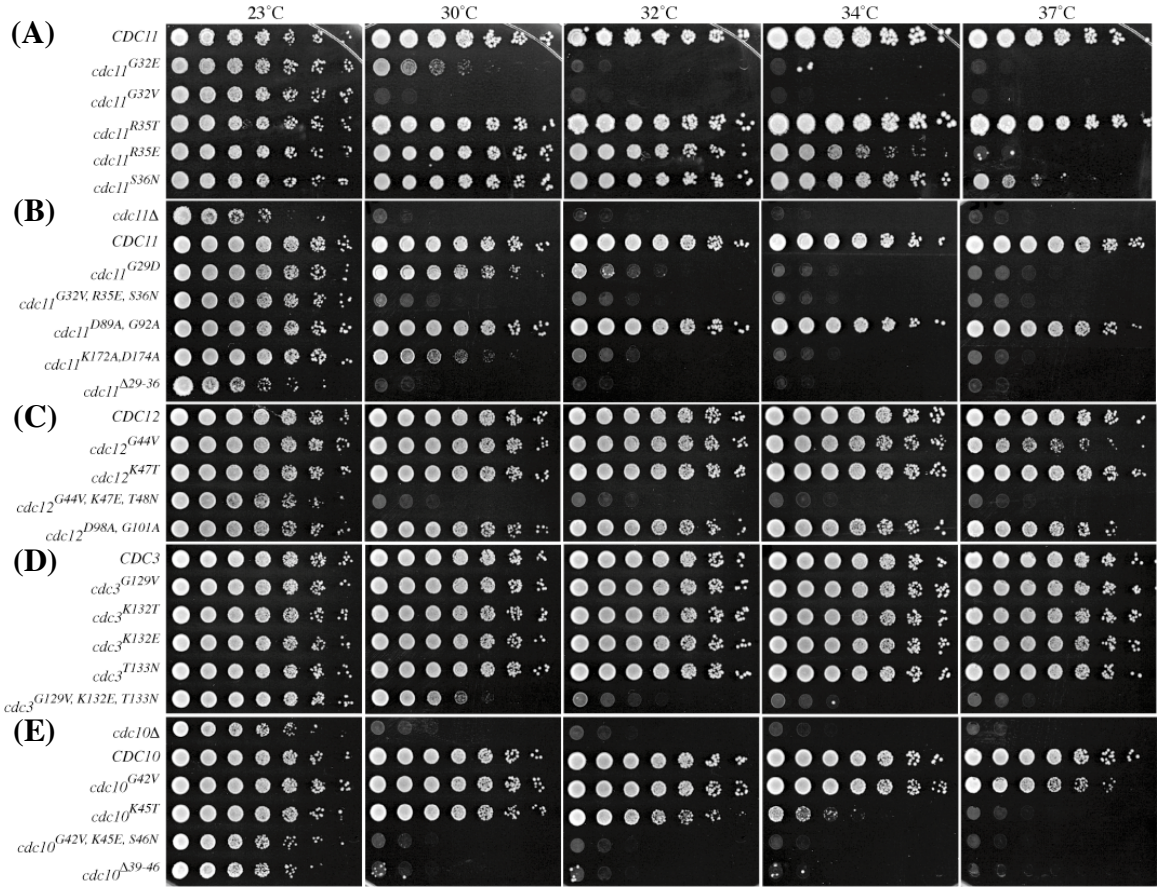

Figure 3. Viability assay of septin P-loop mutants. Septin deletion strains containing the corresponding P-loop mutant were inoculated into YPD media or SD-leu media and grown to saturation at 23˚C. Cells were then plated as 5-fold serial dilutions on appropriate plates and incubated at indicated temperatures for 3 days.

The inviability of septin GTP-binding mutant strains at elevated temperatures could be due to protein instability. To investigate this possibility, we assayed by Western blotting the abundance of wild-type and nucleotide-binding/hydrolysis mutant septins after growth to mid-log phase at 23˚C and after an 8 hr shift to 37˚C. Strains with full viability at 23˚C and 37˚C had wild-type levels of the mutant proteins (Figure 4). With the exception of cdc10pG42V, K45E, S46N, which was absent at both 23˚ and 37˚C, all other septin mutant strains, were present at level similar to that of wild-type septins at both 23˚ and 37˚C. Thus, with the exception of cdc10pG42V, K45E, S46N, the mutations in septin proteins did not destabilize the proteins. Therefore mutant phenotypes are likely to be a result of site-specific mutations rather than absence of the proteins.

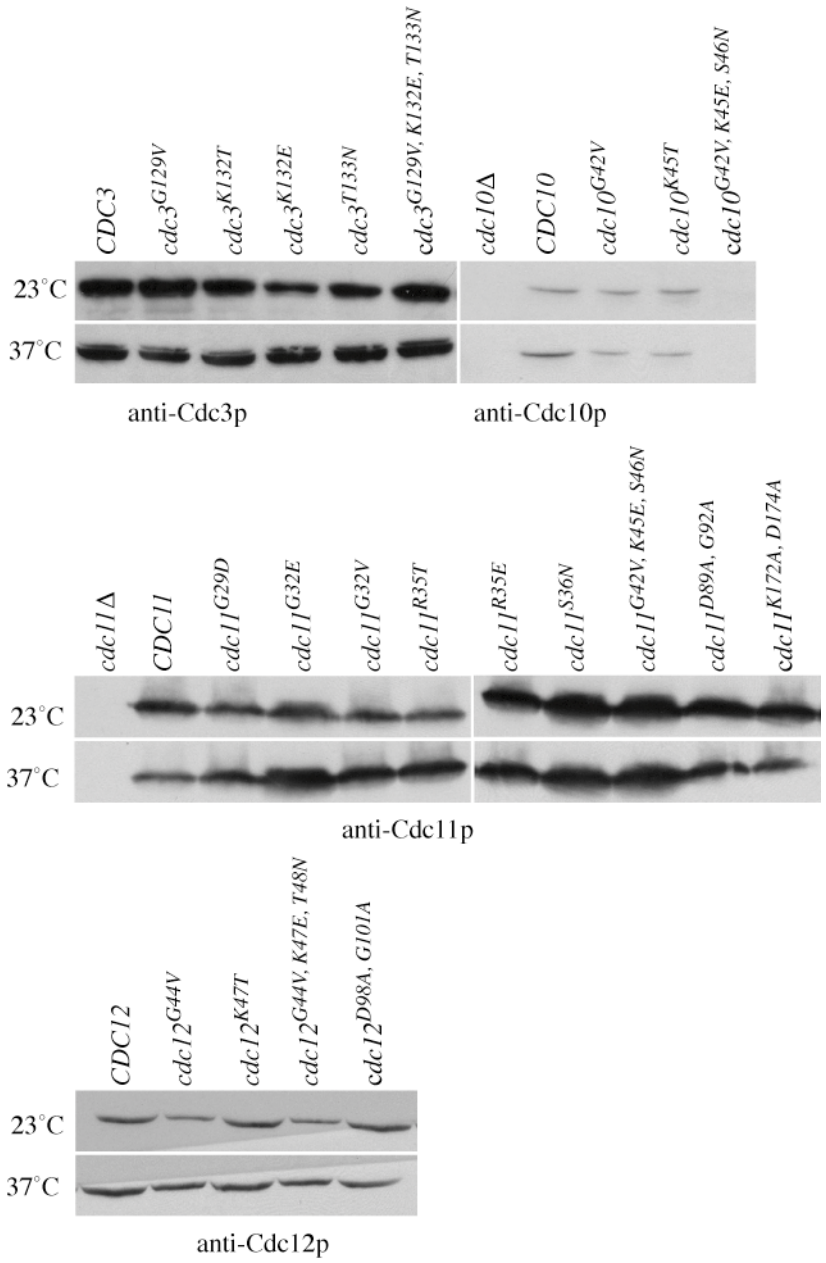

Figure 4. Expression levels of septin P-loop mutant proteins. Total protein extracts were prepared from septin mutant cells grown overnight to mid-log phase (23˚C samples) and then shifted to 37˚C for 8 hours (37˚C samples). 40μg protein was separated by SDS-PAGE, transferred to PVDF membrane, and fusion proteins identified by anti-septin antibodies and ECL.

Mutations in the septin nucleotide-binding domains, at restrictive temperature, result in defective septin localization, cytokinesis/cell separation and cell morphogenesis

In previously identified septin Ts- mutant strains, including *cdc3-1*, *cdc3-6*, *cdc10-1*, *cdc11-1*, *cdc11-6*, *cdc11-7*, *cdc12-5* and *cdc12-6* strains, septins are localized to the mother-bud neck at permissive temperature and become delocalized from the neck after a shift to restrictive temperature. In contrast, at 23˚C, in most, but not all *cdc10*Δ cells, the remaining septins localize to the neck, consistent with the observed slight defect in viability, but become delocalized after a shift to restrictive temperature (Figure 5B). Consistent with their poor viability, at 23˚C the remaining septins localize to the neck in only a small minority (~5%) of *cdc11*Δ cells (Frazier et al., 1998, Figure 5C and unpublished observations), and become delocalized after shift to restrictive temperature. Coincident with loss of neck-localized septins, the cells have defects in cytokinesis, apparently due to defects in neck localization of components involved in the function of the acto-myosin contractile ring and in septum formation (Bi et al., 1998; Lippincott and Li, 1998; Vallen et al., 2000). In addition, disorganized or absent septins result in activation of Swe1p, resulting in elongated cells (Lew, 2003; Longtine et al., 2000; Theesfeld et al., 2003). Together, these data suggest that defects in septin localization result in inviability, likely due to pleiotropic defects in cell growth and division.

Thus, we predicted that septin alleles with reduced viability might display defects in septin localization and that the extent of these defects would be correlated with defects in viability, cell division, and cell morphogenesis. To investigate this possibility, we observed septin localization in wild-type and mutant strains using GFP-tagged septins.

CDC3 strains were transformed with a plasmid expressing Cdc12p-GFP, which is functional at 23˚C and 37˚C, but forms occasional cytoplasmic bars at 37˚C (Martin Schmidt-personal communication and Figure 5A). Wild-type and mutant *CDC10*, *CDC11* and *CDC12* strains were transformed with a plasmid expressing a functional Cdc3p-GFP (Caviston et al., 2003). These strains were grown to mid-log phase at 23˚C and cell morphology and septin localization were observed before and after an 8 hr shift to 37˚C. At both 23˚C and 37˚C, Cdc12p-GFP localized efficiently to the neck in wild-type cells and in *cdc3* single-mutant strains (*cdc3*^{G129V}, *cdc3*^{K12T}, *cdc3*^{K132E} and *cdc3*^{T133N}) (Figure 5A and data not shown). In contrast, Cdc12p-GFP localized to the neck of the triple-mutant *cdc3*$^{G129V, K132E, T133N}$ strain at 23˚C but was delocalized after 8 hr at 37˚C (Figure 5A). As expected, defects in cell morphogenesis and cell division paralleled the defects in septin localization. Like a *cdc10*Δ strain, and consistent with cdc10p*G42V, K45E, S46N* being unstable, a *cdc10*$^{G42V, K45E, S46N}$ showed detectable defects in septin localization at 23˚C (Figure 5B, arrowheads) and the absence of localized septins at 37˚C (Figure 5B), with corresponding defects in cell division and morphogenesis. In the Ts-*cdc10*^{G42V} and *cdc10*^{K45T} strains, septins localize normally at 23˚C but display delocalized septins and defects in cell division and morphogenesis at 37˚C (Figure 5B). Together, these data suggest defects in GTP binding result in defects in localization of Cdc3p and Cdc10p and that the inviability results from the temperature-sensitive localization defects

Examination of *cdc11* and *cdc12* GTP-binding mutant strains also indicated a strong positive correlation between defects in septin localization and defects in viability, cell division and cell morphogenesis. As observed previously (Frazier et al., 1998), septins localize to less than 5% of the necks of *cdc11*Δ cells at 23˚C, consistent with their

greatly reduced viability, and septins are entirely absent from the neck after shift to 37˚C (Frazier et al., 1998 and Figure 5C). At 23˚C, all *cdc11* and *cdc12* strains with mutations in GTP binding motifs showed normal Cdc3p localization, cell division, and cell morphogenesis (Figure 5C and D and data not shown), in agreement with their full viability. Likewise, at 37˚C the fully viable *cdc11*^{R35T} and *cdc12*^{K47T} strains were wild-type for septin localization, cell division, and cell morphogenesis (Figure 5C and D). *cdc11*^{S36N}, which shows reduced viability at 37˚C, showed intermediate defects in septin localization, with Cdc3p being localized to the neck in ~50% of these cells. Furthermore, the localized Cdc3p, in most cases, was abnormal, often localizing as an incomplete collar or apparent patch (arrows, Figure 5C). Finally, after 8 hr at 37˚C the Ts- *cdc11* (*cdc11*^{G29D}, *cdc11*^{G32E}, *cdc11*^{G32V}, *cdc11*$^{G32V, R35E, S36N}$, and *cdc11*$^{K172A, D174A}$) and *cdc12* (*cdc12*^{G44V} and *cdc12*$^{G44V, K47E, T48N}$) strains showed strong defects in septin localization, cell division, and cell morphogenesis (Figure 5C and D). Together, these data suggest that defects in GTP binding of Cdc3p, Cdc10p, Cdc11p or Cdc12p result in temperature-sensitive defects in septin localization which then leads to defects in septin function in cell-cycle progression, cell division and, ultimately, viability.

Defects in nucleotide hydrolysis does not result in septin localization defects

Next, we asked if GTP hydrolysis was required for septin localization or function by assaying the effects of mutations in the G3 domains of Cdc11p and Cdc12p. At 23˚C, Cdc3p localization was normal in both *cdc11*$^{D89A, G92A}$ and *cdc12* $^{D989A, G101}$ cells (Figure 5C and D). At 37˚C septin localization was very mildly perturbed in 20% of the cells and wild-type in 80% of the cells, consistent with their full viability and proper cell division

and morphogenesis. Thus, it appears that GTP hydrolysis by Cdc11p or Cdc12 is not critical for their function in septin localization, cell-cycle progression, or cell division.

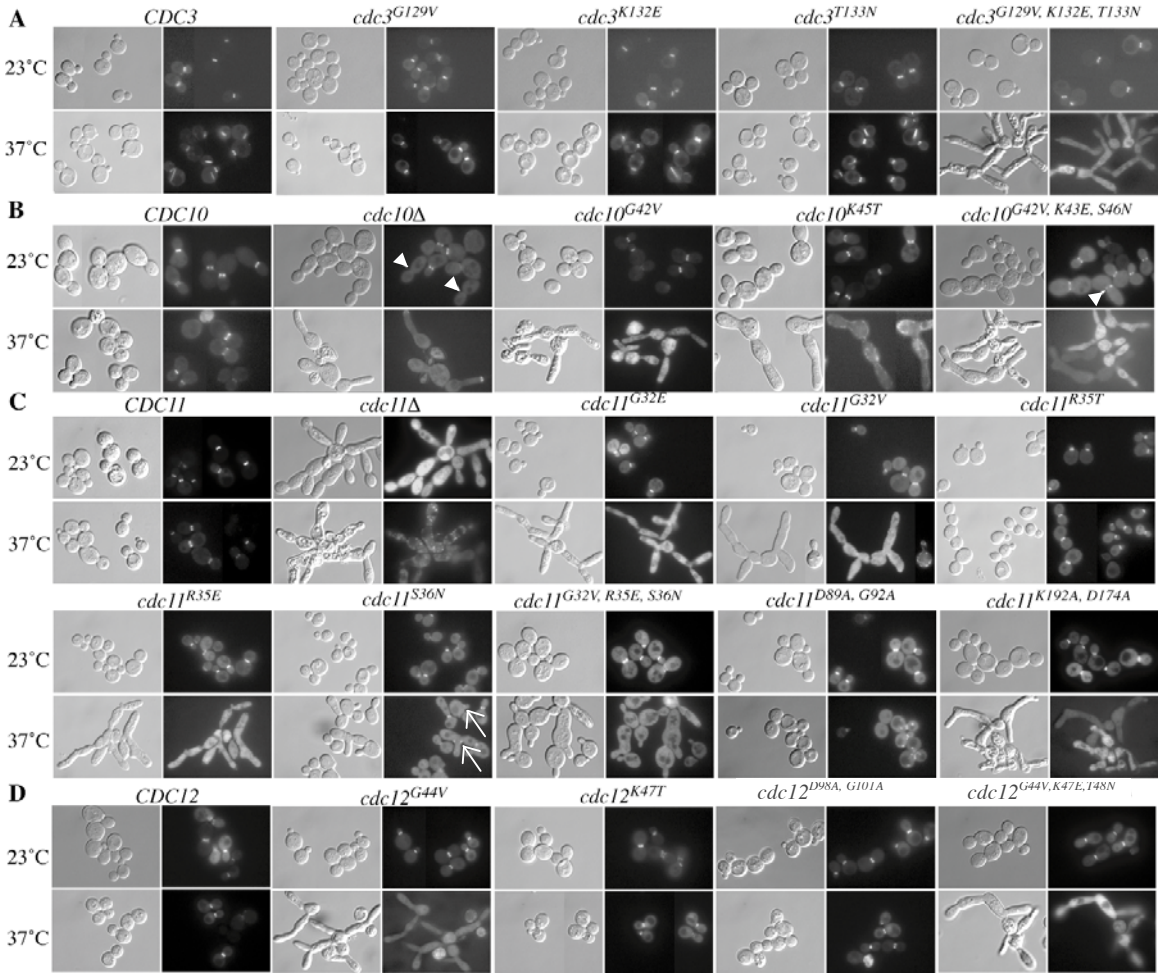

Figure 5. Cell morphology and septin localization of septin P-loop mutants. Septin deletion strains containing P-loop mutants and GFP-tagged Cdc3p or GFP-tagged Cdc12p were inoculated into SD-leu-ura media or SD-leu-his media and grown to mid log phase at 23˚C. Cells were then shifted to 37˚C for 8 hours. Cells at 0 hour and 8 hour at 37˚C were visualized under DIC and FITC channels.

GTP-binding of septins is required for septin ring assembly but not maintenance

Septin localization in yeast occurs in several stages. Based on the frequent localization of a septin cap at the tip of growing buds in strains with defects in septin localization and by the presence of a septin cap at the tip of growing buds in wild-type cells of *Candida albicans*, it was suggested recently that septins initially localize as a transient cortical cap followed by formation of the septin ring at the cortex of unbudded cells. Indeed, real-time imaging has detected the cap and its apparent transition into a cortical ring in wild-type cells (Erfei Bi, personal communication). Genetic and biochemical analyses indicate that the initial localization of septins is mediated by interactions of septins with Gic1p and Gic2p, two related effectors of Cdc42p (Iwase *et. al.*, manuscript in preparation), and I have shown this is likely due to a direct interaction of the septins with the Gic proteins (Appendix B). FRAP analysis of caps in mutant cells and of rings in unbudded wild-type cells indicates that septins in these structures are dynamic, undergoing rapid exchange with non-incorporated septins. During bud emergence, the septins form a continuous collar at the mother-bud neck, which remains until the completion of mitosis. FRAP analyses indicates that septins in the collar are stable, only slowly incorporating exogenous subunits. At the completion of mitosis, the septin collar is split in the middle, forming two discrete rings, with cytokinesis occurring between the rings (Dobbelaere and Barral, 2004). Like the septin ring of unbudded cells, septins in these split rings are highly dynamic.

Our data suggest that the primary defect in GTP-binding mutant septins is defects in septin localization. Decreased GTP binding is likely to result in septin localization defects in one of two ways. First, the mutant septin may be unable localize to the neck at

restrictive temperature at all stages of the cell cycle. Alternatively, the mutant septin may have a defect in initiating or completing one of the steps in the septin localization cycle described above, but be fully functional at other stages To test these possibilities, strains carrying P-loop mutant septins were grown to mid-log phase at 23˚C and septin localization determined before and during a 5 hr incubation at 37˚C (Table IV). As expected, septins remain localized to the neck of a wild-type strain during the entire 5 hr at 37˚C and rapidly disappear from the neck of a *cdc12-6* strain (Adams, 1984). Also as expected, septins remained efficiently localized to the neck in septin mutant strains that did not display septin localization defects after 8 hr at 37˚C. However, unlike the *cdc12-6* strain, septins only slowly delocalized from the neck in all septin P-loop mutant alleles that had temperature-sensitive defects in septin localization, suggesting that perhaps only a specific stage in septin localization is disrupted in these mutants.

To check the step at which the mutant septin is defective, I initially arrested the cells at S phase using hydroxyurea, shifted the cells to 37˚C, and monitored septin in the arrested cells (Table IV). Under these condition, septins remained at the neck, suggesting that maintenance of septin localization is not compromised.

To determine if GTP binding is involved in septin localization early in the cell cycle, cells were arrested in G1 with alpha factor, and released from arrest at 23˚ or 37˚C and septin localization was monitored. As expected, when released at 23˚C septins localize properly as a ring at the incipient bud site and as a septin hourglass in budded wild-type cell at 23˚C and 37˚C (Table V) and in all the mutants at 23˚C (data not shown). In wild type strains 80 min after release, most cells had septin localized to the mother-bud neck of small budded cells. Similar to wild type, the *cdc11*^{R35T} mutant did

have neck localized septin in small-budded cells. The *cdc11*^{R35E} mutant strain that do show viability and morphology defects at 37˚C, had septin localized at the neck. However, septin localization is lost in mid-log phase cells after a 5 hour incubation at 37˚C (Table IV). This is consistent with the viability defects of this mutant and also this mutation being less drastic than other P-loop mutants (For e.g., *cdc11*^{G32V}). The other septin P-loop mutants assayed did show an absence of septin localization or mislocalization to the tip in small-budded cells (Table V). The morphology of the small buds lacking neck-localized septins was slightly elongated (data not shown), consistent with the phenotype of cells with delocalized septins (data not shown). Thus, we conclude that defects in GTP binding does not affect the maintenance of septin localization, but that it does result in defects in the initial localization of septins and assembly of the cortical septin ring.

Table IV. **Septin neck localization defects during log-phase and S-phase arrest**

Strain[c]	Relevant genotype		log phase cells with septin at the neck[a] (%)					hydroxyurea arrested cells[b]	
		˚(C)	0 hr	1 hr	2 hr	3 hr	5 hr	0 min	80 min
M-2437	wild type	23	99	99	99	96	96	n.d.	n.d.
		37	n.a.	94	99	99	89	99	100
M-2909	*cdc3*^{G129V}	37	96	89	78	75	64	n.d.	n.d.
M-2913	*cdc3*$^{G129V, K132E, T133N}$	37	85	39	10	1	0	n.d.	n.d.
M-2894	*cdc10*Δ	37	79	12	5	0	0	n.d.	n.d.
M-2856	*cdc10*^{G42V}	37	97	36	20	6	0	n.d.	n.d.
M-2857	c*dc10*^{K45T}	37	98	15	2	0	0	n.d.	n.d.
M-2452	*cdc11*^{G32E}	37	100	50	16	5	6	92	99
M-2440	*cdc11*^{G32V}	37	99	77	29	23	12	91	94
M-2443	*cdc11*^{R35T}	37	99	96	94	95	94	99	100
M-2446	*cdc11*^{R35E}	37	99	95	82	38	29	96	100
M-2449	*cdc11*^{S36N}	37	97	94	60	53	27	n.d.	n.d.
M-2937	*cdc11*$^{K192A, D174A}$	37	92	26	9	0	0	n.d.	n.d.
M-2864	*cdc12*^{G44V}	37	92	73	52	29	10	n.d.	n.d.
M-2865	*cdc12*^{K47T}	37	91	89	87	82	81	n.d.	n.d.
M-2866	*cdc12*$^{G44V, K47T, T48N}$	37	79	18	1	0	0	n.d.	n.d.
M-2867	*cdc12*$^{D98A, G101A}$	37	97	96	86	87	74	n.d.	n.d.
M-2962	*cdc12-6*	37	91	0	0	0	0	n.d.	n.d.

[a]The indicated strains were grown to mid-log phase in selective medium, and the localization of the GFP-tagged septin determined by epifluorescence microscopy at 23˚C (0 hr) or at the times indicated after the shift from 23˚C to 37˚C. [b]Strains were grown to mid-log phase in YMP medium, arrested with hydroxyurea as described in Materials and Methods and septin localization in large-budded cell determined. For all strains, HU treatment resulted in over 80% of cells arresting growth with large-buds. For each sample, over 200 budded cells were assayed. [c]Strains are indicated in Table I.

