# ROLE OF GTP BINDING IN YEAST SEPTINS

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## ABBREVIATIONS

| | |
|---|---|
| 2-D | 2-Dimensional |
| ATP | Adenosine triphosphate |
| BBF | Bead beating buffer |
| CFP | Cyan fluorescent protein |
| DTT | Dithiothreitol |
| DNA | Deoxyribonucleic acid |
| EB | Elution buffer |
| FRAP | Fluorescence recovery after photo bleaching |
| GAP | GTPase activating protein |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green fluorescent protein |
| GTP | Guanosine triphosphate |
| HPLC | High pressure liquid chromatography |
| kDa | Kilo Dalton |
| µl | micro liter |
| MLL | Mixed lineage leukemia |
| mM | milli Molar |
| NTP | Nucleotide triphosphate |
| pH | -log $[H^+]$ |
| PMSF | Phenyl methyl sulfonyl fluoride |
| PP2A | Protein phosphatase 2A |
| PVDF | Polyvinylidene fluoride |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| RRL | Rabbit reticulocyte lysate |
| SA | Specific activity |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TNT | In vitro transcription and translation |
| UV | Ultraviolet |

# CHAPTER 1

# INTRODUCTION

## 1.1 Septin:

Septins are a highly conserved family of GTP binding proteins found in all eukaryotes, with the exception of plants. Septins were originally identified in *Saccharomyces cerevisiae* in the Hartwell collection of temperature -sensitive mutants defective in various stages of the cell division cycle (Hartwell, 1971). *S. cerevisiae* encodes seven septins: *CDC3, CDC10, CDC11, CDC12, SHS1, SPR28* and *SPR3*. Among septins, there is about 35-90% sequence identity (Gladfelter et al., 2001; Longtine et al., 1996). The size of septins ranges from ~40-75 kDa. The N-terminus of septins contains a nucleotide-binding domain composed of G1 motif (P-loop domain), G3 and G4 motifs while the C-terminal of most septins contains a predicted coiled-coil domain.

In *S. cerevisiae,* Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p are expressed during vegetative growth. These septins initially localize to the cell cortex at the presumptive bud site (Longtine et al., 1996). As the bud emerges, septins rearrange from a cortical ring to an hourglass-shaped collar spanning both sides of the mother-bud neck just under the plasma membrane. This hourglass structure remains until right before cytokinesis when the hourglass structure splits into two distinct rings. These rings persist

at the cortex of the two daughter cells for a short period after the completion of cytokinesis (reviewed in Longtine and Bi, 2003). .

During sporulation, Cdc3p, Cdc10p, Cdc11p, Shs1p, and the sporulation-specific septins Spr3p and Spr28p, are expressed. These septins localize to membrane regions involved in meiosis and spore formation.

Two models have been developed to explain septin function. In the scaffold model , the septin collar at the mother-bud neck is hypothesized to act as a host directing the localization of various non-septin proteins involved in diverse processes including cell wall synthesis (DeMarini et al., 1997), spindle orientation (Kusch et al., 2002) and cytokinesis (Bi et al., 1998).

## 1.2 Functions of septins

### 1.2.1 Cytokinesis:

In yeast, cytokinesis is carried out by two independent, but connected, processes; the contraction of the actomyosin ring and formation of the primary septum (Bi et al., 1998; Lippincott and Li, 1998). The yeast type II myosin, Myo1p, is a component of the actomyosin ring and chitin synthase II (CSII), which is involved in the formation of primary septum, both localize to the yeast mother-bud neck (Lippincott and Li, 1998). Cells that lack septins at the mother-bud neck are unable to undergo cytokinesis due to defects in localization of components of the actomyosin ring and of proteins that deposit the primary septum. However, septins localize apparently normally in cells lacking components of the actomyosin ring or CSII complex, supporting the concept that a septin

"scaffold" localizes proteins involved in cytokinesis, thereby being involved in cytokinesis