Table V. **Septin localization after alpha-factor arrest and release**

				unbudded cells			budded cells		
Strain[c]	genotype°	(C)[a]	(min)[b]	band[d]	cortex[e]	absent	neck	tip[f]	absent
M-2437	wild type	37	0	85	12	0	3	0	0
		37	20	62	7	25	6	0	0
		37	40	3	0	15	82	0	0
		37	80	7	0	12	81	0	1
M-2437	*cdc11*^{G32E}	37	0	75	10	n.d.	14	0	1
		37	20	76	10	n.d.	13	0	1
		37	40	45	10	n.d.	22	0	23
		37	80	8	3	n.d.	12	3	74
M-2443	*cdc11*^{R35T}	37	0	85	0	10	5	0	0
		37	20	74	16	7	3	0	0
		37	40	14	22	3	61	0	0
		37	80	4	2	0	94	0	0
M-2443	*cdc11*^{R35E}	37	0	88	0	11	1	0	0
		37	20	72	10	11	7	0	0
		37	40	22	49	8	21	0	0
		37	80	7	0	3	86	2	2
M-2917	*cdc3*^{G129V,K132E,T133N}	37	0	89	0	6	2	0	3
		37	20	58	18	9	6	2	7
		37	40	20	49	2	3	10	16
		37	80	0	1	0	0	19	79
M-2857	*cdc10*^{K45T}	37	0	81	0	3	11	0	5
		37	20	60	6	12	6	0	16
		37	40	12	43	6	3	20	16
		37	80	0	2	1	0	22	75
M-2864	*cdc12*^{G44V}	37	0	79	0	10	2	0	9
		37	20	63	19	7	3	0	8
		37	40	36	10	19	5	8	22
		37	80	0	0	12	0	6	82
M-2866	*cdc12*^{H44V,K47E,T48N}	37	0	94	2	0	3	0	1
		37	20	67	22	2	5	0	4
		37	40	29	18	10	6	15	22
		37	80	0	0	3	0	11	86

[a]The indicated strains were released into SDC-Leu+Glx pre-warmed at restrictive temperature (37˚C), and the localization of the GFP-tagged septin determined by epifluorescence microscopy. [b]The time in minutes, after release, at which samples were collected and septin localization determined. For each sample, over 100 cells were assayed. [c]Strains are indicated in Table I. [d]Septins localized at the base of the shmoo (mating projection). [e]Septins localized at the tip of the cells like a cap or ring with no visible bud (under DIC). [f]Septins localized at the tip of the small-budded cells.

Bni5p, Cla4p, and Shs1p show additive viability defects with cdc11 P-loop mutations

Surprisingly, septin nucleotide-binding mutant alleles all retained apparently full function for septin localization, cell viability, cell morphogenesis, and cell division at 23˚C. One possible explanation for this result is that these mutations do not disrupt nucleotide binding at 23˚C with disruption of nucleotide binding only occurring at elevated temperatures. However, this seems unlikely as analogous mutations within the P-loop of other proteins, including septins, disrupt nucleotide binding at 23˚C. Thus, an alternative possibility is that nucleotide binding is reduced at 23˚C, but that there is no visible phenotype in the single-mutant strains.

To test this second possibility, we assayed for additive phenotypes, at 23˚C, in septin-mutant strains carrying deletions of *CLA4*, *BNI5*, or *SHS1*. *CLA4* encodes a PAK kinase, which is an effector of the Cdc42p small GTPase. Cla4p may directly phosphorylate Cdc10p, and in *cla4* mutant cells, the septins are often improperly localized, either as bars at the neck or at the tip of the growing bud. *SHS1* encodes a septin that localizes to the mother-bud neck, and cells lacking Shs1p show mild septin localization defects at elevated temperatures. Bni5p localizes to the mother-bud neck where it apparently interacts directly with the septins. In our strain background, *bni5*Δ and *shs1*Δ strains are fully viable up to 37˚C while *cla4*Δ strains show slightly reduced viability at 34 and 37˚C (Figure 6). Deletion of *BNI5* exacerbates the growth defects of *cdc10-1*, *cdc11-6*, and *cdc12-6* (but not of *cdc3-6*) strains at 23˚C while overexpression suppresses the temperature-sensitive viability of *cdc10-1*, *cdc11-6* (*cdc11*^{G32E}) and *cdc12-6* strains but not of a *cdc3-6* strain, suggesting a common but not universal ability to suppress septin defects (Lee et al., 2002). Similarly, overexpression of *BNI5* suppressed

the temperature-sensitive inviability of *cdc11*^{G32V}, *cdc11*^{R35E}, and *cdc11*^{S36N} strains (data not shown) and deletion of *BNI5* enhanced the temperature dependent growth defects of *cdc11* P-loop mutant strains (Figure 6). Thus, Bni5p plays a positive role in septin function, which appears independent of the role of Cdc11p nucleotide binding.

Double mutants were obtained by crossing the haploid *cdc11* P-loop mutants with the haploid *bni5*Δ, *cla4*Δ, or *shs1*Δ strains. Following tetrad dissection, all double-mutant segregants carrying *cdc11* P-loop alleles with *bni5*Δ, *cla4*Δ, or *shs1*Δ were recovered at the expected rates (data not shown), indicating no synthetic lethality at 23˚C. Double-mutant strains were plated as five-fold serial dilutions and incubated at a range of different temperatures (Figure 6). Also, the morphology and septin localization in the single- and double-mutant strains were examined to determine if Cdc11p proteins, with alterations in the P-loop, are fully functional at 23˚C (Figure 7). Compared to the single-mutant parent strains, all double-mutant strains, including those expressing cdc11pR35T, showed increased defects in cell morphology and cytokinesis/cell separation. Thus, we conclude that although single-mutants cdc11p P-loop mutant strains do not display a visible defect at 23˚C, all of these proteins are not fully functional at 23˚C. Thus, it is likely that defects in nucleotide binding are present at 23˚C, but that, possibly due to functional redundancy, these biochemical defects do not result in a detectable phenotype.

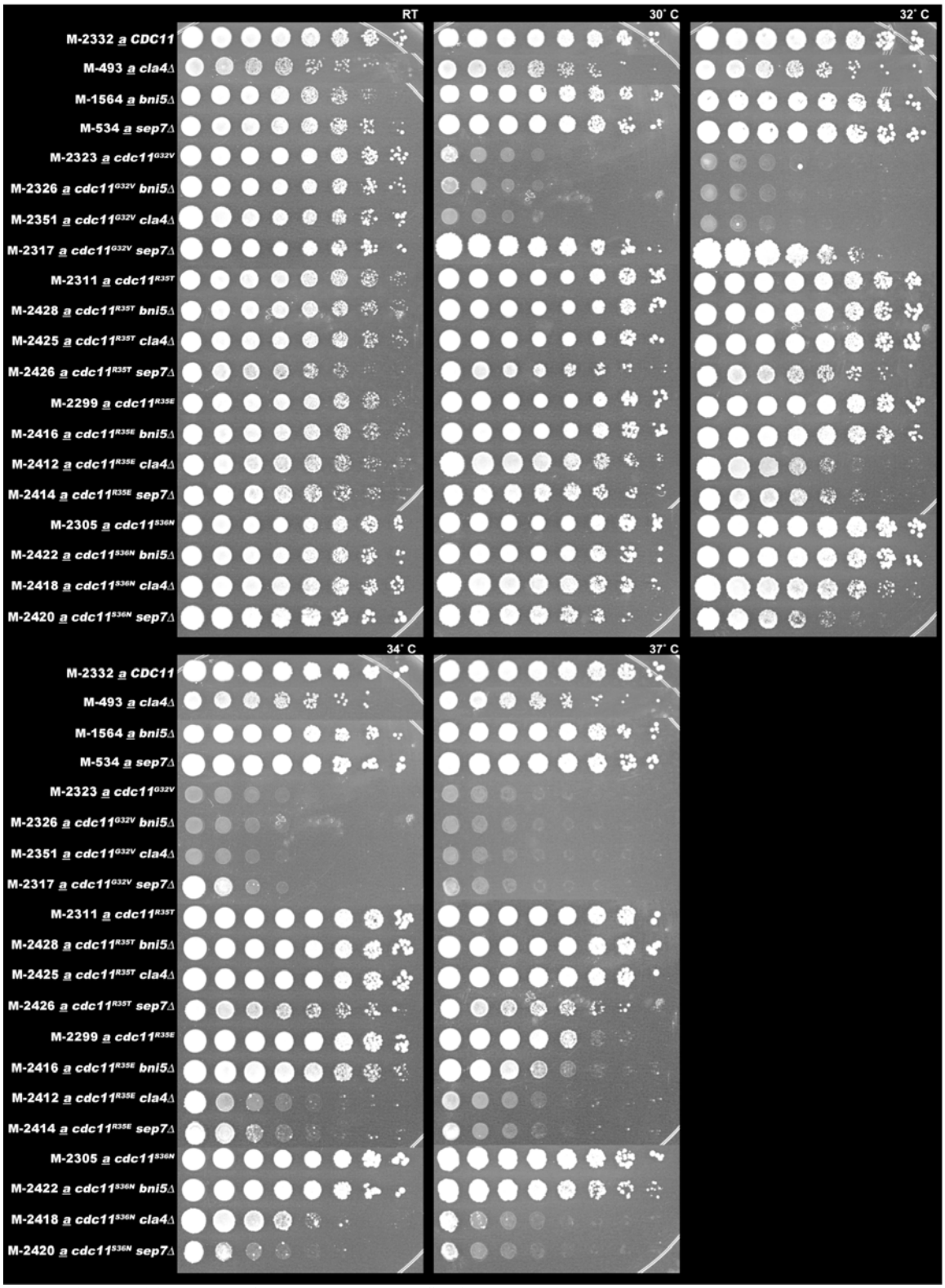

Figure 6. Viability assay of double mutants. Strains containing P-loop mutant genes and deletion of *cla4*, *bni5* or *shs1* were inoculated into SD-leu media and grown to saturation at 23˚C. Cells were then plated as 5-fold serial dilutions SD-leu plates and incubated at indicated temperatures for 3 days.

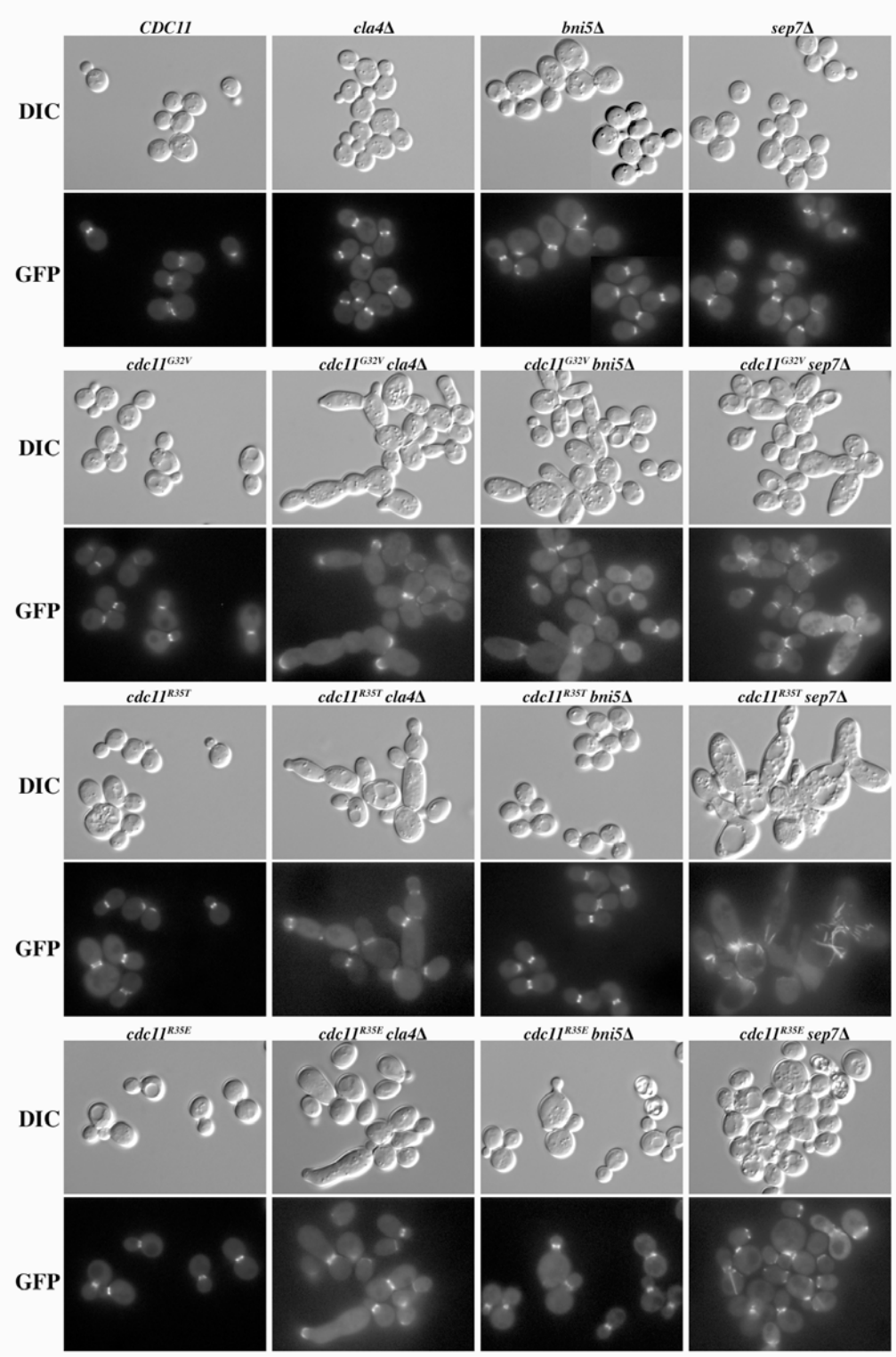

Figure 7. Cell morphology and septin localization of double mutants. Double mutant strains containing P-loop mutant genes and deletion of *cla4*, *bni5* or *shs1* were transformed with *pRS316/GFP-CDC3*. Strains were inoculated into SD-leu-ura media and grown to mid log phase at 23˚C. Cells were visualized under DIC and FITC channels.

GTP binding affects septin-septin interactions

Mutations in the septin P-loop domains, affecting nucleotide binding, confer a temperature-sensitive viability with corresponding defects in morphology and septin localization at restrictive temperatures. To test the filament-formation model, we investigated the role of GTP binding in septin-septin interactions.

First, we carried out 2-hybrid assays to investigate septin-septin interactions. As shown previously (Lee et al., 2002),Wild-type Cdc3p interacts with Cdc11p and Cdc12p in two-hybrid assays (Table VI). DBD-cdc3pG129V,K132E,T133N was unstable and therefore not assayable. cdc3pG129V, cdc3pT133N and cdc3pK132E, which localized and functioned normally, interacted efficiently with Cdc11p and Cdc12p. However, compared to wild-type Cdc3p, these P-loop mutant cdc3p proteins displayed a modest, but consistent, reduction in interaction with Cdc11p and Cdc12p (Table VI), suggesting a defect in septin-septin interactions.

AD-Cdc11p interacts with itself and with Cdc12p while the DBD-Cdc11p fusion shows clear interaction with Cdc3p, Cdc10p, Cdc11p and Cdc12p. To thoroughly investigate the effects of P-loop mutations in cdc11p interactions, both DBD- and AD-fusions were assayed. These data indicate that cdc11pR35T retains septin-septin interactions in the two-hybrid system. However, these interactions are consistently reduced relative to that of Cdc11p, consistent with the detected defect in cdc11pR35T function in double-mutant strains (Figure 7). The cdc11p P-loop mutants (cdc11pR35E and cdc11pS36N) that display intermediate restrictive temperatures and defects in septin localization showed more severe defects in septin-septin interactions. However, it is notable that these mutant proteins lose interactions with Cdc3p, Cdc10p and Cdc11p, but

retain a (reduced) interaction with Cdc12p. This suggests that the strongest, and thus perhaps most direct, interaction of Cdc11p is with Cdc12p. The proteins with the lowest restrictive temperature, cdc11pG32V, cdc11pG32V, R35E, S36N and cdc11pK172A, D174A do not display any septin-septin interactions (Table VI), consistent with these mutations resulting in the lowest restrictive temperature for viability.

Similar results were obtained with cdc12 P-loop mutant proteins. cdc12pG44V shows reduced interactions with Cdc11p and Cdc12p, while cdc12pG44V, K47E,T48N lacks any detectable two-hybrid interactions.

Consistent with its full function, the cdc11p G3 domain mutant (cdc11pD89A, G92A) retains full interaction with Cdc11p and Cdc12p (Table VI).

The lack of interactions observed in the 2-hybrid assay might be real (suggesting a role for GTP binding) or might be false negatives (due to decreased protein levels). To ascertain that the lack of interactions were due to GTP-binding defects, the protein levels of the expressed DBD- and AD- fusions were assayed by western blot analysis (Figure 8 and data not shown). The results showed that mutant proteins showing a lack of interactions in the 2-hybrid assay were expressed at levels similar to both wild-type proteins and mutant proteins that did interact with other septins. These data suggest that the lack of interaction in the 2-hybrid assay is due to decreased ability of GTP binding mutants to interact.

Although the yeast 2-hybrid assay is a good assay for determining if two proteins interact, there is the possibility, especially when assaying yeast proteins, that a third protein that interacts with both the DBD- and AD-fusion proteins serves as a "bridge". Thus, the two-hybrid assay is not a direct test for direct protein-protein interactions. To

assay for direct interactions among septins, we used the rabbit reticulocyte lysate coupled *in vitro* transcription translation system (RRL/TnT) to synthesize septins. These septins were radiolabeled by addition of ^{35}S-Methionine to the RRL/TnT mix. The synthesized septins were allowed to interact, pulled-down using antibodies against one of the septins, and detected by autoradiography (Figure 9).

Cdc11p interacts with Cdc12p but not with Cdc3p or Cdc10p (Figure 9). However, all four septins co-immunoprecipitate as a complex when pulled down using anti-Cdc11p (Figure 9). The cdc11p P-loop mutants, consistent with their temperature-sensitive viability, defective morphology, septin localization and defective in interaction with Cdc12p in the yeast 2-hybrid assay, also did not interact with Cdc12p under these conditions (Figure 10). Finally, the interactions of the N-terminal regions of these mutants with Cdc12p were similar to the *in vitro* interactions of the full-length mutant proteins (Figure 10 and 11). Only Cdc11pN, cdc11pNR35T, and to a lesser extent cdc11pNR35E, interacted with Cdc12p (Figure 11). All the above data consistently suggest that mutation in the nucleotide binding domain disrupts septin-septin interactions that results in subsequent localization defects.

TABLE VI. **Two-hybrid interactions among wild-type and nucleotide-binding/hydrolysis mutant septins**

	β-galactosidase activity (Miller units) with DBD fusion[a]															
DBD fusion plasmid	*pJG4-5*	*CDC3*	*CDC3N*	*CDC3C*	*CDC10*	*CDC11*	*CDC11N*	*CDC11C*	*cdc11G32V*	*cdc11R35T*	*cdc11R35E*	*cdc11S36N*	*cdc11 3nt*	*CDC12*	*CDC12N*	*CDC12C*
pEG202	12	12	3	4	10	5	41	14	15	13	13	8	48	12	10	5
CDC3	19	28	2	21	26	**2803**	**136**	7	10	**931**	8	8	5	**1942**	**618**	8
cdc3G129V	8	11	6	19	16	**361**	85	7	5	**101**	13	5	6	**635**	**235**	15
cdc3K132T	34	23	23	18	43	**869**	**178**	27	19	**366**	41	31	41	**1084**	**311**	43
cdc3T133N	5	18	37	35	13	**663**	**148**	4	14	**215**	32	15	15	**1160**	**205**	23
CDC3N	24	8	2	12	**155**	**227**	50	21	7	44	9	9	38	**249**	**651**	15
CDC3C	14	20	5	4	27	54	82	3	15	52	40	41	6	1853	29	**201**
CDC10	10	**486**	**109**	4	11	**1708**	**366**	29	10	**953**	23	97	10	**333**	41	22
CDC11	19	11	2	3	40	**1892**	60	5	9	**1421**	7	10	9	**1381**	**1843**	6
CDC11N	4	15	5	16	34	**2092**	**2383**	10	24	**1976**	4	14	20	**1440**	**1124**	9
CDC11C	3	14	14	14	7	13	51	28	30	4	1	9	5	15	15	10
cdc11S31V	11	14	n.d.	n.d.	**328**	**2485**	**138**	28	n.d.	n.d.	n.d.	n.d.	n.d.	**3010**	**1970**	13
cdc11G32V	5	2	2	14	10	14	10	14	5	2	2	4	3	16	15	13
cdc11R35T	8	15	10	16	38	**2304**	52	8	9	**836**	10	35	7	**1052**	**717**	7
cdc11R35E	7	15	5	11	7	37	5	10	12	6	8	8	8	**695**	**717**	8
cdc11S36N	4	14	6	11	15	13	3	6	6	7	6	11	10	**1129**	**994**	7
cdc11 3nt	5	21	14	27	9	36	11	6	19	7	9	46	13	16	3	4
cdc11 G3	3	9	n.d.	n.d.	**112**	**2306**	**342**	4	n.d.	n.d.	n.d.	n.d.	n.d	**2021**	**2357**	5
cdc11 G4	10	23	n.d.	n.d.	11	13	8	16	n.d.	n.d.	n.d.	n.d.	n.d	7	11	8
CDC12	7	**157**	5	63	15	**2568**	**954**	11	10	**1820**	**654**	**1622**	10	**2784**	77	7
cdc12G44V	6	**201**	n.d.	n.d.	14	**852**	**311**	5	n.d.	n.d.	n.d.	n.d.	n.d	**666**	n.d.	n.d.
cdc12 3nt	4	14	n.d.	n.d.	7	12	7	10	n.d.	n.d.	n.d.	n.d.	n.d	9	n.d.	n.d.
CDC12N	5	14	15	18	45	**2925**	**527**	44	21	**525**	**128**	78	10	**439**	**196**	11
CDC12C	7	**211**	10	**389**	26	16	40	7	6	5	2	48	8	**1538**	77	**138**

[a]Two-hybrid assays were performed as described in Materials and Methods. At least 4 independent assays each interaction were done using strains grown at 23°C for 16 hr in minimal medium containing leucine ar raffinose and 2% galactose. Shown are the average values in Miller units. Values above 100 and at least 7 higher than the corresponding control (pEG202 with no insert) are deemed positive and are shown in boldf For positive interactions the standard deviations were less than 25% of the average. N and C indicate the N and C-terminal fragments, respectively, of the indicated gene. *cdc11 G3* is *cdc11D89A*, *G92A*, *cdc11 G4* i *cdc11K172A*, *D174A*, *cdc11 3nt* is *cdc11G32V*, *R32E*, *S36N*, *cdc3 nt* is *cdc3G129V*, *K13E*, *T133N* and *cdc 3nt* is *cdc12G44V*, *K47E*, *T48N*. n.d., not done.