Recently, another model for the role of septins in cytokinesis has been hypothesized, by demonstrating a role of septins in restricting the diffusion of membrane-associated proteins. Takizawa and co-workers showed that Ist2p (a transmembrane protein) specifically localized to the bud plasma membrane of the bud diffuses rapidly within the bud. However, GFP-tagged Ist2p did not cross the bud neck and enter the mother-cell membrane. However, after shift of a *cdc12-6* septin mutant to 37˚C, resulting in delocalization of septins from the neck, GFP-Ist2p now diffused across the mother-bud neck and entered the plasma membrane of the mother-cell. These data suggest that membrane-localized septins form a barrier to diffusion across the mother-bud neck. Similarly, Lte1p, a guanine-nucleotide exchange (GEF) protein for the small G protein Tem1p, localizes specifically to the cortex of the bud. However, in *shs1*Δ*/sep7*Δ and *cdc10*Δ strains, with perturbed septin localization, Lte1p is also present in the mother cell. Normally, after the switch to isotropic growth, actin patches and proteins involved in polarized growth are restricted to the cortex of the bud. However, Barral et al. (2000) found that if septins are delocalized then both actin patches and polarity proteins can now be detected in the cortex of the mother cell. Together, these data strongly argue that in yeast septins can serve as a barrier to the movement of transmembrane and membrane-associated proteins.

Recently Schmidt and Nichols (2004) provided evidence for the role of septin in forming a diffusion barrier in mammalian cell. Diffusion of the lipid analog dialkylindocarbocyanine (DiI C18) and the membrane proteins (differing in topology and

lipid association) across the cleavage furrow was measured. The movement of proteins with a cytosolic domain was restricted whereas there was no block in the diffusion of proteins anchored in the outer leaflet of the plasma membrane or of DiI. The pattern of distribution of septin was consistent with a functional role in limiting diffusion, suggesting a possible role in the formation of diffusion barrier in the mammalian cells. Septins are also found on either side of the contractile ring during cytokinesis in *S. pombe*. These data indicate that a septin-dependent barrier to diffusion is a conserved property of cortically localized septins.

Dobbelaere and Barral (2004) identified another function for a septin-based diffusion barrier. During cytokinesis, after the splitting of the septin hourglass structure into two discrete septin rings, they showed that proteins involved in cytokinesis, including the polarizome complex involved in actin filament nucleation, the exocyst involved in secretion, and chitin synthase II involved in formation of the primary septum, all co-localize within the split septin rings. These data suggest that the septins do not serve as a scaffold to recruit cortical proteins, but that septins instead formed a boundary for the localization of these proteins during cytokinesis. To investigate whether or not the proteins localized between the split septin rings were indeed compartmentalized, Dobbelaere et al. did fluorescence recovery after photo bleaching (FRAP) analysis. These data indicated that the proteins localized within the split septin rings indeed are restricted from exchange with proteins outside the neck region. Furthermore, they showed that intact septin rings are required for the restricted movement of the proteins localized between the split septin rings. Thus, another role (at least in yeast) is to provide

a barrier that restricts the localization of proteins involved in cytokinesis to the proper region of the cell.

### 1.2.2 Bud site selection:

Selection of the location of bud emergence ensures proper selection of an axis for cell polarization. Haploid and diploid yeast cells have spatially distinct patterns of budding. In haploid cells the new bud is formed in the axial pattern, being adjacent to the previous bud site (which is also the site of cytokinesis in the preceding cell cycle). In bipolar budding, a characteristic of diploid cells, buds are formed both at the distal and proximal poles. The budding site in the subsequent generation in yeast is determined by spatial landmark proteins localized at the cell poles, including at the mother-bud neck, in the previous cycle. The *BUD3* gene is required for the axial pattern of budding and any mutations of *BUD3* affect the axial pattern. Genes of *BUD8* and *BUD9* encode components of the markers at the distal and proximal poles of the daughter cell and are involved in bipolar budding pattern. When *cdc12* mutant was shifted to restrictive temperature, localization of Bud3p was rapidly lost (Chant, 1999; Schenkman et al., 2002). Bud8p and Bud9p were also shown to be septin dependent for their localization (Chant, 1999; Schenkman et al., 2002). These data indicate that the localization of landmark proteins involved in bud-site selection is septin dependent. Conversely, septin localization is normal in cells lacking bud-site selection proteins. Together, these data are consistent with a model in which the septins serve as a scaffold to localize proteins involved in bud-site selection.