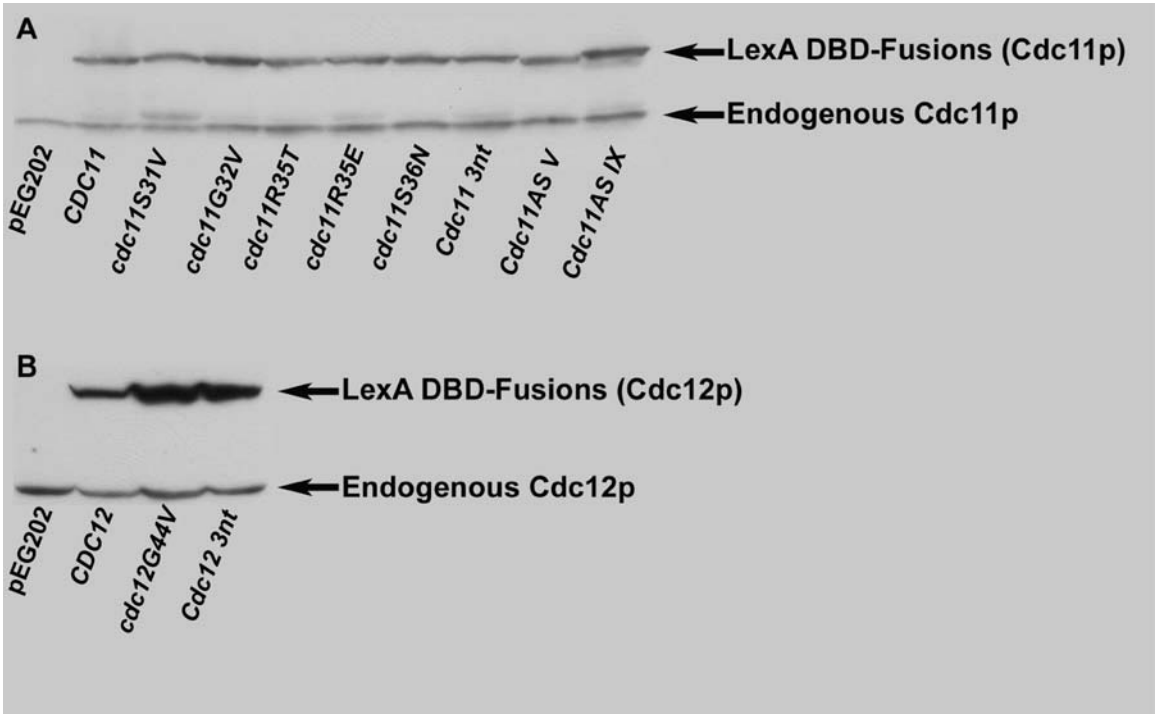

Figure 8. Expression levels of LexA-DBD fusion proteins. Total protein extracts were prepared from strains used for two-hybrid assays (Table 1) expressing LexA-DBD-Cdc11p or LexA-DBD-cdc11p P-loop mutants and LexA-DBD-Cdc12p or LexA-DBD-cdc12p P-loop mutants. 40μg protein was separated by SDS-PAGE, transferred to PVDF membrane, and fusion proteins identified by anti-Cdc11p and anti-Cdc12p antibodies and ECL.

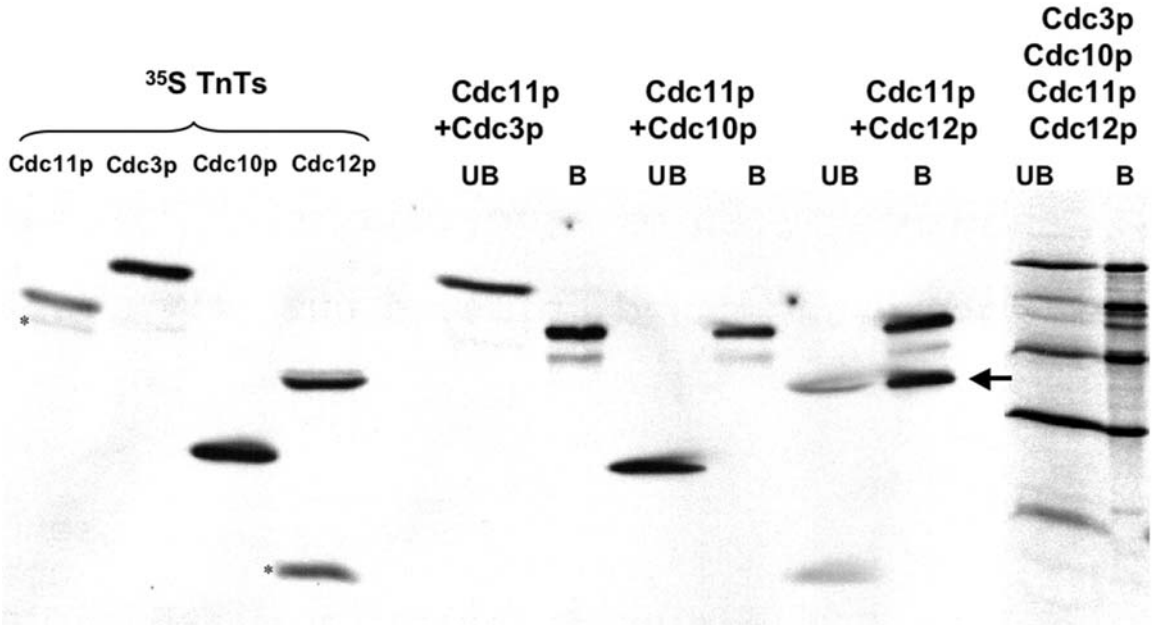

Figure 9. Expression and interaction of Full-length wild-type septins. Cdc3p, Cdc10p, Cdc11p and Cdc12p were synthesized using RRL/TnT and proteins labeled using 35S-methionine. The proteins were allowed to interact, for 1 hour at 23°C, either individually or collectively with Cdc11p and then immunoprecipitated using anti-Cdc11p antibodies. Bound (B) and unbound fractions (UB) were separated by SDS-PAGE, electroblotted to membrane, and detected by autoradiography.

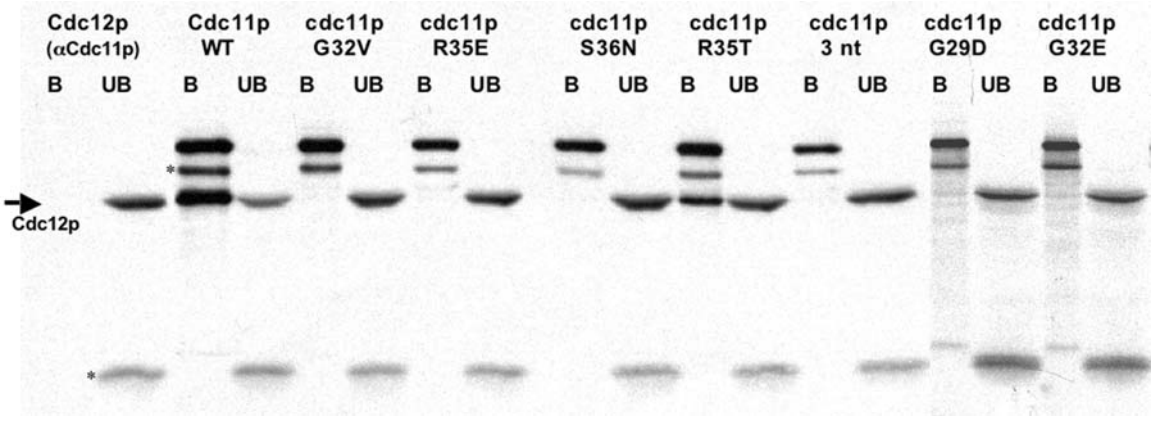

Figure 10. Interaction of Cdc11p and cdc11p P-loop mutants with Cdc12p. Wild-type or P-loop-mutant Cdc11p proteins synthesized in RRL/TnT were assayed as described in Figure 9 for their ability to interact with Cdc12p.

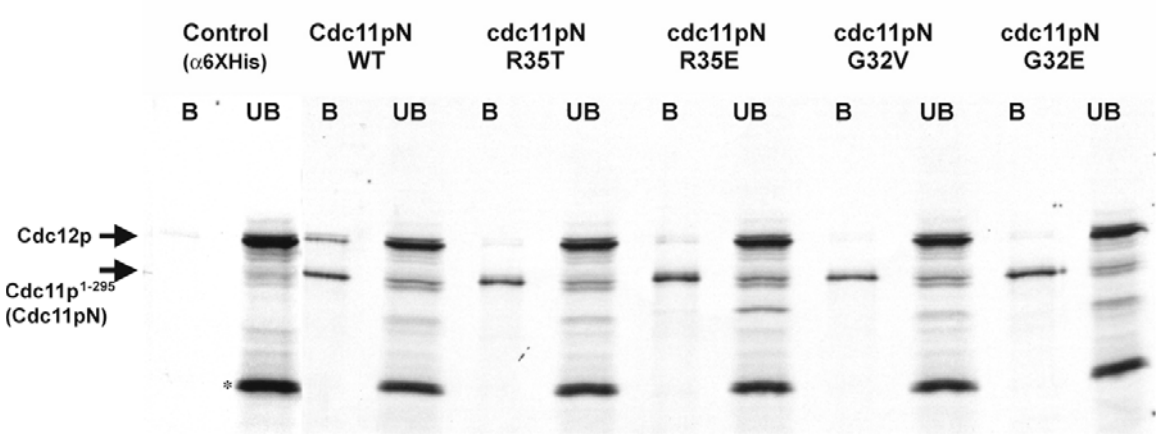

Figure 11. Interaction of the N-terminal of Cdc11p and cdc11p P-loop mutants with Cdc12p. N-terminal regions of Wild-type or P-loop-mutant Cdc11p proteins synthesized in RRL/TnT were assayed as described in Figure 9 for their ability to interact with Cdc12p.

Mutations in the septin P-loop domains do not affect septin-dependent processes

A major role of septins is to serve as a scaffold to direct the proper subcellular localization of septin interacting proteins (Chapter I). In the absence of this septin-directed localization, there are defects in the septin-related process. The data presented above indicate that septins nucleotide binding has a role in septin-septin interactions supporting the "filament formation" model (Figure 1). However, the filament formation model and the "small G-protein" model are not mutually exclusive. That is, it is possible that the status of bound nucleotide may also regulate the interactions of septins with non-septin proteins. Nucleotide-binding mutant cells are fully viable at 23˚C (Figure 3) but appear to display defects in nucleotide binding, as assayed in double-mutant strains. This allowed us to test the small G-protein model for septin function by assaying for defects in septin-dependent processes (and thus of the neck localization of septin-associated proteins) at 23˚C (Table VII).

In yeast, septins are required for the localization of proteins involved in the formation and contraction of the actomyosin ring and in formation of the primary septum. If these proteins are absent or unable to localize to the neck, cells display defects in cytokinesis and/or cell separation. At 23˚C, septin P-loop mutant strains efficiently carry out cytokinesis and cell separation (Table VII), suggesting no defect in localization of proteins involved in cytokinesis or in primary septum formation. Similarly, septins are involved in the neck localization and activation of the Hsl1p kinase, its binding partner Hsl7p, and of the Swe1p protein kinase. In the absence of neck localization of this complex, cells become elongated due to a delay in activation of Cdc28p/Clbp complexes. At 23˚C, septin P-loop mutant strains are not elongated and display normal cell

morphology (Table VII), indicating proper neck localization of the Hsl1p/Hsl7p/Swe1p complex and proper septin-dependent activation of Hsl1p kinase activity (Longtine et al., 2000).

Septins mediate the spatially restricted deposition of chitin in cell wall on the mother side of the neck by directing the asymmetric localization of the chitin synthase III complex. In the absence of septin-mediated localization, chitin is deposited diffusely in the cell while defects in the organization of neck-localized septins can disrupt asymmetric chitin deposition, resulting in symmetric deposition of chitin at the neck (Longtine et al., 1998). To investigate if nucleotide binding by septins has a role in chitin localization, wild-type and septin P-loop mutant strains were grown to mid-log phase at 23˚C and chitin detected by staining with Calcofluor White. Asymmetric chitin deposition at the neck was highly efficient in septin P-loop mutant strains, with over 95% of mutant cells displaying chitin deposition at the mother-side of the neck (Table VII, data not shown). Thus, mutations of the septin P-loop do not disrupt asymmetric chitin deposition at the bud neck, indicating no defect in localization of the chitin synthase III complex.

Yeast chooses the site of budding in a non-random manner, with haploid cells displaying axial bud-site selection and diploid cells displaying bipolar bud site selection. For both the axial and bipolar budding patterns, septins are required for the proper subcellular localization of proteins that direct the site of budding. To determine if septin nucleotide binding plays a role in localization of bud-site selection proteins, haploid and diploid wild-type and septin P-loop mutant cells were stained with Calcofluor White to detect bud scars, which mark sites of previous division, and the efficiency of bud-site selection determined as described in Materials and Methods. At 23˚C, the septin P-loop

mutant strains displayed proper axial and bipolar bud-site selection, indicating no defect in the localization of bud-site selection proteins (Table VII). In agreement with these observations, immunofluorescence experiments indicate that Bud4p properly localizes to the neck of large-budded cells in *cdc11* P-loop mutant strains (data not shown). Thus, mutation of the septin P-loop does not affect axial or bipolar bud-site selection, suggesting disruption of septin nucleotide binding does not disrupt the localization to the neck of proteins involved in bud-site selection.

In response to mating pheromone, yeast form a polarized growth projection (shmoo). Septin proteins localize as a series of diffuse bars at the base of the shmoo, where they may have roles in directing chitin deposition (Ford and Pringle, 1991; Kim et al., 1991; Longtine et al., 1998) and in shmoo morphogenesis. To determine if the septin P-loop mutations affect their roles in response to mating pheromone, wild-type and P-loop-mutant haploid *Mat* a strains were exposed to alpha factor and the efficiency of projection formation, septin localization, and chitin deposition determined. At 23˚C in septin P-loop mutant strains, both Cdc3p and Cdc12p (in case of mutant *cdc3* strains) and chitin localize properly to the base of the shmoo, and shmoos of normal shape are formed as efficiently as in wild-type cells (Table VII). Thus, during shmoo formation, septin nucleotide binding does not appear to have a role in localized chitin deposition, septin localization, or morphogenesis.

A subset of septins, which includes Cdc11p, is expressed during sporulation where they localize to the leading edge of the prospore membrane as it envelops the post-meiotic nuclei and to the entire prospore membrane. Although the role of septins in sporulation is yet unclear, in some strain backgrounds septin mutations result in defects in

sporulation efficiency (Briza et al., 2002; Fares et al., 1996). To determine if *cdc11* P-loop mutants have detectable defects in sporulation, wild-type and mutant cells were inoculated into sporulation medium and the percent of cells containing asci with four or fewer spores determined after 7 days at 23˚C . As shown in Table VII, *cdc11* P-loop mutant strains sporulate as efficiently as isogenic wild-type strains, suggesting no detectable role of nucleotide binding.

In summary, at 23˚C where septin P-loop mutant strains are fully viable, there are no detectable defects in the assayed septin-dependent processes. Thus, we conclude that there is no evidence that septin nucleotide binding or the status of bound nucleotide regulates the neck localization of septin-associated proteins.

Table VII. **Effect of septin P-loop mutations in septin-dependent processes**

Strain[a]	Bud-site seln.		chitin	shmoo		sporulation	cell	cell
	axial[b]	bipolar[c]	dep.[b]	formn[d]	localn[e]	efficiency[f]	sep./cytok[g]	morph.[h]
CDC3[i]	97	n.d.	96	92	93	n.d.	96	97
cdc3^{G129V}[i]	98	n.d.	97	94	91	n.d.	95	97
cdc3^{K132T}[i]	97	n.d.	97	98	94	n.d.	94	96
cdc3^{K132E}[i]	97	n.d.	94	93	89	n.d.	94	98
cdc3^{T133N}[i]	99	n.d.	95	91	91	n.d.	96	98
cdc3^{G129V,K132E,T133N}[i]	95	n.d.	94	90	87	n.d.	91	93
*cdc10*Δ	n.d.	n.d.	n.d.	n.d.	0	n.d.	73	56
CDC10	98	n.d.	91	94	94	n.d.	98	98
cdc10^{G42V}	96	n.d.	89	95	92	n.d.	97	96
cdc10^{K45T}	98	n.d.	93	91	94	n.d.	97	97
cdc10^{G42V,K45E,S46N}	n.d.	n.d.	n.d.	n.d.	0	n.d.	75	51
*cdc10 ploop*Δ	n.d.	n.d.	n.d.	n.d.	0	n.d.	73	59
*cdc11*Δ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5	2
CDC11	94	88	99	88	94	25(29)	99	99
*rsr1*Δ	4	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
*bud9*Δ	4	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cdc11^{G29D}	98	n.d.	95	95	97	n.d.	99	98
cdc11^{G32E}	95	n.d.	98	n.d.	88	27(36)	98	98
cdc11^{G32V}	98	87	99	90	80	25(29)	98	94
cdc11^{R35T}	98	90	99	89	94	25(26)	99	98
cdc11^{R35E}	98	83	98	90	93	21(26)	99	99
cdc11^{S36N}	99	91	99	96	90	24(28)	99	96
cdc11^{G32V,R35E,S36N}	99	n.d.	97	93	98	n.d.	99	95
cdc11AS-V	99	n.d.	98	92	98	n.d.	97	99
cdc11AS-IX	97	n.d.	97	90	**15**	n.d.	91	91
*cdc11 ploop*Δ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CDC12	93	n.d.	94	95	91	n.d.	96	97
cdc12^{G44V}	95	n.d.	95	94	95	n.d.	96	94
cdc12^{K47T}	94	n.d.	95	91	91	n.d.	98	95
cdc12^{G44V,K47E,T48N}	90	n.d.	90	95	**20**	n.d.	91	83
cdc12AS-V	95	n.d.	95	86	92	n.d.	96	92

[a]Strains are indicated in Table IA and B. [b]Haploid *Mat*a cells were grown to mid-log phase at 23°C, stained with Calcofluor White and scored for normal axial budding (cells with 3 or more bud scars, all in a chain) and for normal chitin deposition (chitin localized asymmetrically to the mother side of the neck). [c]Diploid cells were grown to mid-log phase at 23°C, stained with Calcofluor White and scored for normal bipolar budding (cells with 4 bud scars, all at either the proximal or distal poles). [d]For assaying response to mating pheromone, haploid *Mat* a cells were treated with 4 μg/ml alpha factor for 4 hr and the percent of cells forming polarized growth projections (shmoos) determined. [e]The percent of cells displaying normal septin localization at the base of the shmoo was determined using a functional GFP-Cdc3p. [f]To assay sporulation efficiency, cells were grown to saturation and transferred to sporulation medium at 23°C for 7 days followed by determination of the percent of cell bodies containing four spored asci. Parentheses indicate the percent of cell bodies containing 4, 3, or 2 spores. [g]For assaying cell separation and/or cytokinesis, haploid cells were grown to mid-log phase, sonicated for 5 sec and 3 or more cells together were scored as a clump (defective in cell separation). [h]For cell morphology assay, haploid cells treated as done for cell separation and the buds that are had size twice that of mother cell's width was considered elongated. [i]All assays done using α strains (M-380 Bkgd) except for shmoo formation and GFP-CDC12 localization in shmoo cells whereby Mat a strains (M-381 Bkgd) was used. All numbers represent percentages (>200 cells counted per sample).

Discussion

The role of GTP binding and hydrolysis in yeast septins is addressed in this chapter. We predict that GTP binding and/or hydrolysis is important in regulating septin-septin interactions or septins' interactions with septin-associated proteins (Figure 1). The data obtained here is consistent with the role of GTP binding in septin-septin interactions.

Mutations in nucleotide-binding domains (P-loop, G3 and G4) (Figure 2), predicted to disrupt GTP binding and/or hydrolysis, were generated in four of the yeast septins (Figure 2). Consistent with our predictions, defect in GTP binding was demonstrated in these mutants by UV crosslinking assays (Ashok Rajendran, personal communication).

These mutants, when expressed in appropriate septin deletion strains as the sole source of a particular septin with the remaining three being wild type, do not exhibit any detectable phenotype at 23˚C. This suggests, either GTP binding is dispensable for its function at 23˚C or nucleotide binding is not defective at this temperature. The latter seems unlikely, as yeast strains containing P-loop mutated septins and deletion of Cla4p, Bni5p or Sep7p/Shs1p are defective at 23˚C (Figure 6 and 7). A third possibility could be that the other septins (that are wild-type) compensate for the defects of the mutant septin and allow for efficient complex formation and polymerization, or compensate functionally for the defective septin to maintain viability and morphology at 23˚C. Generating double mutant strains with two septin P-loop mutants would give more information on this. However, this also seems unlikely because, genetic analyses suggest that septins have functional differences. Also phylogenetic analyses classify each of

these septins (Cdc3p, Cdc10p, Cdc11p and Cdc12p) into separate classes. Furthermore, the presence of all four of the septins in ~1:1 stoichiometry seems to be a prerequisite for efficient polymerization [Ashok Rajendran, personal communication and (Versele et al., 2004)].

For all mutants of the four septins, GTP binding defects result in temperature-sensitive viability defects (Figure 3). The mutations that have affect GTP binding exhibit a lower viability, while mutations that have little to no defect in GTP binding are similar to wild type in viability (Figure 3). These mutants also have defects in morphology and septin localization that correlate with the temperature-sensitive viability defects (Figure 5). These data suggest a role for nucleotide binding in localization of septins to the mother-bud neck. Furthermore, the localization of the septin complex to the neck is important for its function in viability. Consistent with our observations, the absence of a septin collar at the mother-bud neck was shown by Thorner's lab upon disruption of nucleotide binding in *CDC10* and *CDC12* (Versele and Thorner, 2004). Although unlikely, it cannot be ruled out that the GFP tag of the septins might be causing the localization defects.

From our observations on *CDC11* and *CDC12* and data shown by Thorner' lab using *CDC12*, we conclude that GTP hydrolysis does not have a role in septin localization [(Figure 5) (Versele and Thorner, 2004)]. This is consistent with the binding of nucleotide by septins having a structural role that is similar to that of α-tubulin in microtubule formation (Nogales, 2001). In case of yeast septins, It has been shown that the GTP hydrolysis is limited by a very slow nucleotide turnover (once per cell-cycle) (Vrabioiu et al., 2004), which suggests the absence of a role for GTP hydrolysis in septin

complex formation or localization during the cell-cycle. However, the conserved function of GTP hydrolysis in septin implies a role that is yet to be identified.

Evidences from our lab and Erfei Bi's lab [(Appendix B) (Iwase *et. al.* manuscript in preparation) have shown a role for Gic proteins in the initial localization of septins to the presumptive bud-site. These septins localize as an amorphous cap and undergo a transition to the ring structure followed by the splitting of the ring. So, defects in localization of septin nucleotide-binding mutants may be in one or all the steps. The initial temperature shift assay, whereby cells were monitored at several time points after shift to 37˚C, showed a gradual decrease in cells with localized septins suggesting a defect in one of the steps in localization rather than the mutants being defective in all the steps of septin localization. When *cdc11* p-loop mutant cells were arrested at S phase using hydroxyurea and then shifted to restrictive temperature with continued arrest, the mutant strains retained the septins at the neck suggesting that nucleotide binding is not required for the maintenance of the septin rings at the mother-bud neck (Table IV). The same strains, when arrested at G1 using alpha factor and released from the arrest at restrictive temperature, failed to localize the septins (Table V), suggesting a role for nucleotide binding in the initial assembly of the ring structure.

Consistent with the predicted "filament formation" model (Figure 1), GTP binding appears to have a role in septin-septin interactions. Defects in GTP binding causes defects in septin-septin interactions that result in subsequent defects in localization defects, and thus, defects in septin functions. These interactions were assayed by yeast 2-hybrid assays (Table VI) and *in vitro* co-immunoprecipitation using radiolabeled septins synthesized by RRL/TnT (Figure 10). However, the lack of interactions of mutant

septins in 2-hybrid screens could be due to decreased protein levels or due to absence of the translocation of one of the fusion proteins (wild-type/mutant septin fused to AD/DBD). Though protein levels assayed as in figure 8 (and data not shown) suggest that the lack of interactions is real, it is not possible to eliminate the latter reason for lack of interaction. Monitoring the localization of the fusion proteins by immunofluoresence would possibly be a method to check for the nuclear localization of the fusion proteins.

In addition, septin complexes purified from the P-loop mutant strains by Chris Fields' lab contained sub-stoichiometric amounts of the mutant septin (in the case of the *cdc11* P-loop mutants) (Data not shown), which caused defects in filament formation (Data not shown). The presence of sub-stoichiometric amounts of the mutant septin and defects in filament formation is the likely result of lack of septin-septin interactions. Disruption of filament formation of septin complexes purified from strains containing GTP binding mutations of Cdc10p and Cdc12p have also been demonstrated (Versele and Thorner, 2004). However, in contrast to our data, the authors propose that GTP binding is required for septin filament formation but does not have a role in septin-septin interactions. The authors base their suggestion on data obtained by three different approaches, which are, co-immunoprecipitation of septin complexes from mutant yeast strains, GST pull-down assay using bacterial expressed GST-fusion septins and by co-expression of all septins in *E. coli* at 37˚C. These studies were done only with nucleotide-binding mutants of *CDC10* and *CDC12* septins. In our lab, we have shown that the nucleotide-binding mutant proteins, when co-immunoprecipitated as a complex using antibody against Cdc12p or Cdc11p (from the corresponding mutant strain) do not precipitate along with other wild-type septins at 37˚C. This was shown to be true for four

of the septins, *CDC3*, *CDC10*, *CDC11* and *CDC12* (Ashok Rajendran, personal communication). Also, by utilizing co-immunoprecipitation of radiolabeled septins that were synthesized *in vitro*, we have shown that the mutant proteins do not purify as a complex with other wild-type septins in the case of the *CDC11* P-loop mutants (data not shown). The apparent differences between the data observed by our lab and Thorner's lab may be attributed to two factors. Firstly, the mutations in the P-loop region that were analyzed are different. Thorner's lab report data obtained by mutating the threonine in Cdc12p (cdc12pT48N) and serine in Cdc10p (cdc10pS46N). We did not analyze these mutants. Secondly, the effect of the mutation on nucleotide binding and corresponding defects in morphology, septin localization and septin-septin interactions may vary for each septin. For example, a mutation analogous to cdc12pT48N and cdc10pS46N was generated in Cdc11p, whereby the conserved serine residue was mutated to aspargine (cdc11pS36N). This mutant had defects in morphology and septin localization (similar to their observations) at 37°C. Unlike cdc12pT48N and cdc10pS46N, this mutant protein did not interact with Cdc12p and was present in very low amounts (as compared to Cdc11p) in the complex as shown by co-immunoprecipitation using septins radiolabeled *in vitro* (Figure 10 and data not shown).

Septin nucleotide binding may have role in both septin-septin interactions and septins' interactions with septin-associated proteins. The latter is as predicted by the "small GTPase model" (Figure 1). However, we found that all the septin P-loop mutants were functional and similar to wild type in all septin-dependent processes tested. This suggests nucleotide binding does not have a role in septins' interaction with septin-associated proteins.

References

Adams, A.E., and J.R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *Journal Of Cell Biology*. 98:934-45.

Adams, A.E.M. 1984. Cellular morphogenesis in the yeast *Saccharomyces cerevisiae*. The University of Michigan, Ann Arbor, Michigan.

Amon, A. 2002. Synchronization procedures. *Methods Enzymol*. 351:457-67.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Current Protocols in Molecular Biology. John Wiley and Sons Ltd., New York.

Beites, C.L., H. Xie, R. Bowser, and W.S. Trimble. 1999. The septin CDCrel-1 binds syntaxin and inhibits exocytosis. *Nat Neurosci*. 2:434-9.

Belmont, L.D., A. Orlova, D.G. Drubin, and E.H. Egelman. 1999. A change in actin conformation associated with filament instability after Pi release. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 96:29-34.

Bi, E., P. Maddox, D.J. Lew, E.D. Salmon, J.N. McMillan, E. Yeh, and J.R. Pringle. 1998. Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *Journal Of Cell Biology*. 142:1301-12.

Bi, E., and J.R. Pringle. 1996. *ZDS1* and *ZDS2*, genes whose products may regulate Cdc42p in *Saccharomyces cerevisiae*. *Molecular And Cellular Biology*. 16:5264-75.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature*. 349:117-27.

Briza, P., E. Bogengruber, A. Thür, M. Rützler, M. Münsterkötter, I.W. Dawes, and M. Breitenbach. 2002. Systematic analysis of sporulation phenotypes in 624 non-lethal homozygous deletion strains of *Saccharomyces cerevisiae*. *Yeast*. 19:403-422.

Carroll, C.W., R. Altman, D. Schieltz, J.R. Yates, and D. Kellogg. 1998. The septins are required for the mitosis-specific activation of the Gin4 kinase. *Journal Of Cell Biology*. 143:709-17.

Caviston, J.P., M. Longtine, J.R. Pringle, and E. Bi. 2003. The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. *Molecular Biology of the Cell*. 14:4051-4066.

Craig, D., M.T. Howell, C.L. Gibbs, T. Hunt, and R.J. Jackson. 1992. Plasmid cDNA-directed protein synthesis in a coupled eukaryotic in vitro transcription-translation system. *Nucleic Acids Res*. 20:4987-95.

De Virgilio, C., D.J. DeMarini, and J.R. Pringle. 1996. *SPR28*, a sixth member of the septin gene family in *Saccharomyces cerevisiae* that is expressed specifically in sporulating cells. *Microbiology*. 142:2897-905.

Dobbelaere, J., and Y. Barral. 2004. Spatial coordination of cytokinetic events by compartmentalization of the cell cortex. *Science*. 305:393-6.

Fares, H., L. Goetsch, and J.R. Pringle. 1996. Identification of a developmentally regulated septin and involvement of the septins in spore formation in *Saccharomyces cerevisiae*. *Journal of Cell Biology*. 132:399-411.

Ford, S.K., and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC11* gene product and the timing of events at the budding site. *Developmental Genetics*. 12:281-92.

Frazier, J.A., M.L. Wong, M.S. Longtine, J.R. Pringle, M. Mann, T.J. Mitchison, and C. Field. 1998. Polymerization of purified yeast septins: evidence that organized filament arrays may not be required for septin function. *Journal of Cell Biology*. 143:737-49.

Gietz, D., A. St. Jean, R.A. Woods, and R.H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research*. 20:1425.

Guthrie, C., and G.R. Fink. 1991. Guide to yeast genetics and molecular biology. *In* Methods in Enzymology. Vol. 194. Academic Press, San Diego, CA. 1-933.

Gyuris, J., E. Golemis, H. Chertkov, and R. Brent. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*. 75:791-803.

Haarer, B.K., and J.R. Pringle. 1987. Immunofluorescence localization of the *Saccharomyces cerevisiae CDC12* gene product to the vicinity of the 10-nm filaments in the mother-bud neck. *Molecular And Cellular Biology*. 7:3678-87.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast: IV. Genes controlling bud emergence and cytokinesis. *Experimental Cell Research*. 69:265-276.

Hoffenberg, S., L. Nikolova, J.Y. Pan, D.S. Daniel, M. Wessling-Resnick, B.J. Knoll, and B.F. Dickey. 1995. Functional and structural interactions of the Rab5 D136N mutant with xanthine nucleotides. *Biochem Biophys Res Commun*. 215:241-9.

Hwang, Y.W., and D.L. Miller. 1987. A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. *J Biol Chem*. 262:13081-5.

Jeong, J.W., D.H. Kim, S.Y. Choi, and H.B. Kim. 2001. Characterization of the *CDC10* product and the timing of events of the budding site of *Saccharomyces cerevisiae*. *Molecules to Cells*. 12:77-83.

Kim, H.B., B.K. Haarer, and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC3* gene product and the timing of events at the budding site. *Journal Of Cell Biology*. 112:535-44.

Kinoshita, M., S. Kumar, A. Mizoguchi, C. Ide, A. Kinoshita, T. Haraguchi, Y. Hiraoka, and M. Noda. 1997. Nedd5, a mammalian septin, is a novel cytoskeletal component interacting with actin-based structures. *Genes and Development*. 11:1535-47.

Krieg, P.A., and D.A. Melton. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res*. 12:7057-70.

Lee, P.R., S. Song, H.S. Ro, C.J. Park, J. Lippincott, R. Li, J.R. Pringle, C. De Virgilio, M.S. Longtine, and K.S. Lee. 2002. Bni5p, a septin-interacting protein, is required for normal septin function and cytokinesis in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 22:6906-20.

Lew, D.J. 2003. The morphogenesis checkpoint: how yeast cells watch their figures. *Curr Opin Cell Biol*. 15:648-53.

Lillie, S.H., and J.R. Pringle. 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *Journal of Bacteriology*. 143:1384-94.

Lippincott, J., and R. Li. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *Journal of Cell Biology*. 140:355-66.

Longtine, M.S., H. Fares, and J.R. Pringle. 1998. Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *Journal of Cell Biology*. 143:719-36.

Longtine, M.S., C.L. Theesfeld, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 20:4049-61.

McGrath, J.P., D.J. Capon, D.V. Goeddel, and A.D. Levinson. 1984. Comparative biochemical properties of normal and activated human ras p21 protein. *Nature*. 310:644-9.

McMillan, J.N., M.S. Longtine, R.A. Sia, C.L. Theesfeld, E.S. Bardes, J.R. Pringle, and D.J. Lew. 1999. The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Molecular and Cellular Biology*. 19:6929-39.

Mino, A., K. Tanaka, T. Kamei, M. Umikawa, T. Fujiwara, and Y. Takai. 1998. Shs1p: a novel member of septin that interacts with Spa2p, involved in polarized growth in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications*. 251:732-6.

Muller-Reichert, T., D. Chretien, F. Severin, and A.A. Hyman. 1998. Structural changes at microtubule ends accompanying GTP hydrolysis: information from a slowly hydrolyzable analogue of GTP, guanylyl (alpha,beta)methylenediphosphonate.

Proceedings of the National Academy of Sciences of the United States of America. 95:3661-6.

Nogales, E. 2001. Structural insight into microtubule function. *Annu Rev Biophys Biomol Struct*. 30:397-420.

Petracek, M.E., and M.S. Longtine. 2002. PCR-based engineering of yeast genome. *Methods Enzymol*. 350:445-69.

Pringle, J.R. 1991. Staining of bud scars and other cell wall chitin with calcofluor. *Methods In Enzymology*. 194:732-5.

Pringle, J.R., A.E. Adams, D.G. Drubin, and B.K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods In Enzymology*. 194:565-602.

Pringle, J.R., R.A. Preston, A.E. Adams, T. Stearns, D.G. Drubin, B.K. Haarer, and E.W. Jones. 1989. Fluorescence microscopy methods for yeast. *Methods In Cell Biology*. 31:357-435.

Saraste, M., P.R. Sibbald, and A. Wittinghofer. 1990. The P-loop: a common motif in ATP- and GTP-binding proteins. *Trends in Biochemical Science*. 15:430-434.

Schmidt, G., C. Lenzen, I. Simon, R. Deuter, R.H. Cool, R.S. Goody, and A. Wittinghofer. 1996. Biochemical and biological consequences of changing the specificity of p21ras from guanosine to xanthosine nucleotides. *Oncogene*. 12:87-96.

Sprang, S.R. 1997. G protein mechanisms: insights from structural analysis. *Annu Rev Biochem*. 66:639-78.

Theesfeld, C.L., T.R. Zyla, E.G. Bardes, and D.J. Lew. 2003. A monitor for bud emergence in the yeast morphogenesis checkpoint. *Mol Biol Cell*. 14:3280-91.

Vallen, E.A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in cytokinesis in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*. 11:593-611.

Versele, M., and J. Thorner. 2004. Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J Cell Biol.* 164:701-15.

Vrabioiu, A.M., S.A. Gerber, S.P. Gygi, C.M. Field, and T.J. Mitchison. 2004. The majority of the Saccharomyces cerevisiae septin complexes do not exchange guanine nucleotides. *J Biol Chem.* 279:3111-8.

Weijland, A., G. Parlato, and A. Parmeggiani. 1994. Elongation factor Tu D138N, a mutant with modified substrate specificity, as a tool to study energy consumption in protein biosynthesis. *Biochemistry*. 33:10711-7.

Yu, B., V.Z. Slepak, and M.I. Simon. 1997. Characterization of a Goalpha mutant that binds xanthine nucleotides. *J Biol Chem*. 272:18015-9.

Zhong, J.M., M.C. Chen-Hwang, and Y.W. Hwang. 1995. Switching nucleotide specificity of Ha-Ras p21 by a single amino acid substitution at aspartate 119. *J Biol Chem*. 270:10002-7.

CHAPTER III

ALANINE-SCANNING MUTAGENESIS OF THE CDC11p SEPTIN IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Introduction

Septins are important in a variety of cell cycle events in both yeast and in mammalian cells (reviewed in chapter I). One important aspect of septin function is the interactions of septins to form heteromeric septin-septin complexes. In addition, the interactions of septins with non-septin proteins are clearly essential to many (and perhaps all) aspects of septin function. In chapter II, I presented data I obtained, which suggest that the major role of septin GTP binding is to promote septin-septin interactions. However, as GTP-binding motifs tend to be in the protein's interior, it is probable that there are other amino acids on the protein surface involved in the physical contact with other septins. Similarly, it is probable that there are regions on the septins that are involved in interactions with non-septin proteins.

Thus, in addition to the GTP-binding motifs, it is of interest to identify regions of the septin proteins that are involved in septin function. Computational methods predicts a C-terminal coiled-coil structure in most septins. The predicted C-terminal

coiled-coil region of Cdc11p is dispensable for septin localization (Versele et al., 2004), interaction with Cdc12p (Chapter II, Figures 8 and 11) and viability (Lee et al., 2002). However, strains expressing the truncated Cdc11p protein have reduced growth rates, reduced septin localization to the neck, and defects in the neck localization of Shs1p, suggesting this domain has an important, though not essential, role in Cdc11p function (Casamayor and Snyder, 2003). Data from our lab (M. Longtine, personal communication) has shown that the predicted C-terminal coiled-coil domains of Cdc3p and Cdc12p are essential for viability. It is possible that these regions are involved in septin-septin interactions. In mammalian cells, investigators identified a region with conserved positively charged amino acids just N-terminal to the P-loop. Work from Bill Trimble's lab (Zhang et al., 1999) and Mike Snyder's lab (Casamayor and Snyder, 2003) has indicated that this region is important in the ability of mammalian and yeast septins to bind phosphatidylinositol phospholipids. In yeast, mutagenesis of this region results in defects in phospholipid binding and in septin localization, leading to the suggestion that phospholipid binding may have an important role in septin localization (Casamayor and Snyder, 2003).

Because septins appear to interact with multiple septin-associated proteins, and with multiple other septins, it is probable that septins contain multiple regions involved in septin-septin and septin-non-septin protein-protein interactions. Thus, the identification and characterization of these regions is essential to a full understanding of septin function. To identify functionally important regions of the Cdc11p septin, we use an "alanine-scanning" mutagenesis approach. Alanine scanning mutagenesis is a technique to identify interacting regions of proteins (DeLano, 2002). In this method, clusters of

charged residues, which are present typically on the surface of the proteins, are mutated to alanine by site-directed mutagenesis. The alanine residue results in the removal of all side chain atoms beyond the β-carbon (Morrison and Weiss, 2001), while still allowing proper folding as alanine allows a wide range of conformations of the polypeptide backbone (Gibbs and Zoller, 1991). In yeast, this approach has been used to investigate functionally important regions of actin and Cdc42p (Kozminski et al., 2000; Richards et al., 2000; Wertman et al., 1992).

We searched for regions of the vegetatively expressed septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p), which contain conserved stretches of charged amino acids (lysine, arginine, glutamic acid, or aspartic acid). The rationale is that the septins likely have a conserved structure, with conserved regions exposed to the surface. Some, but perhaps not all, of these regions would display regions of charged amino acids. Conversely, it is unlikely for stretches of charged amino acids to be on the interior of the protein and to have major roles in protein folding. This analysis identified ten regions, termed ASI, ASIII, ASIV, ASVI-VIII, and ASX-XIII. Region ASII is excluded from this analysis as it is the P-loop region. Similarly, regions ASV and ASIX are excluded as they are the Cdc11p G3 and G4 domains, involved in nucleotide-binding and hydrolysis and analyzed in Chapter II. The ASI domain encompasses the predicted phospholipid binding domain while ASIII, IV, VI-VIII, and X-XIII are in previously uncharacterized regions (Figure 1). The mutagenesis of these regions in Cdc11p and my analysis of the function of these mutant proteins in septin localization and function are the subject of this chapter.

Septin	ASI			ASII			ASIII		
Cdc11p	12	RKRK-HLKR	19	29	GQSGSGRS	36	62	TEIDLQLREE	71
Cdc3p	108	QWHRRSIKN	116	126	GPDGIGKT	133	180	HKVKIKSYES	189
Cdc12p	23	QRYKIVNEE	31	41	GESGLGKT	48	74	KTVEIDITRA	83
Cdc10p	21	QIEHRLLKK	29	39	GQSGLGKS	46	73	KTTEMKISTH	82
Sep7p	13	RRKK-EHKR	20	30	GPAGTGKT	37	108	TSLELGGNKD	117

Septin	ASIV			ASV			ASVI		
Cdc11p	74	ELEDDEGVKI	83	89	DTPG	92	111	RHQYDEILLE	120
Cdc3p	192	E---ENGVKLNLN	201	204	DTEG	207	227	DSRFDQYLDA	236
Cdc12p	86	E---EKHFELRVN	95	98	DTPG	101	120	DDQHDSYMRQ	129
Cdc10p	85	V---EDRVRLNIN	94	97	DTPG	100	119	KEQHSQYLRK	128
Sep7p	120	KPEMNEDDTV	129	135	MTHG	138	157	EQQFDIVLAE	166

Septin	ASVII			ASVIII			ASIX		
Cdc11p	125	RRNPRFKDG	133	149	LKEIDVEFI	157	171	SKSD	174
Cdc3p	241	NR-HSINDKR	249	264	LKPLDLKFMQ	273	286	AKSD	289
Cdc12p	134	YR-TKKFDLR	142	157	LKPIDIETMK	166	179	AKAD	182
Cdc10p	133	QRERFITDT	141	157	LSRLDVEALK	166	179	GKSD	182
Sep7p	171	KRNPRFEDT	179	195	LREVDVELMK	204	217	TRAD	220

Septin	ASX			ASXI			ASXII		
Cdc11p	178	RDELKLNKKL	187	208	EISDEDYETN	217	233	EVYEMGGDVG	242
Cdc3p	293	DEEILSFKKT	302	322	NDDAENSHLS	331	346	NDIVENYSGN	355
Cdc12p	186	AQELQQFKSR	195	225	NPDSAAVEHA	234	249	EKKFDNGQGT	258
Cdc10p	186	LDERTEFREL	195	214	ELTDEELELN	223	238	ENEIEINGET	247
Sep7p	224	KEELTQFRKN	233	254	DDDLESMEEN	263	278	DTRDSEGRYV	287

Septin	ASXIII		
Cdc11p	254	LDVEDSSISD	263
Cdc3p	367	IEVDNDNHSD	376
Cdc12p	270	VEIENDSHCD	279
Cdc10p	258	INVEDINQCD	267
Sep7p	295	ISIDDDKISD	304

Figure 1. Regions determined for alanine scanning mutagenesis. The Clustal W program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page=/NPSA/npsa_clustalw.html) was used to identify charged amino acids clustered in a region conserved among the vegetatively expressed yeast septins. The identified regions are labeled as Alanine Scanning (AS) I-XIII. Charged amino acids(underlined) in the identified regions of Cdc11p were mutated to alanine for characterization.

Materials and methods

Strains and genetic techniques

The alanine scanning mutants (Table 1) generated, are expressed on low-copy number plasmids in the corresponding *cdc11* deletion strain of YEF473 (Bi and Pringle, 1996). All haploid strains used are *Mat* a except for YML141 (Gyuris et al., 1993) which is *Mat* α (Table I). Strains derived from *cdc11-6* are all diploids. Standard yeast and E. coli media and genetic techniques are as described in Chapter II. Plasmids carrying mutated *cdc11* genes were transformed into appropriate yeast septin gene deletion strains carrying the wild-type *CDC11* gene on a plasmid with the *URA3* selectable marker and transformants selected on SD–leu-ura plates. Two individual transformants were then picked and inoculated into SD–leu-ura media and grown to saturation at 23ºC. Serial dilutions were then spotted onto SD–leu, SD-leu–ura, and SD–leu+5' FOA plates and incubated at 23ºC to select for cells that had lost the *URA3* plasmid carrying the *CDC11* allele, resulting in strains expressing only the mutated *cdc11* allele. For all mutants, viable colonies were obtained from SD–leu+5' FOA plates and used for further analysis.

The construction of *cdc11ASVIII* allele varies slightly from construction of other alleles. Hence, it appears inviable at 23˚C on SD-leu-ura plate (Figure 3, Plate -Leu-Ura). To generate this strain, the *cdc11* deletion strain containing *CDC11* on a plasmid with a *URA3* gene as selectable marker was transformed with the centromeric plasmid containing *cdc11ASVIII* and *LEU2* as a selectable marker. Then the transformed cells were plated on SD-leu plate to select preferably for cells containing only *cdc11ASVIII*. Furthermore, the isolated transformants was inoculated into SD-leu media and grown to

saturation before plating them as five-fold serial dilutions on selective plates. This process of using only SD-leu media ensured the enrichment of *cdc11ASVIII* allele. Due to absence of *URA3* (*pRS/CDC11* has a *URA3* marker), these strains are inviable in media lacking uracil.

Plasmid construction

Plasmids used in this study are listed in Table II. PCR amplifications were done using the Expand High-Fidelity PCR kit (Roche, Indianapolis, IN). Restriction enzymes were from Promega (Madison, WI), Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA). Nucleotide sequencing for verification of introduction of the desired mutation, and the lack of undesired mutations, was done by the core facility of Biochemistry and Molecular Biology Department at Oklahoma State University. For all plasmids, the entire region amplified by PCR was sequenced.

The low-copy-number plasmid, *YCp111/CDC11* was made by cloning ~3.9kb SalI/PstI fragment containing *CDC11* from *pALTER/CDC11* (provided by Mark Longtine). cdc11p alanine scanning mutations were introduced into YCp111/CDC11 by PCR of the region of CDC11 using one primer to introduce the desired mutation, followed by cotransformation of the mutagenized PCR product and *YCp111/CDC11* plasmid linearized by digestion with *Hpa*I into strain YML97. Transformants were selected on medium lacking leucine. To identify plasmids that had undergone homologous recombination, plasmid and yeast genomic DNA were prepared by scraping ~100 µl of transformed yeast cells off the plate and resuspending them in 200 µl buffer containing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0 and 1 mM

EDTA pH 8.0, + 200 µl phenol: chloroform (1:1). The cells were lysed by rapid vortexing with glass beads for 4 min at 23ºC followed by centrifugation at high speed in a microfuge for 10 min. The aqueous supernatant, containing the plasmid and chromosomal DNA, was precipitated and resuspended in 40 μl of 10 mM Tris pH 7.5, 1 mM EDTA pH 8.0 and 1μl was electroporated into DH5α *E.coli* (Life Technologies, Rockville, MD) followed by selection on LB medium containing ampicillin. Plasmid DNA was purified and sequenced. Two-hybrid plasmids containing the alanine-scanning mutations were generated by PCR amplification of the mutant regions using appropriate YCp111/cdc11 plasmids as template and *in vivo* recombination of the PCR products with *Not*I/*Bsp*EI-digested *pEG202/CDC11*. The primers used to generate mutations and for DNA sequencing are listed in Table III.

Viability, morphology and septin localization assays

For viability assays, mutant strains and wild-type control strains were inoculated into 5 ml SDC-Leu media. Starting with equal amounts of cells, five-fold serial dilutions were plated on the appropriate selective solid medium, followed by incubation at the temperatures indicated for 4 to 6 days. To assay cell morphology and septin localization, strains were inoculated into synthetic media lacking leucine and grown overnight at 23˚C to mid-log phase. Samples were then collected before (0 hr) and after (8 hr) a shift of the cultures to 37˚C. Cells were observed by DIC microscopy for analysis of cell morphology and by epifluorescence microscopy to localize a GFP-tagged Cdc3p using a Nikon Eclipse E800 (Tokyo, Japan) equipped with a Hamamatsu single CCD camera (Tokyo, Japan) with image processing with MetaMorph and Photoshop software.

Yeast two-hybrid assay

Two-hybrid interactions were done as described by Brent and coworkers (Ausubel et al., 1995; Gyuris et al., 1993). Strain YML140 (Table I) was transformed with *pEG202* and derivatives which express the GAL4 DNA-binding domain followed by selection on SDC-HIS medium. *pJG4-5* and derivatives, which express the GAL4 transcriptional activation domain, were transformed into strain YML141 and transformants selected on SDC-TRP medium. Transformed YML140 and YML141 strains were mated on YPD plates and selected on minimal media+leucine to generate diploid strains carrying both a DBD- and an AD-plasmid. The diploid strains were inoculated in 3 ml minimal media containing leucine, 1% raffinose and 2% galactose and grown for 12 hr at 30˚C. Quantitative assays to determine β-galactosidase activity, was done for 3-6 cultures for each plasmid combination, using methods described previously (De Virgilio et al., 1996).