### 1.2.3 Morphogenesis and cell cycle progression:

In response to perturbation of the actin cytoskeleton, yeast cells activate the "morphogenesis" checkpoint, in which the Cdc28p Cdk complexed with B-type cyclins is phosphorylated by the checkpoint-activated kinase Swe1p on tyr 19 of Cdc28 (Lew, 2000). This phosphorylation inactivates Cdc28p/Clb complexes, arresting cells prior to mitosis. In septin mutants, Swe1p is activated, and (because actin remains polarized at the bud tip) results in elongated cells. Work from several labs (Barrel et al., 1999: Shulewitz et al., 1999. Longtine 2000) indicate that Swe1p and its negative regulators, Hsl1p and Hsl7p localize to the mother-bud neck in a septin-dependent manner. In the absence of neck localized septins, Hsl1p and Hsl7p no longer repress Swe1p activity, and the resulting active Swe1p results in a delay in cell-cycle progression and the elongated-bud phenotype typical of septin mutant strains. The absence of Hsl1p, Hsl7p, or Swe1p does not affect septin localization. Thus, septins appear to serve as a scaffold to localize a complex of proteins involved in the regulation of cell cycle progression.

### 1.2.4 Spindle orientation:

Localization of septins and an actomyosin contractile ring (cleavage apparatus) at the cell cortex (Field and Kellogg 1999; Field et al. 1999) determines the plane of cleavage in *S.cerevisiae*. The presence of the cleavage apparatus is necessary for the positioning of the nucleus to the bud-neck. The microtubule organizing centers (spindle pole bodies) are present at the nuclear envelope and the spindle formation occurs inside the nucleus. Spindle positioning in the bud neck is essential, as spindle elongation causes the equal distribution of chromosomes between the daughter and the mother cell. The

proper position of the spindle, relative to the cleavage apparatus, depends on the capture and shrinkage of the astral microtubules emanating from the spindle pole bodies. By using a temperature sensitive *cdc12-1* mutation, it was shown that septin function was required for proper nuclear migration and positioning of the spindle relative to the cleavage plane (Kusch et al., 2002). This work also found that both Tub1-GFP and Kar9-GFP show transient interactions with the bud neck and that these interactions typically resulted in movement of the nucleus towards the neck. Thus, it appears as if septins interact (directly or indirectly) with microtubules and have a role in proper spindle orientation and migration prior to mitosis.

**1.2.5 Secretion:**

In all cells, secretion of new material to the plasma membrane is essential for cell growth prior to cell division. In *S. cerevisiae*, bud emergence occurs by the specific and targeted delivery of new membrane and cell wall components to the growing bud. The general pathway of secretion is fairly well characterized; in polarized secretion, vesicles are carried along actin cables by Myo2p, a type V myosin, and are tethered to the sites of secretion by a multiprotein complex called the exocyst (Novick et al., 1995). The final vesicle fusion step, conserved in yeast, is mediated by formation of a SNARE complex, which involves the vesicle-associated v-SNAREs Snc1p or Snc2p associating with the plasma membrane- associated t-SNARES, Sso1p/Sso2p, and with the SNAP 25 homolog, Sec9p (Sollner et al., 1993). The Munc-18 homolog, Sec1p, plays a positive role in vesicle fusion by binding to productive preassembled trans-SNARE complexes (Carr et al., 1999).

The current knowledge of septin function in secretion comes largely from the studies of mammalian septins, especially in regards to function in neuronal secretions. Several lines of evidence suggest the involvement of septins in secretion; the mammalian septin *CDC10* co-immunoprecipitates with the Sec6/8 (exocyst) complex (Hsu et al., 1998); the neuronal septin Sept5 was shown to bind directly to the t-SNARE, syntaxin 1-A (Beites et al., 1999); and cell fractionation and immunoelectron and immunofluorescent microscopic studies indicate a possible association of septins with secretory vesicles (Beites et al., 1999; Dent et al., 2002; Kinoshita et al., 2000). Secretion assays using cultured HIT-T15 and platelets from mouse lacking Syntaxin-4 indicated a negative role for septins in secretion (Beites et al., 1999; Blomberg et al., 1999). In accordance with these results, it was recently observed that α-SNAP, which is involved in disassembling cis-SNARE complexes present after vesicle fusion, shows competitive binding to SNAREs with septins, suggesting that septin interactions with SNARE proteins may play a negative role in secretion (Beites et al., 2005).