Table I. **Strains used in this work**

Strain	Relevant genotype	Source
YML-95	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63*	Bi and Pringle, 1996
YML-140	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34*	Gyuris et al., 1993
YML-141	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34*	Gyuris et al., 1993
YNT-95	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111*	Chapter II
YNT-97	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/CDC11*	This Study
YNT-181	*a/α cdc11-6/cdc11-6* with *YCp111*	This Study
YNT-183	*a/α cdc11-6/cdc11-6* with *YCp111/CDC11*	This Study
M-2301	*a/α* $cdc11^{R35E}/cdc11^{R35E}$	Chapter II
M-2325	*a/α* $cdc11^{G32V}/cdc11^{G32V}$	Chapter II
M-2334	*a/α CDC11/CDC11*	Chapter II
M-2344	*a/α cdc11-6/cdc11-6*	Chapter II
M-2858	YNT-97 with *pRS316/GFP-CDC3*	This Study
M-2878	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS I*	This Study
M-2879	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS III*	This Study
M-2880	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS VIII*	This Study
M-2897	YNT-95 with *pRS316/GFP-CDC3*	This Study
M-2914	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS IV*	This Study
M-2915	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS V*	Chapter II
M-2916	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS VI*	This Study
M-2917	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS IX*	Chapter II
M-2918	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS X*	This Study
M-2919	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS XI*	This Study
M-2920	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS XII*	This Study
M-2921	*a ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63 cdc11Δ::TRP1* with *YCP111/cdc11AS XIII*	This Study
M-2931	M-2878 with *pRS316/GFP-CDC3*	This Study
M-2932	M-2879 with *pRS316/GFP-CDC3*	This Study
M-2933	M-2914 with *pRS316/GFP-CDC3*	This Study
M-2934	M-2915 with *pRS316/GFP-CDC3*	Chapter II
M-2935	M-2916 with *pRS316/GFP-CDC3*	This Study
M-2936	M-2880 with *pRS316/GFP-CDC3*	This Study
M-2937	M-2917 with *pRS316/GFP-CDC3*	Chapter II
M-2938	M-2918 with *pRS316/GFP-CDC3*	This Study
M-2939	M-2919 with *pRS316/GFP-CDC3*	This Study
M-2940	M-2920 with *pRS316/GFP-CDC3*	This Study
M-2941	M-2921 with *pRS316/GFP-CDC3*	This Study
M-2944	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS I*	This Study
M-2945	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS III*	This Study
M-2946	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS IV*	This Study
M-2947	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS V*	This Study
M-2948	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS VI*	This Study
M-2949	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS VII*	This Study
M-2951	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS IX*	This Study
M-2952	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS X*	This Study
M-2953	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS XI*	This Study
M-2954	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS XII*	This Study
M-2955	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS XIII*	This Study
M-2959	*a/α cdc11-6/cdc11-6* with *YCp111/cdc11AS VIII*	This Study
M-2969	M-2334 with *YCp111/cdc11AS III* and *pMS76*	This Study
M-2970	M-2344 with *YCp111/cdc11AS III* and *pMS76*	This Study
M-2971	M-2325 with *YCp111/cdc11AS III* and *pMS76*	This Study
M-2972	M-2301 with *YCp111/cdc11AS III* and *pMS76*	This Study
2H-1	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202*	Chapter II
2H-25	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/CDC11*	Chapter II
2H-71	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS V*	Chapter II
2H-73	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS IX*	Chapter II
2H-81	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS IV*	This Study
2H-83	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS VI*	This Study
2H-85	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS VII*	This Study
2H-87	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS VIII*	This Study
2H-89	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS X*	This Study
2H-93	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS XIII*	This Study
2H-99	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS III*	This Study
2H-101	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5*	Chapter II
2H-117	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC3*	Chapter II
2H-123	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC10*	Chapter II
2H-125	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC11*	Chapter II
2H-131	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/CDC12*	Chapter II
2H-148	*a his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pJG4-5/*$cdc11^{G32E}$	Chapter II
2H-501	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS XI*	This Study
2H-503	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS XII*	This Study
2H-505	α *his3 trp1 ura3-52 leu2::pLEU2-LEXAop6* with *pSH18.34* and *pEG202/cdc11AS I*	This Study

Table II. **Plasmids used in this study**

Plasmid	Characteristics	Source
YCplac111	Low-copy *LEU2 CEN4/ARS1*,	Gietz and Sugino, 1988
YCplac111/CDC11	Low-copy *LEU2 CEN4/ARS1, CDC11*	Chapter II
YCplac111/cdc11AS I	Low-copy *LEU2 CEN4/ARS1, cdc11AS I* ($cdc11^{R12A,K13A,R14A,K15A,K18A,R19A}$)	This Study
YCplac111/cdc11AS III	Low-copy *LEU2 CEN4/ARS1, cdc11AS III* ($cdc11^{E63A,D65A,R69A,E70A,E71A}$)	This Study
YCplac111/cdc11AS IV	Low-copy *LEU2 CEN4/ARS1, cdc11AS IV* ($cdc11^{E74A,E76A,D77A,D78A,E79A,K82A}$)	This Study
YCplac111/cdc11AS V	Low-copy *LEU2 CEN4/ARS1, cdc11AS V* ($cdc11^{D89A,G92A}$)	This Study
YCplac111/cdc11AS VI	Low-copy *LEU2 CEN4/ARS1, cdc11AS VI* ($cdc11^{R111A,D115A,E116A,E120A}$)	This Study
YCplac111/cdc11AS VII	Low-copy *LEU2 CEN4/ARS1, cdc11AS VII* ($cdc11^{R125A,R126A,R129A,K131A,D132A,R134A}$)	This Study
YCplac111/cdc11AS VIII	Low-copy *LEU2 CEN4/ARS1, cdc11AS VIII* ($cdc11^{K150A,E151A,D153A,E155A,R158A}$)	This Study
YCplac111/cdc11AS IX	Low-copy *LEU2 CEN4/ARS1, cdc11AS IX* ($cdc11^{K172A,D174A}$)	This Study
YCplac111/cdc11AS X	Low-copy *LEU2 CEN4/ARS1, cdc11AS X* ($cdc11^{R178A,D179A,E180A,K182A,K183A,K186A}$)	This Study
YCplac111/cdc11AS XI	Low-copy *LEU2 CEN4/ARS1, cdc11AS XI* ($cdc11^{E208A,D211A,E212A,D213A,E215A}$)	This Study
YCplac111/cdc11AS XII	Low-copy *LEU2 CEN4/ARS1, cdc11AS XII* ($cdc11^{E233A,E236A,D240A}$)	This Study
YCplac111/cdc11AS XIII	Low-copy *LEU2 CEN4/ARS1, cdc11AS XIII* ($cdc11^{D255A,E257A,D258A,D263A}$)	This Study
pEG202/	2μ, *HIS3*, *lexA* DNA Binding Domain (DBD)	Ausubel et al., 1995
pEG202/CDC11	2μ, *HIS3*, *lexA* DBD, *CDC11*	Chapter II
pEG202/cdc11AS I	2μ, *HIS3*, *lexA* DBD, *cdc11AS I* ($cdc11^{R12A,K13A,R14A,K15A,K18A,R19A}$)	This Study
pEG202/cdc11AS III	2μ, *HIS3*, *lexA* DBD, *cdc11AS III* ($cdc11^{E63A,D65A,R69A,E70A,E71A}$)	This Study
pEG202/cdc11AS IV	2μ, *HIS3*, *lexA* DBD, *cdc11AS IV* ($cdc11^{E74A,E76A,D77A,D78A,E79A,K82A}$)	This Study
pEG202//cdc11AS V	2μ, *HIS3*, *lexA* DBD, *cdc11AS V* ($cdc11^{D89A,G92A}$)	Chapter II
pEG202//cdc11AS VI	2μ, *HIS3*, *lexA* DBD, *cdc11AS VI* ($cdc11^{R111A,D115A,E116A,E120A}$)	This Study
pEG202//cdc11AS VII	2μ, *HIS3*, *lexA* DBD, *cdc11AS VII* ($cdc11^{R125A,R126A,R129A,K131A,D132A,R134A}$)	This Study
pEG202//cdc11AS VIII	2μ, *HIS3*, *lexA* DBD, *cdc11AS VIII* ($cdc11^{K150A,E151A,D153A,E155A,R158A}$)	This Study
pEG202/cdc11AS IX	2μ, *HIS3*, *lexA* DBD, *cdc11AS IX* ($cdc11^{K172A,D174A}$)	Chapter II
pEG202/cdc11AS X	2μ, *HIS3*, *lexA* DBD, *cdc11AS X* ($cdc11^{R178A,D179A,E180A,K182A,K183A,K186A}$)	This Study
pEG202/cdc11AS XI	2μ, *HIS3*, *lexA* DBD, *cdc11AS XI* ($cdc11^{E208A,D211A,E212A,D213A,E215A}$)	This Study
pEG202/cdc11AS XII	2μ, *HIS3*, *lexA* DBD, *cdc11AS XII* ($cdc11^{E233A,E236A,D240A}$)	This Study
pEG202/cdc11AS XIII	2μ, *HIS3*, *lexA* DBD, *cdc11AS XIII* ($cdc11^{D255A,E257A,D258A,D263A}$)	This Study
pJG4-5PL	2μ, *TRP1*, transcriptional Activation Domain (AD)	DeMarini et al., 1997
pJG4-5PL/CDC3	2μ, *TRP1*, transcriptional AD, *CDC3*	De Virgilio et al., 1996
pJG4-5PL/CDC10	2μ, *TRP1*, transcriptional AD, *CDC10*	De Virgilio et al., 1996
pJG4-5PL/CDC11	2μ, *TRP1*, transcriptional AD, *CDC11*	De Virgilio et al., 1996
pJG4-5PL/CDC12	2μ, *TRP1*, transcriptional AD, *CDC12*	De Virgilio et al., 1996
pJG4-5PL/cdc11^{G32E}	2μ, *TRP1*, transcriptional AD, $cdc11^{G32E}$	Chapter II
pMS76	Low-copy *HIS3, CEN,* CDC12-GFP	Schmidt et al., 2003
pRS316/CDC3-GFP^{S65T}	Low-copy *URA3, CEN,* CDC3-GFP^{S65T}	Caviston et al., 2003

Alanine scanning mutants in the centromeric plasmids were generated by PCR based mutagenesis and in vivo recombination of the PCR products with HpaI digested YCp111/CDC11. Two-hybrid plasmids containing the mutants were generated by PCR amplification of the mutant regions using appropriate centromeric plasmids as template and in vivo recombination of the PCR products with NotI/BspEI digested pEG202/CDC11.

Table III. **Oligonucleotides used in this study**

	Name	Sequence
1.	ML 687	5'-TTTCAGTTCATCTCTTGTCAAGGAATC-3'
2.	ML 688	5' ATGTCCGGAATAATTGACGCATCTTCTGCATTAGCAGCCGCAGCGCATTTGGCAGCAGGTATAACCTTCACT-3'
3.	ML 689	5' ACGACAATCTTGTTACCCACAGATACGTCCACAGCAATAGCCTTACAATTGGCAGCGGCGACGGTCGAATTAGAA-3'
4.	ML 725	5'-GAAATAGACTTACAATTGAGAGAGGAGACGGTCGCATTAGCAGCTGCTGCAGGTGTCGCGATACAACTTAATATC-3'
5.	ML 691	5'-GATGAAGGTGTCAAGATTCAACTTAATATCATCGCTACTCCGGCATTCGGTGATTCTCTC-3'
6.	ML 692	5'-TCTCCATCTTTCGAAATCATTTCCGACTACATTGCCCACCAATATGCTGCAATCTTATTGGCAGAAAGTCGTGTGAGA-3'
7.	ML 693	5'-TATGATGAAATCTTATTGGAAGAAAGTCGTGTGGCAGCAAACCCAGCATTTGCGGCCGGCGCAGTTCATTGTTGTCTT-3'
8.	ML 694	5'-CTTTACTTAATCAACCCAACTGGCCACGGTTTAGCAGCGATTGATGTGGCATTCATCGCACAGTTGGGATCCTTA-3'
9.	ML 695	5'-TTAATCAACCCAACTGGCCACGGTTTA-3'
10.	ML 696	5'-TTTATTCAGTTTCAGTTCATCTCTTGTCAAGGAAGCTGATGCGCTGATCACAGGGAT-3'
11.	ML 697	5'-CCAGTTCCATCTGTCGATATCCTCCATGATCAATGCTGCATTCAGTGCCAGTGCAGCTGCTGTCAAGGAATCTGA-3'
12.	ML 698	5'-AAAAGGTAGAAGTGTACGTAGGTACATATTGGTTGCGTAAGCTGCGGCTGATATTGCATCTTCATCGAAAGG-3'
13.	ML 699	5'-CCATGGATACTTTCTGCCACGAATTGTCCCAACAGCGCCGCCCATTGCGTAAACTGCATTGGATCCAATAAT-3'
14.	ML 700	5'-AGAGATCAATAACGCATTCCTTAAGATAACAAAAGCAGAGATCGATGAAGCTGCCACGGCCAGTATGCCCCATGG-3'
15.	ML 747	5'-AATTGGCAAGTTCCATCTGTC-3'
16.	ML 748	5'-ATAAATACTTTGTGCGGTCAGCAA-3'
17.	ML 749	5'-CAACTGTCTGATGAATTCCACATC-3'
18.	ML 750	5'-CTCGGTTCTATATCTTTCATATAA-3'
19.	ML 751	5'-GCCGCCCATTTCGTAAACTTCATTGGATCCAATAAT-3'
20.	ML 582	5'-GACTGGCTGGAATTCCCGGGGATCCGTCGACCATGGCGGCCGCTCGAGATGTCCGGAATAATTGAC-3'
21.	ML 244	5'-GATGAAGATGAAATATCA-3'
22.	ML 1018	5'-CCAATTGTCGTAGATCTTCGT-3'

Primer 1(forward) was used with primers 2 to 14 (reverse) to generate alanine scanning mutants I - XIII respectively. Primers 15 to 19 were used as sequencing primers to check the mutations. Primer 20 (forward) was used with primers 15 and 18 (both reverse) to clone alanine-scanning mutants into pEG202 plasmids. Primers 21 and 22 were used as sequencing primers to check the pEG202 constructs. Underlined nucleotides in primers 2 to 14 denote the mutated residues in the gene.

Results

Complementation to *cdc11-6* allele

The *cdc11-6* allele is a temperature-sensitive allele isolated in a screen for cell-division-cycle mutants (Hartwell, 1971). The *cdc11-6* allele ($cdc11^{G32E}$) results in an amino-acid change in the Cdc11p P-loop causing a defect in interactions with Cdc12p (see Chapter II) and defects in cell division and cell morphogenesis at 37˚C. To investigate if the *cdc11AS* mutations we generated result in defects in cdc11p function, *YCp111*, *YCp111/CDC11* and *YCp111/cdc11AS*-mutation plasmids were transformed into a *cdc11-6/cdc11-6* diploid strain and transformants selected on SDC-leu medium. Transformants were efficiently obtained with the *YCp111* and *YCp111/CDC11* control plasmids as well as for all ten *cdc11* alanine-scanning mutants. The *cdc11-6* strain expressing vector, wild-type *CDC11* or the *cdc11ASI*, *III, IV, VI-VIII, and X-XIII* mutants grew with equal viability and at indistinguishable growth rates at 23˚C (Figure 2), indicating that none of the cdc11AS mutations result in dominant negative effects on viability at this temperature.

Six mutants (*cdc11ASIII*, *IV*, *VI*, *VIII*, *XI* and *XII*) complemented *cdc11-6* for viability at all temperatures (Figure 2), being indistinguishable from a strain expressing wild-type Cdc11p (Figure 2). This suggests that these mutations likely do not affect the interaction of Cdc11p with Cdc12p.

Two mutants, *cdc11ASI* and *cdc11ASVII*, both showed full viability at 23˚C in the *cdc11-6* strain, but at the intermediate temperatures of 27 and 30˚C, displayed reduced growth compared to that of the *cdc11-6* strain carrying YCp111 vector (Figure 2). As for

the *cdc11-6* strain carrying *YCp111*, *cdc11-6* carrying *YCp111*/*cdc11ASI* and *cdc11ASVII* was inviable at 32˚C and above. The *cdc11ASI* region is in the phospholipid binding domain of Cdc11p [(Casamayor and Snyder, 2003) Figure 1], important in binding phospholipids *in vitro* and, likely, *in vivo*. The *cdc11ASVII* region is a previously uncharacterized region (Figure 1). The dominant-negative effects of these mutations suggest that they play an active role in disrupting septin localization and/or function. The lack of complementation with *cdc11-6* suggests that they are also likely defective in interaction with Cdc12p.

The *cdc11ASX* and *cdc11ASXIII* alleles did not complement *cdc11-6* for its growth defect at restrictive temperatures (Figure 2, Plates 30˚C, 32˚C, 34˚C and 37˚C). One possibility could be that the defects of these mutations are redundant with that of cdc11pG32E that is defective, primarily, in interaction with Cdc12p.

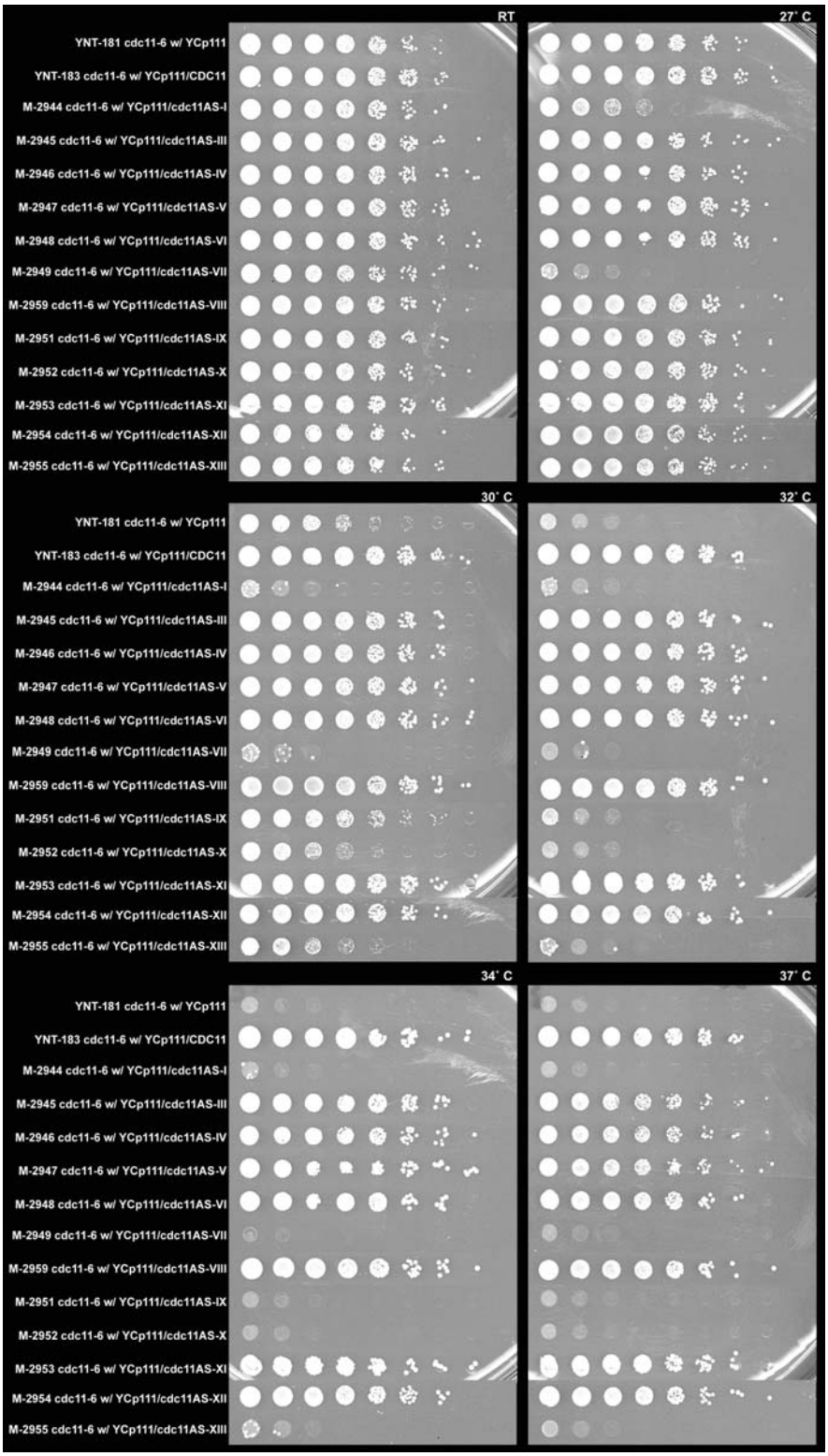

Figure 2. Complementation of *cdc11*AS mutants to *cdc11-6* strain's viability at different temperatures. Cells were grown in SD–Leu and 5-fold serial dilutions were plated on –Leu plates and incubated at the temperatures indicated for 3 days.

Dominant-lethal allele

Using a plasmid-shuffle technique, we were able to efficiently introduce all the *cdc11AS* alleles into a *cdc11*Δ strain with the notable exception of *cdc11ASVII* (Figure 3). In this method, a *cdc11*Δ strain carrying a *URA3*-marked plasmid containing wild-type *CDC11* is transformed with a *LEU2*-marked plasmid carrying the *cdc11AS* allele, and cell carrying both plasmids selected for by growth on medium lacking uracil and lacking leucine. To select for cells that have lost the *URA3*-marked plasmid by mitotic mis-segregation, the doubly transformed cells are spotted onto medium containing leucine and uracil and 5' FOA. Cells expressing Ura3p convert the 5' FOA to a toxic compound and are unable to grow. Thus, the cells that grow are those that only contain the *LEU2*-marked plasmid (expressing the *cdc11AS* allele). After this initial finding, the result was verified by assaying eight independent transformants carrying *YCp111/cdc11ASVII* (Figure 4). All of these cells were inviable on SDC-leu+5'FOA plates, with the rare appearance of apparent suppressor colonies. Thus, when present as the only cdc11p protein in the cell, the cdc11ASVII mutation results in dominant lethality at 23˚C. When present as a heterozygote with cdc11pG32E, the cdc11pASVII results in dominant negative viability at 30˚C. The explanation for this dominant negative effect is unclear and will require further investigation. However, one possibility is that cdc11pASVII may interact with Cdc12p, but that this interaction may be non-productive.

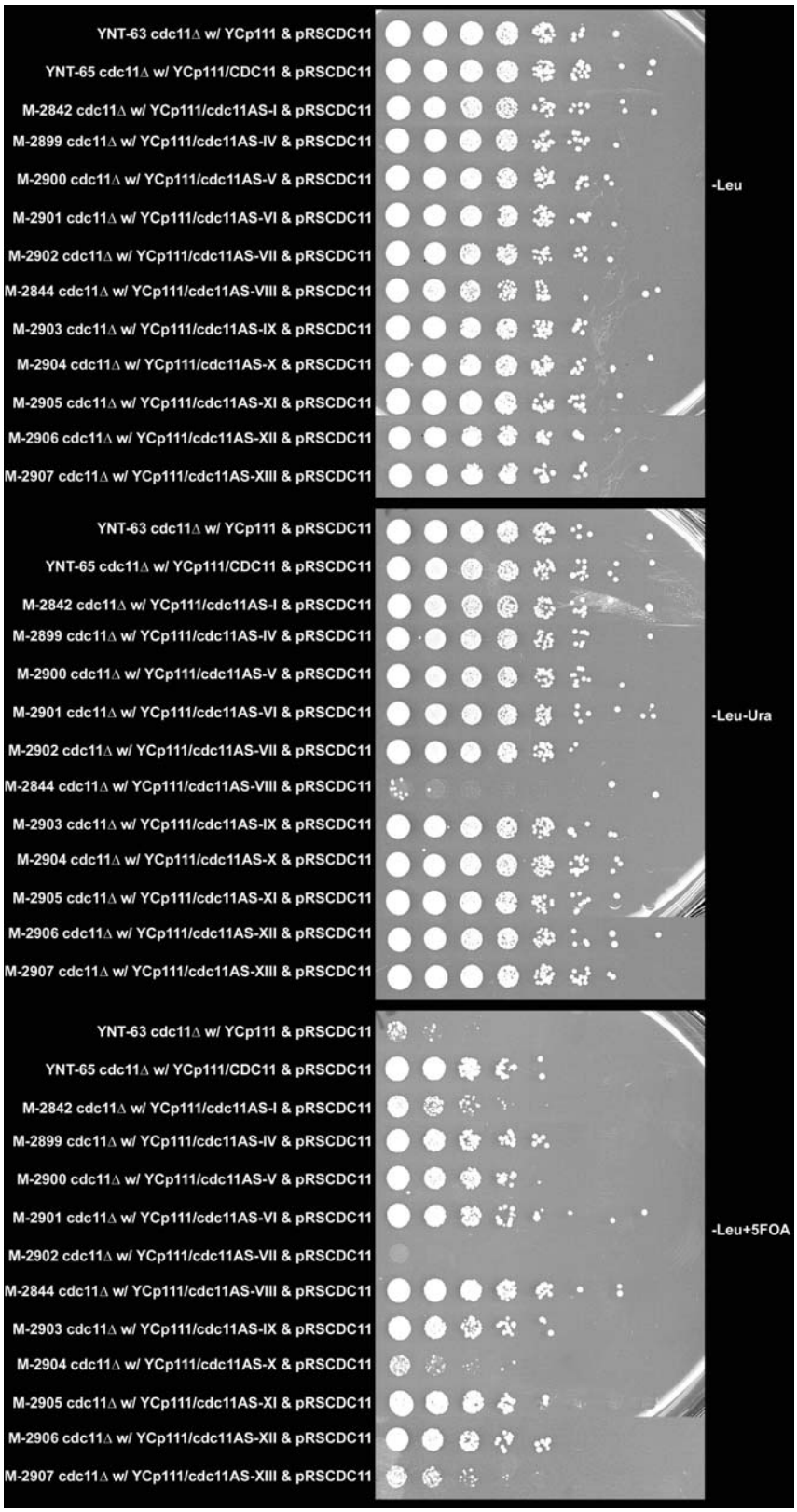

Figure 3. Plasmid shuffle technique to obtain alanine-scanning mutants. Cells were grown in SD–Leu and 5-fold serial dilutions were plated on SD plates as indicated and incubated at 23˚C for 3 days. The variation in viability of *cdc11ASVIII* on SD-leu-Ura is due to variation in generating the transformants and is explained in materials and methods.

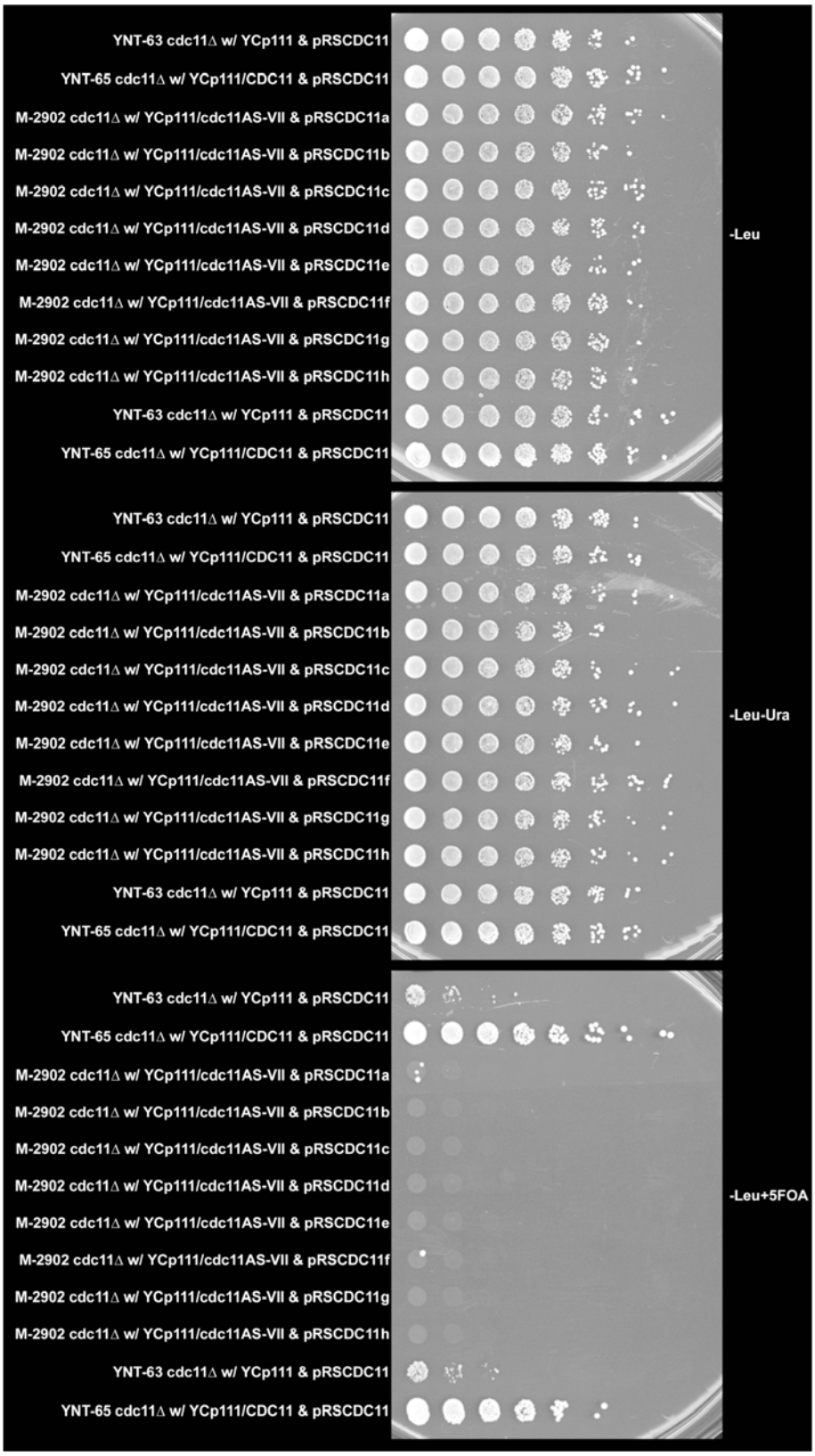

Figure 4. Dominant-lethal phenotype of *cdc11*AS*VII* mutant. Eight isolated transformants were inoculated into SD-leu-ura media and grown to saturation at 23˚C. Cells were then plated as 5-fold serial dilutions SD plates as indicated and incubated at 23˚C for 3 days.

Conditional mutant alleles

Haploid *cdc11* deletion strains expressing the *cdc11* alanine scanning alleles were assayed for viability defects at 23˚C, 27˚C, 30˚C, 32˚C, 34˚C and 37˚C (Figure 5). Five-fold serial dilutions of mutant cultures were plated on SD-leu plates and incubated at indicated temperatures. Two of the alleles, *cdc11ASI* and *cdc11ASX*, showed reduced viability at 23˚C, with viability similar to that of the cdc11Δ strain carrying vector. Thus, these two alleles appear to be non-functional. This is consistent with the lack of complementation of the *cdc11-6* mutation by these two alleles (Figure 2). *cdc11ASI* contains mutations in the phospholipid-binding domain and are expected to disrupt phospholipid binding. Thus, as observed by Casamayor et al. (Casamayor and Snyder, 2003), phospholipid binding appears to be important for Cdc11p function.
In addition to not being functional in the *cdc11*Δ strain, *cdc11ASX* did not complement the *cdc11-6* allele (Figure 2), suggesting that amino acids 178 to 187 are important in Cdc11p function.

The *cdc11ASXIII* showed a temperature sensitive phenotype as compared to the wild-type strains (Figure 5). This allele is comparable to a *cdc11* deletion strain at temperature 27˚C and above. However, it differed from the deletion strain at 23˚C, whereby the viability of these alleles was similar to that of wild-type strain. This region in this allele probably is not required for viability at 23˚C or, similar to the P-loop mutants (Chapter II), does not have a detectable defect in viability at this temperature. The viability of the remaining alleles, *cdc11ASIII*, *cdc11ASIV*, *cdc11ASVI*, *cdc11ASVIII*, *cdc11ASXI* and *cdc11ASXII*, were comparable to that of wild-type strains. This suggests

that these alleles are similar to wild-type in their function for viability (Figure 5 and data not shown).

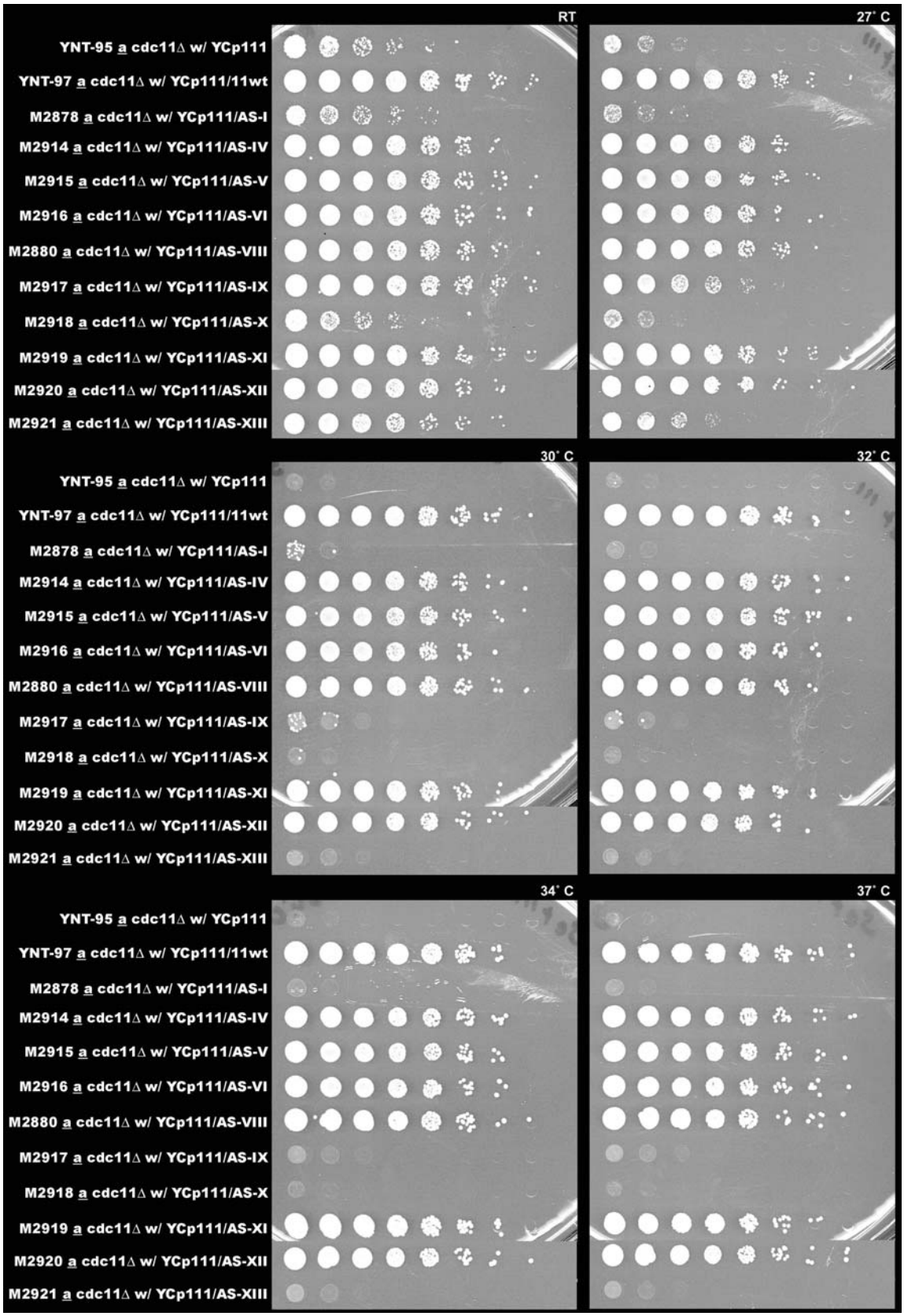

Figure 5. Viability assay of *cdc11*AS mutants. *cdc11* deletion strains containing alanine-scanning mutants were inoculated into SD-leu media and grown to saturation at 23˚C. Cells were then plated as 5-fold serial dilutions SD-leu plates and incubated at indicated temperatures for 3 days.

Morphology and septin localization

To determine if the growth defects of the alanine scanning mutants correlated with defects in septin localization and cell division and morphogenesis, *cdc11AS* mutant strains expressing GFP-Cdc3p were grown to mid-log phase in SD-leu-ura at 23˚C for 12 hr and cells observed by DIC and fluorescence microscopy before and after an 8 hr shift to 37˚C (Figure 6). *cdc11ASI* and *cdc11ASX* strains were defective in septin localization and cell morphogenesis and division at 23˚C (Figure 6, 0 hr). In both *cdc11ASI* and *cdc11ASX* strains, GFP-Cdc3p localized to the bud tip on the majority of the cells (Figure 6, 0 hr, arrows).

Consistent with the correlation between septin localization defects causing inviability, all *cdc11AS* mutant strains that are inviable at 37˚C (*cdc11ASI*, *cdc11ASX*, and *cdc11ASXIII*) show delocalized Cdc3p after 8 hr at 37˚C (Figure 6). Also as expected, in all strains with defects in septin neck localization, the cells displayed defects in cell division and cell morphogenesis. The other *cdc11* alanine scanning mutants (*cdc11ASIII, cdc11ASIV*, *cdc11ASVI*, *cdc11ASVIII*, *cdc11ASXI* and *cdc11ASXII*) that were fully viable at 37˚C showed correspondingly full function in septin localization and function (Figure 6 and data not shown).

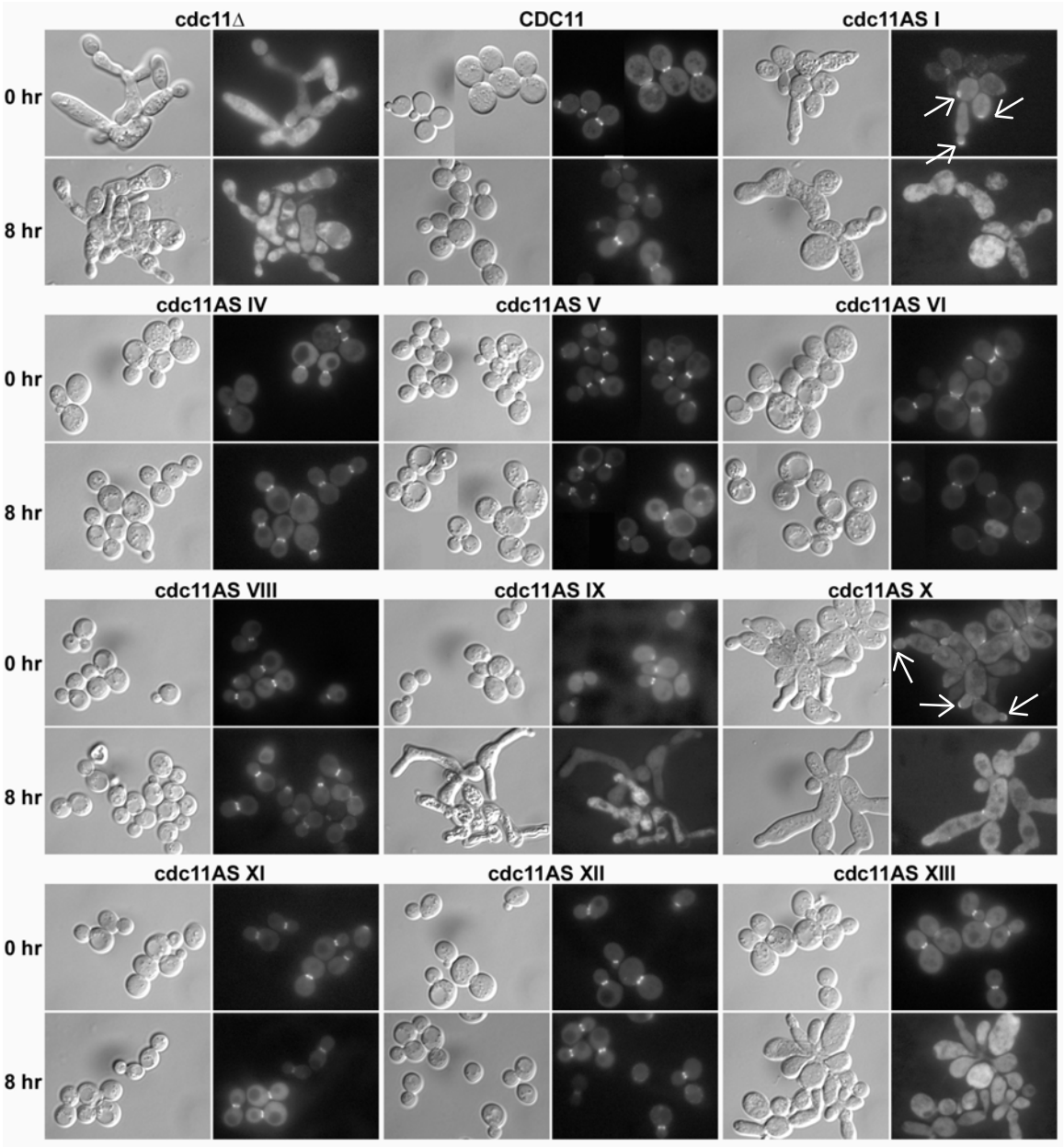

Figure 6. Cell morphology and septin localization of *cdc11*AS mutants. *cdc11* deletion strains containing alanine scanning mutants and GFP-tagged Cdc3p were inoculated into SD-leu-ura media and grown to mid log phase at 23˚C. Cells were then shifted to 37˚C for 8 hours. Cells at 0 hour and 8 hour at 37˚C were visualized under DIC and FITC channels.

Yeast two-hybrid assay

Previously, we observed a strong correlation among defects in septin-septin interactions and temperature-sensitive septin localization, viability, morphogenesis and cell division. To determine if the defects in function of the cdc11 alanine-scanning mutations may be due to defects in interaction of Cdc11p with the other septins, I used the yeast two-hybrid assay (Table IV). Consistent with the correlation shown by septin P-loop mutants, the temperature-sensitive alanine scanning mutant, *cdc11ASXIII* (Figure 5) and defective morphology at restrictive temperature (Figure 6) did not interact with other wild-type septins (Table IV). Even the *cdc11ASI* and *cdc11ASX* alleles that were similar to *cdc11* deletion strains in viability (Figure 5) and morphology (Figure 6) did not interact with the other wild-type septins (Table IV). The remaining alleles that were similar to wild-type in viability, morphology and septin localization did show an interaction with wild-type septins (Table IV).

Also, the alanine-scanning alleles *cdc11ASI* and *cdc11ASVII* depict a dominant negative phenotype in *cdc11-6* background. This dominant phenotype might be due to either the sequestering of cdc11pG32E by cdc11pASI or cdc11pASVII or by hindering another process. The sequestration of cdc11pG32E predicts a likely interaction of the two alanine scanning mutants with cdc11pG32E. To test the above two hypotheses, I assayed for two-hybrid interactions among alanine-scanning mutant proteins (cdc11pASI and cdc11pASVII) with cdc11G32Ep (Table V). The two alleles that depict dominant negative phenotype do not interact with cdc11G32Ep (Table V), suggesting a disruption of some process other than sequestering cdc11pG32E.

Table IV. **Two-hybrid interactions of alanine scanning mutants and wild-type septins**

AD / DBD	*pJG4-5*	*CDC3*	*CDC10*	*CDC11*	*CDC12*
pEG202	7	5	7	4	3
CDC11	3	9	**59**	**837**	**888**
cdc11ASI	2	3	3	29	12
cdc11ASIII	4	29	**147**	**1478**	**2279**
cdc11ASIV	4	8	**52**	**1012**	**999**
cdc11ASV	3	9	**112**	**2306**	**2021**
cdc11ASVI	3	10	34	**901**	**1265**
cdc11ASVII	5	3	4	3	2
cdc11ASVIII	2	6	32	**764**	**899**
cdc11ASIX	10	23	11	13	7
cdc11ASX	13	24	8	47	4
cdc11ASXI	4	21	**61**	**1146**	**1179**
cdc11ASXII	4	16	**76**	**1154**	**1380**
cdc11ASXIII	2	2	4	8	1

Two-hybrid assays were done for ≥3 replicates for each interaction. Strains (Table I) were grown at 23˚C in minimal medium containing leucine, 1% raffinose and 2% galactose. The top row depicts wild-type septin proteins fused to the transcriptional activation domain (AD) and the first column shows the alanine-scanning mutants fused to the DNA binding domain (DBD). The average β-galactosidase activity in Miller units is shown.

Table V. **Two-hybrid interactions of cdc11AS mutants with *cdc11-6*(*cdc11*^{G32E})**

pJG4-5 / pEG202	*pJG4-5*	*cdc11*^{G32E}	*CDC11*
pEG202	6	4	n.d.*
CDC11	6	13	775
cdc11AS I	7	5	9
cdc11AS VII	8	6	n.d.*

Two-hybrid assays were done as above. The average values, in miller units, for the β-galactosidase activity are shown.
n.d.* = Not done

Discussion

The *cdc11ASI* allele has residues mutated in the phospholipid-binding domain. This allele shows a dominant-negative phenotype in *cdc11-6* background (Figure 2). In the *cdc11-6* allele, the mutant cdc11pG32E is defective in its interaction with Cdc12p. The presence of a stable protein at 37˚C and the absence of septins at the neck at this temperature suggest that Cdc11p-Cdc12p interaction is important for its neck localization. However, cdc11pG32E and Cdc12p coimmunoprecipitate as complex (suggesting an interaction) at permissive temperature (Ashok Rajendran, personal communication) and the *cdc11-6* allele is viable at this temperature. So, cdc11pASI might perturb the cdc11G32Ep-Cdc12p interaction and/or disrupt the localization of the cdc11pG32E-Cdc12p complex and thus cause a dominant negative effect for viability. Previous evidence show that mutation in the phospholipid-binding domain disrupts septin localization (Casamayor and Snyder, 2003). From our work and from work by Snyder's lab, it becomes likely for cdc11pASI to interact with either cdc11pG32E or Cdc12p, so that it could sequester the individual protein and/or the complex. However, this mutant protein does not interact with cdc11G32Ep or Cdc12p as shown by two-hybrid assay (Table IV and V). The most plausible explanation at this stage would be that this mutant protein might somehow enhance the defects in conjunction with the cdc11pG32E, thereby decreasing the viability of the *cdc11-6* strain. The *cdc11AI* allele is also defective in viability in a *cdc11*Δ background (Figure 5). cdc11ASIp as the sole source of cdc11p looks like a deletion strain suggesting that the protein is not functional or unstable. The stability of the mutant protein will be checked by monitoring the protein

levels by western blotting. However, certain evidences do suggest that this mutant protein is expressed and are stable at both permissive and restrictive temperatures. Primarily in case of alanine scanning mutants under *cdc11-6* background, the *cdc11ASI* allele, causes a dominant effect on *cdc11-6* strains (Figure 2). If the proteins were unstable at restrictive temperatures, then these alleles should be similar to the control *cdc11-6* allele with just the centromeric plasmid. The second evidence is the localization of GFP-Cdc3p to the tips of majority of the *cdc11*Δ (with cdc11pASI) cells (Figure 6, Arrows). A similar septin localization of GFP-Cdc3p (and the mutant cdc11p) is seen in the P-loop mutants at semi-permissive temperatures (Unpublished data - Our Lab). It is likely that cdc11pASI is also expressed and is mislocalized to the tip.

The *cdc11ASVII* allele also has dominant negative effect in *cdc11-6* strain and is lethal in a *cdc11*Δ strain. There is a possibility that this allele might also disrupt some essential pathway.

Most defects in viability, morphology, septin localization and septin-septin interactions observed here were shown previously for other septin mutants (Chapter II). It has also been shown that usually there is a correlation among the defects, i.e., a septin mutant if depicting a defect in viability or temperature-sensitive phenotype, will have a defective morphology at restrictive temperature with absence of the septins at the neck and also fails to interact with other septins (Chapter II). Three of the alanine scanning mutants, two defective in viability at 23˚C (*cdc11ASI* and *cdc11ASX*) and one with temperature sensitive phenotype (*cdc11ASXIII*), agree with this phenomenon. However, the septin localization to the tip (in case of *cdc11ASI* and *cdc11ASX* at 23˚C) should be further studied. The morphology and septin localization assay of the alanine-scanning

mutants predominantly shows one morphological defect, if any, and two types of septin localization defects. All mutants (*cdc11ASI*, *cdc11ASX* and *cdc11ASXIII*) that are defective in morphology, at 23˚C and/or 37˚C, show an elongated bud morphology. This morphological defect has been observed in most septin mutants. It has been shown earlier that a delay in isotropic bud growth is also seen due to disruption of septin-mediated localization of Swe1p and its negative regulators Hsl1p and Hsl7p (Longtine et al., 2000). This delay in isotropic bud growth leads to elongated bud morphology. It is also shown, by two-hybrid assay, that the septin Cdc11p directly interacts with Hsl1p (Hanrahan and Snyder, 2003). The morphological defect, in case of alanine scanning mutants, is probably a result of the absence of septins at the neck, thereby not providing a scaffold for the proteins involved in the growth switch, rather than a defect in septin interactions with the proteins involved in the growth switch.