In yeast, the plasma membrane t-SNAREs Sso1p and Sso2p localize throughout the periphery of the plasma membrane while several other components involved in targeted secretion localize to the region of polarized growth (the bud tip, the bud periphery, or to the mother-bud neck during cytokinesis). In a recent study Gladfelter et al. (2005) observed that temperature sensitive *cdc42*$^{V36T,K94EA}$ cells displayed wide necks with misorganized septins at the neck and mislocalized septins at the bud tip (Gladfelter et al., 2002). However in *cdc42* $^{V36T, K94EA}$ *cdc12-6* double-mutant cells, which lack localized septins wide bud necks were not observed. These results suggest that, at least in yeast, septins may play a positive role in directing secretion. Recent work from our lab

(Manivannan Subramaniyan, personal communication) suggests that the yeast Cdc3p septin may interact with the yeast plasma-membrane t-SNAREs, Sso1p and Sso2p. Thus, it appears as if septin-SNARE interactions may be conserved, but the exact function of these interactions may differ between mammalian cells and yeast.

### 1.2.6 Mating:

In response to mating pheromone, cells develop a characteristic pointed mating projection, known as Shmoo. Shmoos form prior to fusion of cells of the opposite mating type and formation of the diploid cell. Septins are found as diffused band at the base of shmoos, (Ford and Pringle, 1991; Kim et al., 1991) and Giot and Konoka (1997) reported that cells carrying a temperature-sensitive mutation (cdc12-6) were defective in shmooing. In addition, Cdc12p interacts with Afr1p, the protein induced by mating pheromone, and improves the ability of Afr1p to promote shmoo morphogenesis. Thus, septins appear to have a role in mating, though further work needs to be done to clarify this possible role.

## 1.3 Filament formation and dynamics of septin complexes:

Septins purified from *S. cerevisiae* (Frazier et al., 1998; Versele et al., 2004), *D. melanogaster* (Field et al., 1996) and mammals (Hsu et al., 1998) form filaments *in vitro*. However, the role of filament formation and nuclear organization of septin complexes remain unclear. Septins purified from wild type *S. cerevisiae* cells under low salt conditions, formed filaments measuring 7-9 nm in diameter. Extensive filament pairing was also observed. However, *cdc10Δ* or *cdc11Δ* strains at 23˚C did not form long or

paired filaments *in vitro* and filamentous appearing structures were not visible *in vivo* (Frazier et al., 1998). Nevertheless, in *cdc10*Δ strains septins are largely functional, suggesting that long filaments are not necessary for septin function.

Dynamics of septin localization and assembly is important in understanding septin function. Cdc42p, a rho-family GTPase, and its activating factor Cdc24p, are required for septin delivery to the future bud site. Gladfelter et al. (2002), proposed a model in which Cdc42 resembles the EF-Tu/EF-1α GTPase and hydrolysis of Cdc42p-bound GTP has a direct role in septin ring formation. Caviston et al. (2003), proposed another model in which Cdc42 acts as a Ras-like molecular switch along with its GTPase activity protein (GAPs). According to the model of Caviston et al. (2003), septins are first recruited to the presumptive bud after which assembly of the ring takes place. Cdc42 and its GAPs are required for the above processes. Recently, a patch structure of septins was shown to form before it transforms into the ring (Erfei Bi, personal communication). The mechanism of transition of septin patch into a cortical ring in unbudded cells and into the septin hourglass at the neck of budded cells is not clearly understood. It has been suggested that GTP binding to Cdc10p and Cdc12p and septin phosphorylation of septin by Cla4, a PAK kinase, may be necessary for the transition into the septin collar (Versele and Thorner, 2004).

EM studies on yeast septins showed 10 nm striations at the bud neck but striations were not seen in unbudded cells (Byers and Goetsch, 1976). The striations are absent during late cell cycle, which may be due to the splitting of septin collar into a double ring structure. Fluorescence Recovery After Photo bleaching (FRAP) analysis revealed that septin rings are dynamic and unstable before bud emergence but that septins in the

hourglass are non-dynamic while septin rings after hourglass splitting are again dynamic (Dobbelaere et al., 2003). These studies indicate that septin ring consists of less organized septin assembly compared to the septin collar, which may have a more stable and high order organization.

### 1.4 Importance of septin organization in its function:

At elevated temperatures, temperature sensitive yeast septin mutants have elongated bud morphology and defective septin structure (Lew, 2000; McMillan et al., 1999). Proteins encoded by *BN15*, *CLA4*, and *GIN4* are important in septin organization as deletion of any of these genes results in abnormal septin localization and organization and in elongated buds (Longtine et al., 2000; Sreenivasan et al., 2003). Moreover, perturbation in septin organization causes the arrest of cells in the G2/M phase of the cell cycle indicating that septin mis-organization results in a cell cycle delay. The function of septins as a barrier to diffusion likely requires highly complex organization. However, Longtine et al. (1998), showed that for at least some septin functions, abnormal septin organization at the neck can still result in significant septin function. In *gin4Δ* cells septins functioned efficiently to localize proteins involved in bud-site selection and chitin deposition, even though septin organization was clearly abnormal.