The two types of septin localization, mentioned earlier, are mislocalization to the tips at 23˚C in case of *cdc11ASI* and *cdc11ASX* alleles and the absence of septins at restrictive temperature in all temperature-sensitive alleles. Based on recent evidences of the Cdc42p effectors Gic1p and Gic2p interacting with septins (Erfei Bi, personal communication and Chapter II, Appendix B), it is suggested that the Gic1p and Gic2p are required for initial localization of septins to the presumptive bud-site. Also, at semi-permissive temperatures, the septin P-loop mutants mislocalize to the tip (Chapter II). These evidences suggest the possibility that the septins upon interaction with Gic1p and Gic2p localize to the tip and then persist at the tip due to their inability to interact with each other to form ring structure. Consistently, the *cdc11AS I* and *cdc11AS X* allele that show septin mislocalized to the tip do not interact with Cdc12p in the two-hybrid assay.

The two-hybrid interaction data is consistent with the idea of mutants defective in viability and morphology fail to interact with other wild-type septins. The alanine-scanning mutants, *cdc11ASI*, *cdc11ASVII*, *cdc11ASX* and *cdc11ASXIII* do not interact with wild-type septins (Table IV). Previous data have also shown that the disruption of nucleotide binding of Cdc11p, by mutating the P-loop domain, disrupts their interaction with Cdc12p (Chapter II). It is surprising that any mutation in Cdc11p perturbs its interaction with Cdc12p, thereby presumably prevent the neck localization of septins. One possible explanation would be the presence of multiple regions on the protein surface that engage in charged-charge interactions with other proteins. It is not uncommon among proteins to have this feature. In fact reviews on the Ras superfamily of GTP binding proteins and the Rho-family GTPases have suggested multiple regions in the proteins to be involved in interaction with their cognate partners (Corbett and Alber, 2001; Dvorsky and Ahmadian, 2004). The fact that I have mutated only the conserved charged residues that are predicted to be on the surface implies that most of these regions, if not all, might be the regions interacting directly with Cdc12p. Hence, the primary output of these mutations would be abrogating the interaction of cdc11p with Cdc12p, thus causing subsequent defects in septin localization, morphology and viability.

The data above is analyzed and discussed based on two assumptions. Firstly, mutated residues of the septin, Cdc11p are predominantly on the surface of the protein. Secondly, the mutated proteins are expressed and are stable at all temperatures. The first assumption can be clarified upon obtaining structural data on septins. The stability of the proteins can be monitored easily by checking the proteins levels at 23˚C and 37˚C. This will be done in the future and will be included in the manuscript to be submitted for

publishing in a journal. However, certain evidences do suggest that these proteins are expressed and are stable at both permissive and restrictive temperatures. Primarily in case of alanine scanning mutants under *cdc11-6* background, the *cdc11ASI* and *cdc11ASVII* alleles, cause a dominant effect on *cdc11-6* strains (Figure 2). If the proteins were unstable at restrictive temperatures, then these alleles would be similar to the control *cdc11-6* allele with just the centromeric plasmid. The remaining alleles (with the exception of *cdc11ASX*) do complement *cdc11-6* for growth defect at all temperatures suggesting that the mutant proteins are stable at restrictive temperatures to complement the growth.

All the mutant alleles express the proteins and are also stable at 23˚C in case of conditional mutant alleles. However, the temperature sensitive phenotype of *cdc11ASXIII* alleles might be real or due to unstable protein, whereby the strains would similar to a *cdc11* deletion strains, hence inviable.

The stability of proteins, cdc11ASpI and cdc11ASpX are in question in case of the deletion strains containing these proteins as their sole source of Cdc11p. In chapter II, deletion strains containing only the P-loop mutants that showed a temperature-sensitive viability also showed septin mislocalization to the tip at semi-permissive temperature. This localization was monitored using GFP tagged Cdc3p. Also, immunofluorescence data using anti-Cdc11p showed the presence of mutant cdc11p at the bud-tip (data not shown). Similarly, in this case, there is a possibility for the presence of cdc11ASpI and cdc11ASpX at the tip.

At this point, it becomes important to mention that the data presented in this chapter is work still in progress and the caveats mentioned above will be properly addressed and answered prior to submission of the manuscript.

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Current Protocols in Molecular Biology. John Wiley and Sons Ltd., New York.

Bi, E., and J.R. Pringle. 1996. *ZDS1* and *ZDS2*, genes whose products may regulate Cdc42p in *Saccharomyces cerevisiae*. *Molecular And Cellular Biology*. 16:5264-75.

Casamayor, A., and M. Snyder. 2003. Molecular dissection of a yeast septin: distinct domains are required for septin Interaction, localization, and function. *Molecular and Cellular Biology*. 23:2762-2777.

Corbett, K.D., and T. Alber. 2001. The many faces of Ras: recognition of small GTP-binding proteins. *Trends Biochem Sci*. 26:710-6.

De Virgilio, C., D.J. DeMarini, and J.R. Pringle. 1996. *SPR28*, a sixth member of the septin gene family in *Saccharomyces cerevisiae* that is expressed specifically in sporulating cells. *Microbiology*. 142:2897-905.

DeLano, W.L. 2002. Unraveling hot spots in binding interfaces: progress and challenges. *Curr Opin Struct Biol*. 12:14-20.

Dvorsky, R., and M.R. Ahmadian. 2004. Always look on the bright site of Rho: structural implications for a conserved intermolecular interface. *EMBO Rep*. 5:1130-6.

Gibbs, C.S., and M.J. Zoller. 1991. Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J Biol Chem*. 266:8923-31.

Gyuris, J., E. Golemis, H. Chertkov, and R. Brent. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*. 75:791-803.

Hanrahan, J., and M. Snyder. 2003. Cytoskeletal activation of a checkpoint kinase. *Mol Cell*. 12:663-73.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast: IV. Genes controlling bud emergence and cytokinesis. *Experimental Cell Research*. 69:265-276.

Kozminski, K.G., A.J. Chen, A.A. Rodal, and D.G. Drubin. 2000. Functions and functional domains of the GTPase Cdc42p. *Mol Biol Cell*. 11:339-54.

Lee, P.R., S. Song, H.S. Ro, C.J. Park, J. Lippincott, R. Li, J.R. Pringle, C. De Virgilio, M.S. Longtine, and K.S. Lee. 2002. Bni5p, a septin-interacting protein, is required for normal septin function and cytokinesis in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 22:6906-20.

Longtine, M.S., C.L. Theesfeld, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 20:4049-61.

Morrison, K.L., and G.A. Weiss. 2001. Combinatorial alanine-scanning. *Curr Opin Chem Biol*. 5:302-7.

Richards, K.L., K.R. Anders, E. Nogales, K. Schwartz, K.H. Downing, and D. Botstein. 2000. Structure-function relationships in yeast tubulins. *Mol Biol Cell*. 11:1887-903.

Versele, M., B. Gullbrand, M.J. Shulewitz, V.J. Cid, S. Bahmanyar, R.E. Chen, P. Barth, T. Alber, and J. Thorner. 2004. Protein-protein interactions governing septin

heteropentamer assembly and septin filament organization in Saccharomyces cerevisiae. *Mol Biol Cell.* 15:4568-83.

Wertman, K.F., D.G. Drubin, and D. Botstein. 1992. Systematic mutational analysis of the yeast ACT1 gene. *Genetics*. 132:337-50.

Zhang, J., C. Kong, H. Xie, P.S. McPherson, S. Grinstein, and W.S. Trimble. 1999. Phosphatidylinositol polyphosphate binding to the mammalian septin H5 is modulated by GTP. *Current Biology*. 9:1458-67.

APPENDIX A

TRYPSIN NICKING ASSAY

Introduction

Coupled transcription and translation (TnT) in rabbit reticulocyte lysate (RRL) or wheat germ extract is a useful technique for detecting protein-protein interactions (Jagus and Beckler, 1998). In Chapter II, I used this method to synthesize radiolabeled wild-type and mutant septins and used these radiolabeled proteins to investigate septin-septin interactions. In this work, I characterized septin-septin interactions and found that mutations in the P-loop disrupted septin-septin interactions, suggesting septin nucleotide binding has a role in septin-septin interactions. However, one caveat to this conclusion is that the lack of interaction of P-loop-mutant septins is due to structural instability of the mutant protein. To investigate this possibility, I used a mild proteolytic nicking assay (Hartson et al., 1998) to determine if the P-loop mutant cdc11p proteins displayed a significantly different structure than wild-type Cdc11p. This method has been successfully used to investigate changes in protein structure, including the identification of regions of Hsp90 whose conformation is changed by molybdate (Hartson et al., 1999) and also to assay the effect of the drug, geldanamycin on structure of a kinase, Hck499F (Scholz et al., 2001).

The partial protease digestion (or "protease nicking") approach to assay for changes in protein structure is based on the principle that regions of the protein that are susceptible to protease are not all equally susceptible to the enzyme when the protein has formed its three-dimensional structure. For example, potential cleavage sites in the interior of the protein are likely to be cleaved less efficiently than potential cleavage sites on, or near, the protein exterior. This is due to simple stearic hindrance of the protease recognizing, and acting on, the sites on the interior of the protein. Similarly, potential cleavage sites at amino acids in other types of secondary or tertiary structure will show a range of susceptibility to cleavage.

In this assay, I compared the pattern of partial protease digestion (to generate a protein "fingerprint") of the Cdc11p wild-type and cdc11p P-loop-mutant proteins. We expect that if a mutation results in significant folding defects, then the mutant and wild-type proteins will display different fingerprints due to their different three-dimensional structures and alterations in sensitivity of sites to the protease. Conversely, identical (or very similar) fingerprints would suggest that the mutant protein(s) form a tertiary structure very similar to the wild-type Cdc11p protein. This result would strongly suggest that the defect in interactions of the P-loop mutant Cdc11p with Cdc12p is due to specific defects in GTP binding, rather than being caused by an overall defect in proper folding of the mutant cdc11p proteins.

Materials and methods

Rabbit reticulocyte lysate coupled in vitro transcription and translation reactions were done as described in Chapter II. After completion of the RRL/TnT reaction, aurintricarboxylic acid (ATA) was added to a final concentration of 75μM for 1 hr at 37˚C to stop protein synthesis. The lysates were chilled on ice for at least 10 min prior to partial proteolysis. One volume of the chilled lysate containing the synthesized, ^{35}S-labelled wild-type or P-loop-mutant septin was mixed with one volume of trypsin digestion buffer containing 10mM Tris-HCl, pH 7.4, 150mM NaCl, 4mM $CaCl_2$ and 0.1mM EDTA. The mixture was vortexed briefly and various concentrations of TPCK-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ) were added to the mixture. Digestion was allowed to proceed for six minutes on ice and was stopped by adding boiling SDS-PAGE sample buffer. The digested protein products were then separated by 10% SDS-PAGE, transferred to PVDF membrane, and the proteins and protein fragments detected by autoradiography.

Results and discussion

The partial protease digestion products of a protein usually separate into discrete-sized protein fragments. During partial digestion, sites that are readily accessible (often because they are on the surface of the folded protein) are cleaved more rapidly than other, less accessible sites. Thus, the banding pattern resulting from partial protease digestion is related to the three dimensional structure of the protein. Changes in banding pattern

typically reflect changes in protein folding. However, it is important to note that a mutation that adds, or removes, a protease digestion site will result in a slight change in the resulting "fingerprint".

The partial protease digestion fingerprints of wild-type and P-loop-mutant Cdc11p septins are shown in Figure 1. Each panel shows an assay performed on two mutants, with a wild-type Cdc11p control digestion that was carried out at the same time as the mutant protein digestions. The assays were restricted to three samples per experiment to reduce as much as possible variations in digestion due to the times involved in sample manipulation. As expected, there is a slight variation in the precise rates of digestion of the wild-type controls between experiments. These slight variations in the kinetics of digestion are the result of variations due to unavoidable variations in pipetting and times of sample manipulation. However, the protease fingerprint (which bands appear, and their order of appearance) is very reproducible (Figure 1, Four Cdc11p samples), suggesting the assay accurately reflects protein structure.

The full-length Cdc11p has a predicted molecular mass of 48 kDa but migrates similarly to the 60 kDa marker on our SDS-PAGE gels (Figure 1). In all assays, full-length Cdc11p migrates as a doublet at 60 kDa and slightly below (Figure 1A, Cdc11p, Arrowheads, compare with corresponding doublets at 60 kDa in D, G and J). These bands are confirmed to be a product(s) of *CDC11* by western blot using anti-Cdc11p (data not shown). Apart from this doublet, in all lanes of Cdc11p, at 0 μg/μl concentration of trypsin, there is a third major band at 30 kDa (Figure 1A, D, G and J, Lane 0 μg/μl). Several bands of lighter intensity can also be seen at ~50 kDa, 45 kDa, 40 kDa and 25 kDa (Figure 1A, D, G and J, Lane 0 μg/μl). It is expected that these bands

are due to truncation products of Cdc11p generated in the RRL/TnT reaction, or that they are due to improper initiation of translation.

At 2-16 μg/μl concentration there is a reduction in the full-length Cdc11p and the simultaneous appearance of two major bands between 40 kDa-50 kDa. However, we do not see the expected appearance of a new band that migrates between 10 kDa and 20 kDa. This might be due to a released 10 kDa-20 kDa peptide lacking a methionine residue (and thus not being labeled with ^{35}S). Indeed, analysis of the Cdc11p sequence identifies a peptide of ~17 kDa is obtained when trypsin cleaves the protein simultaneously at arginine 69 and lysine 186. The resulting peptide does not contain a methionine residue so would not be detected by autoradiography. Using an N-terminally tagged Cdc11p (6XHis-Cdc11p) for digestion and probing the blots with anti-His antibodies would provide more information on this point.

An additional band of~35 kDa starts appearing at 4μg/μl or 8 μg/μl lanes in all Cdc11p lanes (Figure 1A, arrow, compare with corresponding band in D, G and J). The 35 kDa band appears in parallel with decrease of the 50 kDa and 40 kDa bands described above, suggesting that it is a product of cleavage of these polypeptides. Perhaps the cleavage results in two ~30 kDa peptides that co-migrate, or in the resultant 30 kDa peptide and other cleavage products not identified due to size or low levels (or the absence) of methionine residues. At the highest concentration of trypsin, there are five major bands, as detected by autoradiography, (Figure 1, Cdc11p, Lane 48 μg/μl) of 55 kDa, 40 kDa, 35 kDa, just below 25 kDa and ~10 kDa (Figure 1A, asterisks).

In case of cdc11pG32V, cdc11pS36N, cdc11pG29D and cdc11pG32E, the fingerprints are very similar to that of wild-type Cdc11p (Figure 1A-C and G-I, compare

Cdc11p with cdc11pG32V, cdc11pS36N, cdc11pG29D and cdc11pG32E). In all cases, the order of appearance of new bands and reduction of previously existing bands is similar to that of the wild-type Cdc11p, as described above. Briefly, there is the initial presence of the 60 kDa doublet present at 0 μg/μl of trypsin (Figure 1B, C, H and I-all 0 μg/μl lanes). As digestion progresses, there is the appearance of two major bands between 40 kDa and 50 kDa with decreasing intensities of the 60 kDa doublet (Figure 1B, C, H and I-all 2 μg/μl lanes). Appearance of 35 kDa band coincides with decreasing intensity of the 30 kDa band (Figure 1B, C, H and I-all 4 μg/μl and 8 μg/μl lanes) and finally, five major bands are seen at the highest concentration of trypsin (Figure 1B, C, H and I-all 48 μg/μl lanes). It is notable that the cdc11pG32V, cdc11pG32E, and cdc11pG29D alleles result in a strongly defective protein (see Chapter II). The similarity of the fingerprints of wild-type Cdc11p and the P-loop mutant cdc11pG32V, cdc11pS36N, cdc11pG29D and cdc11pG32E proteins strongly suggests that the mutation in the Cdc11p P-loop do not result in significant defects in the overall folding of Cdc11p.

In case of mutant proteins, cdc11pR35T, cdc11pR35E and cdc11pG32V,R35E,S36N, there are common and reproducible differences in their fingerprints compared to that of wild-type Cdc11p (Figure 1E, F and K-all lanes). In case of these mutants, the 60 kDa doublets and the major bands between 40 kDa-50 kDa are more prominent and of equal amounts. The increased levels of the 60 kDa doublets in the absence of trypsin suggests that these mutants may be more susceptible to early termination of translation, or to translation starting at in inappropriate site. It seems likely that the appearance of the doublet of equal intensities at 40 kDa and 50 kDa upon trypsin treatment is a result of cleavage of the equal intensity doublet peptides at ~60 kDa

(in wild-type Cdc11p these bands are not of equal intensities). However, there is also another difference between the fingerprints of these cdc11p P-loop mutants and that of wild-type Cdc11p. Compared to Cdc11p, there is no appearance of bands just below the lower band at ~60 kDa and also no appearance of a band between the 40 kDa and 50 kDa doublet.

One possibility for the absence of these bands is that cdc11pR35T, cdc11pR35E and cdc11pG32V, R35E, S36N have undergone a more significant structural change than the other cdc11p P-loop-mutant proteins. However, we find this unlikely, as there is no correlation between the digestion pattern observed for these mutant proteins and their ability to function. For example, cdc11pR35T is nearly fully functional under all conditions tested, while cdc11pR35E has an intermediate defect in Cdc11p function and cdc11pG32V,R35E,S36N has a dramatic defect in septin function. Thus, it seems unlikely that these proteins contain a common defect in folding. Alternatively, we note that all three of these mutant proteins (but none of the ones that look identical to Cdc11p) contain a mutation of R35 that disrupts a potential trypsin cleavage site. Moreover, in cdc11pR35T and cdc11pR35E, there is a similarity in the presence of 30 kDa band (Figure 1D, E and F-black circles, Lanes 0 μg/μl, 2 μg/μl and 4 μg/μl) and the appearance of 35 kDa band (Figure 1Figure 1D, E and F, Lanes 8 μg/μl, 16 μg/μl and 48 μg/μl) as compared to their corresponding Cdc11p. In cdc11pG32V,R35E,S36N, the 30 kDa band appears to run at a slightly lower size and also persists at a higher concentration of trypsin as compared to its corresponding Cdc11p (Figure 1J and K, Compare lane 8 μg/μl of cdc11pG32V,R35E,S36N and the corresponding Cdc11p). Also, there is no appearance (light band even if present) of the ~35 kDa band in this mutant suggesting the

protection of a cleavage site, thereby making it resistant to trypsin digestion. This suggests the protein is more stabilized as compared to the wild-type Cdc11p.

These data suggest that mutation of the Cdc11p P-loop does not result in a global change in the structure of proteins. This result suggests that the defects in these proteins in interaction with Cdc12p and in septin function is not due to overall folding defects but rather are due to specific defects in interaction with Cdc12p.,

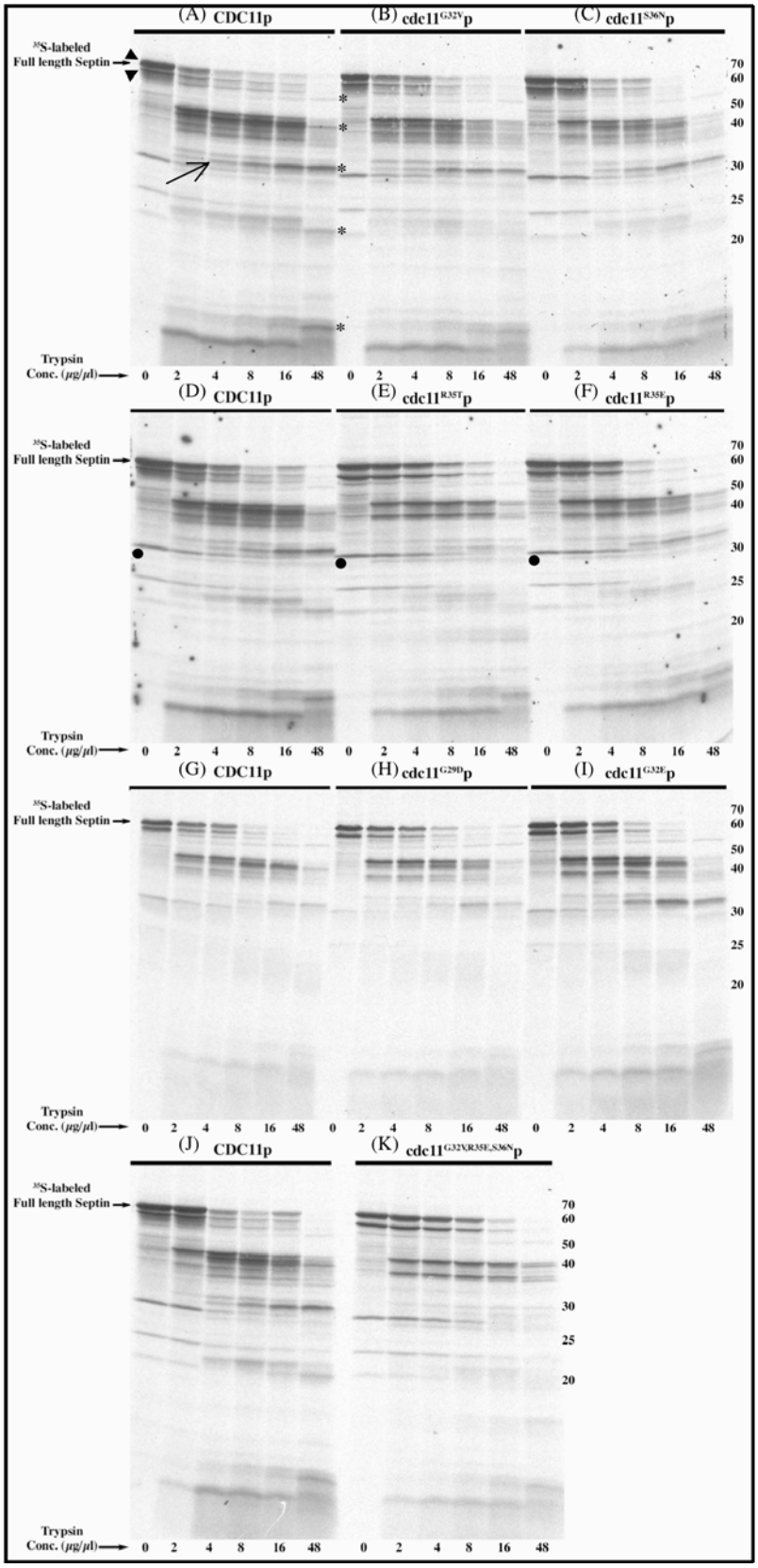

Figure 1. Peptide fingerprints of partially digested, ^{35}S-labelled wild-type Cdc11p and cdc11p P-loop-mutant proteins. RRL/TnT-synthesized proteins, as indicated, were subjected to partial proteolysis using various concentrations of trypsin. Samples were separated by SDS-PAGE, transferred to PVDF membrane and subjected to autoradiography for 24 hr. The molecular masses (kDa) of standards are indicated on the right.

References

Hartson, S.D., E.A. Ottinger, W. Huang, G. Barany, P. Burn, and R.L. Matts. 1998. Modular folding and evidence for phosphorylation-induced stabilization of an hsp90-dependent kinase. *J Biol Chem.* 273:8475-82.

Hartson, S.D., V. Thulasiraman, W. Huang, L. Whitesell, and R.L. Matts. 1999. Molybdate inhibits hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. *Biochemistry.* 38:3837-49.

Jagus, R., and G.S. Beckler. 1998. Overview of eukaryotic in vitro translation and expression systems. Current Protocols in Cell Biology. John Wiley and Sons, New York. 11.1.1-11.1.13. pp.

Scholz, G.M., S.D. Hartson, K. Cartledge, L. Volk, R.L. Matts, and A.R. Dunn. 2001. The molecular chaperone Hsp90 is required for signal transduction by wild-type Hck and maintenance of its constitutively active counterpart. *Cell Growth Differ.* 12:409-17.