### 1.5 Septins in other organisms:

#### 1.5.1 Septins in *D. melanogaster:*

The genome of *D. melanogaster* encodes five septins - Pnut, Sep1, Sep2, Sep4 and Sep5 (Fares et al., 1995; Field et al., 1996; Neufeld and Rubin, 1994) and (Adam et

al., 2000). Pnut, the first septin identified in *D. melanogaster*, is thought to be involved in cytokinesis. Pnut mutants die as pupae with small imaginal discs due to the defects in cytokinesis (Neufeld and Rubin, 1994). In addition to Pnut, Sep1 and Sep2 are also involved in cytokinesis. *In vitro*, reconstitution of septin complexes isolated from *D. melanogaster* embryos, Pnut, Sept1 and Sept7 were found in stoichiometric amounts (Field et al., 1996). Though the role of Sep4 and Sep 5 in *D. melanogaster* is not clear, it is believed that these septins may be involved in non-cytokinetic processes like vesicle trafficking and exocytosis in non-dividing neuronal cells.

**1.5.2 Septins in mammals:**

Septins are a multigene family in mammals comprising of 12 septins (*SEPT*1-12), some of which produce multiple splice variants (Macara et al., 2002; Surka et al., 2002). Mammalian septins are predicted to be involved in vesicle transport and exocytosis (Beites et al., 1999). Some septins, such as Sept 5/CDCrel-1, are expressed almost exclusively in the brain (Caltagarone et al., 1998; Xue et al., 2000). Sep 5 is also a substrate for Parkin, an E3 ubiquitin ligase involved in Parkinson's disease.

Sept 2 and sept 5 were shown to interact with the SNARE protein syntaxin (Beites et al., 1999). Sept 2, Sept 4, Sept 6 and Sept 7 associate with the exocyst complex, which is involved in polarized secretion (Kinoshita et al., 1998). Septins were also presumed to be involved in regulating exocytic events, but the mechanism is still not clear (Beites et al., 1999).

Septins are also involved in various human diseases and neurodegenerative disorders (Kartmann and Roth, 2001). Septin genes are found in genes encoding MLL

(mixed lineage leukemia). The experimental results of (Liauw et al., 2002) shows that sept 7 expression is down-regulated in Alzheimer's disease. Sept 5 over-expression in the brain results in dopamine neurodegeneration (Dong, 2003). Sept 1, Sept2 and Sept 4 accumulate in filamentous deposits called neurofibrillary tangles (Kinoshita et al., 1998). Sept 4 is found in the α-synuclein, a protein responsible for the formation of pathological filamentous aggregates scientifically known as lewy bodies (Ihara et al., 2003). These data indicate that septins play an essential role in human brain function.

### 1.5.3 Septins in *C. elegans:*

UNC-59 and UNC-61 are the two septins encoded in the *C.elegans* genome. These septins are not involved in embryonic cytokinesis. These septins are reported to be involved in post-embryonic neuroblast cell division (Nguyen et al., 2000). These septins were first isolated as uncoordinated (unc) mutants in *C. elegans* (Brenner, 1974). Mutations in septins caused uncoordination in newly hatched larvae in the absence of cytokinetic defect (Finger et al., 2003) suggesting roles for septins in addition to cytokinesis. Possible roles include roles in axon guidance and migration during *C. elegans* embryogenesis (Choi et al., 2003). These data, and the fact that septins are not expressed in neurons of the adult worm, led the authors to conclude that septins have a developmental, rather than a functional, role in the *C. elegans* nervous system.

# CHAPTER 2

# ROLE OF NUCLEOTIDE BINDING IN YEAST SEPTINS

## 2.1 P-Loop in septins:

All septins possess an N-terminal P-loop nucleotide-binding domain, which is characteristic of GTP and ATP binding proteins. It has been reported that *D. melanogaster* (Field et al., 1996), human (Kinoshita et al., 1997b) and yeast septins (Field et al., 1996) bind GTP. The conserved glycines in the P-loop are necessary for proper folding of this nucleotide-binding pocket; the conserved lysine forms a bond with the phosphate, and the conserved serine/threonine aids in the co-ordination with $Mg^{2+}$. Mutation of these residues disrupt nucleotide binding and/or hydrolysis of a wide variety of nucleotide binding proteins (Bourne et al., 1991).