APPENDIX B

INTERACTION OF SEPTINS WITH GICp PROTEINS

Introduction

In *S. cerevisiae*, the Cdc42p Rho-type small GTPase plays a central role in cell polarity. Cdc42p is activated by the Cdc24p guanine nucleotide exchange factor (GEF) that exchanges GDP for GTP resulting in activation of Cdc42p (Johnson, 1999). *S. cerevisiae* encodes Rga1p, Rga2p, and Bem3p, three redundant GTPase activating proteins (GAPs) that enhance the hydrolysis of GTP by Cdc42p, thus inactivating Cdc42p. Cdc42p function is mediated by a set of proteins, called effector proteins that interact specifically with the GTP-bound form of Cdc42p. Effector proteins bound to GTP-Cdc42p then participate in a wide variety cellular processes, including actin polarization and septin recruitment to the cortex at the incipient bud site (Johnson, 1999).

Among the 10 or so known Cdc42p effector proteins (Johnson, 1999), two are the related proteins, Gic1p and Gic2p (Johnson, 1999). Gic1p and Gic2p were identified by a search of the *S. cerevisiae* genome for proteins that contain Cdc42/Rac-interactive binding (CRIB) domains (Brown et al., 1997). Gic1p and Gic2p are 39% identical and 54% similar in their amino acid sequences (Chen et al., 1997). Consistent with their identification as likely CRIB-domain containing proteins, genetic studies suggested that

Gic1p and Gic2p play a positive role in Cdc42p function (Chen et al., 1997). Also, as expected, Gic1p and Gic2p specifically interact with the GTP-bound form of Cdc42p and this interaction involves the CRIB domains of Gic1p and Gic2p and the effector domain of Cdc42p (Chen et al., 1997).

Gic1p and Gic2p colocalize with Cdc42p to the incipient bud-site, to the tip of small buds, and to the mother-bud neck of medium and large-budded cells (Brown et al., 1997; Chen et al., 1997). By assaying for budding defects in double and triple-mutant strains, it was suggested that Gic1p and Gic2p, along with the Rsr1p-Cdc24p complex, promote and maintain the localization of protein complexes at the budding site (Kawasaki et al., 2003). One role of the Gic proteins appears to be in promoting polarization of the actin cytoskeleton as, at restrictive temperature, double-mutant g*ic1*Δ *gic2*Δ strains arrest growth as unbudded cells with a depolarized actin cytoskeleton (Brown et al., 1997).

One phenotype of a number of mutants with defects in normal septin localization is the abnormal localization of septins to the tips of a subset of cells with small buds. Among the mutants that display this phenotype are cells lacking Cla4p, a Cdc42p effector PAK kinase, and strains carrying certain mutations in Cdc42p or lacking subsets of the Cdc42p GAPs (Caviston et al., 2003; Cvrckova et al., 1995; Gladfelter et al., 2002; Smith et al., 2002; Versele and Thorner, 2004; Weiss et al., 2000). Together, these data suggest that, in the absence of GTP-hydrolysis or of Cla4p activity, septins localize to the cell cortex, but rather than transitioning into a ring that remains at the mother-bud neck, the septins colocalize to the tip of the growing bud. We hypothesize that if septins are unable to efficiently form a ring, but interact with proteins involved in their initial cortical

localization, then they localize to the tip of growing buds and that this localization may involve Cdc42p activity.

As described in Chapter 2, we found that at semi-permissive temperature, mutations in Cdc3p, Cdc10p, Cdc11p or Cdc12p P-loop domains results in the frequent localization of septins to the tip of small buds. These results suggest the possibility that in the absence of high-affinity septin-septin interactions the septins continue to localize with Cdc42p, perhaps by a continued interaction with a Cdc42p effector protein(s) that is at the tip of the bud. In agreement with this idea, a large-scale two-hybrid study found that Gic2p interacts with Cdc12p (Drees et al., 2001). This observation, coupled with the fact that Cdc42p is required for septin localization to the incipient bud site, raised the possibility that the Gic proteins may be the effectors of Cdc42p that mediate septin localization to the cell cortex.

We have been evaluating this idea in collaboration with the lab of Erfei Bi at the University of Pennsylvania Medical School. This manuscript is in preparation. The Bi lab confirmed that septin localization to the cell cortex of unbudded cells requires GTP-bound Cdc42p and that septins are unable to localize to the cell cortex of unbudded cells in a double-mutant *gic1*Δ *gic2* Ts- strain at restrictive temperature. Moreover, the Bi lab showed that septins interact with both Gic1p and Gic2p by two-hybrid assays and by co-immunoprecipitation. These data strongly suggest that the Gic proteins bound to GTP-Cdc42p are directly involved in septin localization at the incipient bud site. In my work on this project, I assayed for direct interactions between the septins and the Gic proteins using in vitro pull-down assays. As described below, this work shows that the Gic proteins interact directly with septins, showing the highest affinity for Cdc12p.

Materials and methods

Plasmids construction:

The plasmids used in this study are shown in Table I. The plasmids used for *in vitro* transcription and translation are described in Chapter II. The plasmids for expression of maltose-binding proteins (MBP) fusions of Gic1p and Gic2p in *E. coli* were constructed by Erfei Bi's lab, by sub-cloning the *BamHI*/*SalI* fragment containing *Gic1* ORF or the *EcoRI*/*SalI* fragment containing the *Gic2* ORF into the corresponding sites of *pMal-c* vector (New England Biolabs, Beverly, MA). Restriction sites flanking the *GIC* ORFs were introduced by PCR.

Protein expression and purification:

Plasmids for expression of MBP (*pMAL-c*) or MBP-Gic1p (*pMAL-GIC1*) or MBP-Gic2p (*pMAL-GIC2*) fusion proteins were transformed into *E coli* BL21 (Promega, Madison WI) and cells were grown at 23°C to mid-log phase (OD_{600} ~0.4) in medium containing ampicillin. Protein expression was induced for 5 hr by the addition of IPTG to a final concentration of 1mM at 23˚C, and cells were harvested, frozen in liquid nitrogen and stored at -70°C. To purify MBP and MBP-Gic fusion proteins, cell pellets of *E. coli* expressing MBP, MBP-Gic1p, and MBP-Gic2p were resuspended in TBS (10mM Tris pH 8.0 150mM NaCl) with 1% Triton X-100 (Sigma) and protease inhibitors (Complete, Roche). Cells were lysed by three rounds of sonication followed by incubation on ice for 30 min and centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was collected, centrifuged again at 12,000 rpm for 10 min at 4°C, and transferred to a tube

containing 200 µl bed-volume of amylose resin (New England Biolabs). After 1 hr at 4°C with gentle mixing, the beads were washed three times with 1 ml each wash of TBS with 5mM EDTA, 5mM DTT, and 0.1% Triton X-100, followed by the addition of 200μl TBS containing 1% Triton X-100.

***In vitro* transcription and translation:**

Genes expressing full-length Cdc3p, Cdc10p, Cdc11p, and Cdc12p were used to synthesize the proteins using plasmids described in Chapter II. ^{35}S-labeled septin proteins were produced using a rabbit reticulocyte lysate coupled *in vitro* transcription and translation (RRL/TnT) system, as described in Chapter II.

***In vitro* binding assay:**

To aid in elimination of background binding to MBP, 35μl of RRL/TnT-produced septins were pre-cleared by incubation with MBP-bound to amylose resin for 20 min at 23˚C and 20 μl of the supernatants were transferred to tubes containing equal amounts of amylose-resin-bound MBP, MBP-Gic1p or MBP-Gic2p. After incubation for 1 hr at 23˚C with gentle mixing, 5 µl of the supernatant was collected as the unbound fraction. The beads were washed four times with 1 ml each time of TBS with 5mM EDTA, 5mM DTT and 0.1% Triton X-100, and the supernatant removed yielding the bead-bound(bound) fraction. Bound and unbound fractions were separated by 10% SDS-PAGE and transferred on to a PVDF membrane. Ponceu S staining verified similar amounts of bound MPB or MBP-Gic fusion proteins. The PVDF membranes were then

subjected to autoradiography and to Western blot analysis to detect Cdc3p,Cdc11p and Cdc12p.

Antibodies used for Western blotting were rabbit anti-Cdc11p (Santa Cruz Biochemicals, Santa Cruz CA), affinity purified rabbit anti-Cdc3p antibodies (kindly provided by Manivannan Subramanyian) and affinity purified rabbit antibodies raised against the C-terminal 14 amino acids of Cdc12p (kindly provided by Manivannan Subramanyian). Bound anti-septin antibodies were detected using HRP-conjugated secondary antibodies and enhanced chemilumincescence (Amersham).

Table I. **Plasmids used in this study**

Plasmid	Characteristics	Source
pSP64T(S)XBBSE/CDC3FL	Amp^r Vector for SP6 coupled transcription/translation with *CDC3*	Chapter II
pSP64T(S)XBBSE/CDC10FL	Amp^r Vector for SP6 coupled transcription/translation with *CDC10*	Chapter II
pSP64T(S)XBBSE/CDC11FL	Amp^r Vector for SP6 coupled transcription/translation with *CDC11*	Chapter II
pSP64T(S)XBBSE/CDC12FL	Amp^r Vector for SP6 coupled transcription/translation with *CDC12*	Chapter II
pMal-Gic1	Amp^r Vector for Maltose binding protein fused to Gic1p	This Study
pMal-Gic2	Amp^r Vector for Maltose binding protein fused to Gic2p	This Study

Plasmids for analysis of Gic1p and Gic2p interaction with septins.

Results and discussion

To investigate if the Gic proteins interact directly with the septins, I carried out *in vitro* binding assays. Maltose-binding protein (MBP) and MBP fused to Gic1p and Gic2p were purified after expression in *E. coli* and incubated with RRL/TnT-synthesized, ^{35}S-labeled septins. Cdc10p showed non-specific binding to MBP (data not shown), and was not analyzed further. MBP-Gic1p and MBP-Gic2p both showed strong and consistent interactions with Cdc12p (Figure 1), suggesting that they directly interact with Cdc12p (Figure 1). In contrast, the Gic proteins showed a weak interaction with Cdc3p and no detectable interaction with Cdc11p. Together, these data suggest that Gic1p and Gic2p directly interact with the septins with the interaction being mediated largely by an interaction with Cdc12p

Together, two-hybrid assays and co-immunoprecipitation experiments (Erfei Bi, personal communication) and my direct biochemical experiments indicate that Gic proteins interact with the septins. The requirement of the GTP-Cdc42p and the Gic proteins for septin localization to the cell cortex, suggests that GTP-Cdc42p binding to the Gic proteins promotes their interaction with septins and mediate the localization of septins to the cell cortex at the incipient bud site. Septins are frequently localized to the tip of small buds in septin P-loop mutant strains (Chapter II) and in other mutants with defects in septin ring assembly. Based on these observations, we hypothesize that the localization of septins at bud tips reflects their continued interaction with tip-localized Gic proteins. We speculate that in the lack of high affinity septin-septin interactions (due, for example, to defects in GTP binding) septins at the cell cortex are unable to form

a cortical ring and instead remain associated with the Gic proteins as they localize to the bud tip. Consistent with this idea, we note that like the Gic in medium and large budded cells, which do not contain localized Gic proteins, septins also no longer localized to the tip of these buds.

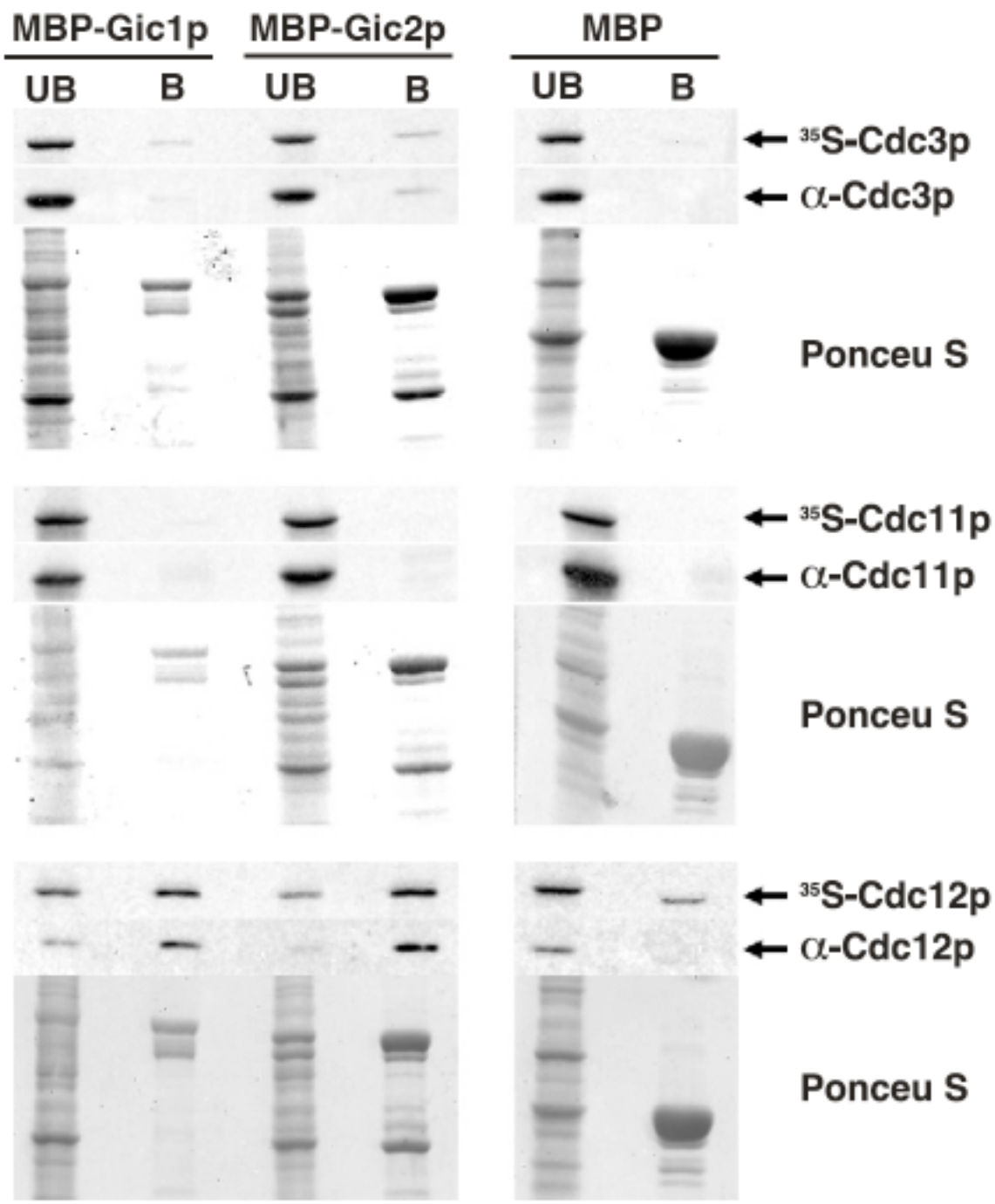

Figure 1. Gic1p and Gic2p interact directly with the septin Cdc12p *in vitro*. MBP or fusions of MBP to full-length Gic1p or Gic2p were expressed in *E. coli* and purified using amylose resin. ^{35}S-labeled full-length septins (Cdc3p, Cdc11p, and Cdc12p) were produced using RRL/TnT and pre-cleared using amylose-resin bound MBP. The pre-cleared septins were then incubated with resin-bound MBP, MBP-Gic1, or MBP-Gic2. Bound (B) and unbound (UB) fractions were collected, separated by SDS-PAGE, and transferred to PVDF membrane. Ponceu S staining indicated equivalent amounts of fusion protein. Septins present in the bound (B) or unbound (UB) fractions were detected by autoradiography and Western blotting, as indicated.

References

Brown, J.L., M. Jaquenoud, M.P. Gulli, J. Chant, and M. Peter. 1997. Novel Cdc42-binding proteins Gic1 and Gic2 control cell polarity in yeast. *Genes And Development*. 11:2972-82.

Caviston, J.P., M. Longtine, J.R. Pringle, and E. Bi. 2003. The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. *Molecular Biology of the Cell*. 14:4051-4066.

Chen, G.-C., Y.-J. Kim, and C.S.M. Chan. 1997. The Cdc42 GTPase-associated proteins Gic1 and Gic2 are required for polarized cell growth in *Saccharomyces. cerevisiae*. *Genes Dev*. 11:2958-2971.

Cvrckova, F., C. De Virgilio, E. Manser, J.R. Pringle, and K. Nasmyth. 1995. Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes and Development*. 9:1817-30.

Drees, B.L., B. Sundin, E. Brazeau, J.P. Caviston, G.C. Chen, W. Guo, K.G. Kozminski, M.W. Lau, J.J. Moskow, A. Tong, L.R. Schenkman, A. McKenzie, 3rd, P. Brennwald, M. Longtine, E. Bi, C. Chan, P. Novick, C. Boone, J.R. Pringle, T.N. Davis, S. Fields, and D.G. Drubin. 2001. A protein interaction map for cell polarity development. *Journal of Cell Biology*. 154:549-71.

Gladfelter, A.S., I. Bose, T.R. Zyla, E.S.G. Bardes, and D.J. Lew. 2002. Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *Journal of Cell Biology*. 156:315-326.

Johnson, D.I. 1999. Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiology And Molecular Biology Reviews*. 63:54-105.

Kawasaki, R., K. Fujimura-Kamada, H. Toi, H. Kato, and K. Tanaka. 2003. The upstream regulator, Rsr1p, and downstream effectors, Gic1p and Gic2p, of the Cdc42p small GTPase coordinately regulate initiation of budding in *Saccharomyces cerevisiae*. *Genes Cells*. 8:235-250.

Smith, G.R., S.A. Givan, P. Cullen, and G.F. Sprague, Jr. 2002. GTPase-activating proteins for Cdc42. *Eukaryotic Cell*. 1:469-480.

Versele, M., and J. Thorner. 2004. Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J Cell Biol*. 164:701-15.

Weiss, E.L., A.C. Bishop, K.M. Shokat, and D.G. Drubin. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nature Cell Biology*. 2:677-85.

CHAPTER VI

SUMMARY

The data presented in this thesis begins by showing the role of nucleotide-binding in regulating septin-septin interactions (Chapter II). The data was obtained by generating mutations in the nucleotide binding domains of four septins (Cdc3p, Cdc10p, Cdc11p and Cdc12p) and by characterizing these mutants by assaying for defects in various septin functions and in septin-septin interactions. In Chapter III, I discuss the analysis of additional regions that might be involved in interactions with other septins and with septin-associated proteins. In this work, I used an alanine-scanning mutagenesis approach. In two appendices, I discuss partial trypsin digestion assays done to investigate the folding of P-loop mutant septins and I discuss collaborative work, which suggests that septin localization to the cortex early in the cell division cycle involves direct interactions with Gicp proteins .

Nucleotide binding of septins regulates septin-septin interactions

Nucleotide-binding and hydrolysis defective mutants of septins were initially characterized for defects in viability, morphology and septin localization. At 23˚C, none of these mutants display a detectable phenotype. However, many display temperature-sensitive viability with corresponding defects in septin localization. These defects both

correlate with the extent of defects in nucleotide binding as shown by Ashok Rajendran (personal communication). This data also suggest that GTP hydrolysis by Cdc11p and Cdc12p does not have role in septin function. By performing arrest and release assays, we show that GTP binding is important the initial assembly of the ring but is not required for the maintenance of septin rings at the mother-bud neck. Data obtained by *in vitro* assays, show that nucleotide-binding defects cause defects in septin-septin interactions.

Functional domains of Cdc11p

In this work, we identified regions of Cdc11p with conserved charged residues. These charged residues were mutated to alanine and the resulting mutant proteins analyzed for septin localization and function. The rationale for this type of mutagenesis is that the charged residues are likely to be on the surface of the proteins and might be involved in direct interactions with other septins or septin-associated proteins. Mutating these residues would provide information about the role of Cdc11p in a particular function(s) of septins.

Initial characterization of the regions show one of them to be lethal (*cdc11ASVII*), two to be defective in viability at all temperatures (*cdc11ASI* and *cdc11ASX*) and one of to be temperature-sensitive for viability (*cdc11ASXIII*). These mutants also showed corresponding defects in morphology, septin localization and interactions with other septins. The septin localization defects in AS I and AS X differ from other mutants and the significance of these is discussed.

Trypsin nicking assay

In Chapter II, radiolabeled septins were synthesized using RRL/TnT and used for assaying for septin-septin interactions. The nucleotide-binding mutant proteins of Cdc11p do not interact with Cdc12p. One caveat of this experiment is that the absence of interactions among mutant and wild-type septins could be due to structural instability of the mutant proteins. Therefore, by performing a partial trypsin digestion assay and comparing the proteolytic fingerprints of the mutant proteins we demonstrate that cdc11p P-loop mutant proteins do not have a structure that is dramatically different from that of wild-type Cdc11p. This work is described in appendix A.

Interaction of septins with Gicp proteins

Erfei Bi's lab has shown that the Gic proteins, effectors of the Cdc42p GTPase, are necessary for the initial localization of septins to the presumptive bud-site (Iwase *et. al.* manuscript in preparation). In collaboration with the Bi lab, I carried out in vitro protein-protein interaction assays that demonstrate the septins likely directly interact with the Gicp proteins.

Concluding remarks

Until recently, the role of nucleotide binding of septins was not clear. My data suggest that a major role of GTP binding is in allowing/regulating septin-septin interactions. These interactions are important for septins filament formation and localization to the mother-bud neck. There are additional regions in the proteins that are

functionally significant and the alanine-scanning mutagenesis I carried out, are a beginning in our identification of other functionally important regions of the septins.

VITA

Satish Nagaraj

Candidate for the degree of

Doctor of Philosophy

Thesis: CHARACTERIZATION OF FUNCTIONAL DOMAINS OF *SACCHAROMYCES CEREVISIAE* SEPTINS.

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Bangalore, India, On November 14, 1973, the son of Nagaraj and Sudha.

Education: Graduated from Sri Kumaran Childrens Home, Bangalore, India in June 1989; received Bachelor of Science degree in Microbiology from Bangalore University, India in May 1994; received Master of Science in Microbiology from Bangalore University, India in May 1997. Completed the requirements for the Doctor of Philosophy degree with a major in Biochemistry and Molecular Biology in May, 2005.

Experience: Employed as a Technical Executive in XCyton Diagnostics Limited, India, 1997; employed as a Application Specialist in Millipore India Ltd, India, 1998; employed as a Project Assistant in the center for Reproductive Biology and Molecular Endocrinology in Indian Institute of Science, India from 1998-1999; employed as a Graduate Research Assistant in the department of Biochemistry and Molecular Biology, Oklahoma State University, 1999 to present.

Professional Memberships: Genetics Society for America, Biochemistry & Molecular Graduate Biology Student Association, Graduate and Professional Student Government Association

Name: Satish Nagaraj Date of Degree: May, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHARACTERIZATION OF FUNCTIONAL DOMAINS OF *SACCHAROMYCES CEREVISIAE* SEPTINS.

Pages in Study: 161 Candidate for the degree of Doctor of Philosophy

Major Field: Biochemistry and Molecular Biology

S. cerevisiae expresses 5 septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p and Shs1p) during mitotic growth. Septins interact with each other, colocalize to the cortex at the mother-bud neck, and are required for the localization and function of proteins involved in cytokinesis, bud-site selection and chitin deposition. Septin nucleotide binding and/or hydrolysis may regulate the interaction of septins with septins or non-septins. We assayed septin mutations predicted to disrupt nucleotide binding. In contrast to cells lacking a particular septin, at 23˚C septin nucleotide-binding mutant cells are fully viable and show efficient completion of septin-dependent processes suggesting septin nucleotide binding is not required for the function of septin-interacting proteins. Septin nucleotide-binding mutations show temperature-sensitive viability, which correlates with the predicted defects in nucleotide binding and with the extent of defects in septin localization. Two-hybrid and in vitro assays show septins interact directly with each other in a nucleotide-binding-dependent manner. Thus, septin nucleotide binding promotes septin-septin interactions and septin localization. To identify the particular regions in septins involved in protein-protein interactions, I used alanine-scanning mutagenesis to mutate charged residues (presumed to be on the surface of the protein) in Cdc11p. I describe the effects of these mutations on cell viability, cell division, cell morphogenesis and on septin-septin interactions.

Advisor's Approval Dr. Mark. S Longtine