## 2.2 Septin nucleotide binding:

The role of septin nucleotide binding and/or hydrolysis is poorly understood. Various models have been hypothesized on the role of GTP binding and hydrolysis. First is the polymerization model, where GTP binding and hydrolysis is required for the polymerization of septins into filaments, that GTP binding may play a solely structural role, simply being required for proper septin folding. In yet another model it has been proposed that GTP binding aids in the formation of heteromeric complex (complex assembly model) rather than polymerization. Since all septins posses a conserved GTP-

binding domain, septins can be compared with GTPase-signaling proteins and thereby septin function can be related to their ability to bind and hydrolyze GTP. *In vitro* studies by Field et al., 1996 showed that purified *D. melanogaster* septin complexes strongly bound to GTP/GDP and had GTPase-activity, but this GTP binding/exchange of *D. melanogaster* septin was found to be very slow. Individual recombinant septins and mammalian septins showed faster GTP binding/exchange kinetics *in vitro*. Cdc3p, Cdc10p, Cdc11p and Cdc12p immunopurified from *S. cerevisiae* bind guanosine nucleotides in stoichiometric amounts, with a GDP: GTP ratio of approximately 2.2:1. Despite the different number of septin polypeptides in the septin complex, the nucleotide: septin ratios and the GDP:GTP ratios are highly conserved in *S. cerevisiae, D. melanogaster* and mammals. This evolutionary conservation suggests that GTP binding may be necessary for stabilizing the septin polypeptides and that nucleotide binding plays a structural role, analogous to the role of GTP for ∝-tubulin.

Versele et al. (2004) studied the GTPase function of yeast septins. In their work, which used yeast septins expressed in E. coli, only Cdc10p and Cdc12p bound and hydrolyzed in vitro. A cdc12 mutant (*cdc12Δ*$^{339\text{-}407}$) that lacked the coiled-coil domain, had a five-fold increase in the rate of GTP hyrolysis as compared to full-length Cdc12p. They also studied the effect of GTP binding to *CDC12* and *CDC10*. They made mutants in which GTP-binding or GTP hydrolysis was specifically abrogated. Using these mutants, the authors found that GTP hydrolysis might not play an important role in vivo in septins. Furthermore, they have shown that GTP binding may be essential for the proper assembly of the septin collar at the bud neck, although GTP binding did not appear, in their conditions, to be required for efficient formation of heteromeric septin

complexes. A double-mutant strain with *cdc10 cdc12* GTP-binding defective alleles displayed a more dramatic defect in septin localization and function than either single mutant, suggesting redundancy of function. By reconstituting wild-type and mutant septin complexes in vitro and by examining the filament formation by electron microscope the authors found that under low salt condition, wild-type septins formed long filaments where as GTP-binding deficient mutant failed to form long filaments, further indicating that GTP binding may be involved in the formation of higher order septin complexes.

The role of GTP binding in mammalian septins has also been demonstrated in forming hetero-oligomeric polymers. (Kinoshita et al., 1997a) showed Nedd5 (SEPT2) mutants lacking GTP-binding activity disrupted Nedd5 containing fibers and failed to organize into filamentous like structures, implying that GTP binding is required for its assembly. They also demonstrated that immunological depletion of SEPT2 interfered with cytokinesis. Kinoshita et al later demonstrated that SEPT2, 6 and SEPT7 formed filaments in the presence of anillin (actin-binding protein). The involvement of Sept5 (CDCrel-1) in exocytosis has been demonstrated in which it was found that transfection of HIT-T15 cells with dominant negative mutation (defective in GTP-binding) promoted secretion, where as wild type CDCrel-1 reduced secretion. SEPT2, SEPT6 and SEPT9 have also been shown to bind GTP (Kinoshita et al., 1997; Sheffield et al., 2003; Robertson et al., 2004).

The role of nucleotide binding in septin function has been complicated by conflicting results; GTPase activity of bacterially expressed recombinant Sept2 was reported to promote septin polymerization; Mendoza et al. (2002) expressed a single

recombinant septin polypeptide (xenopus Sept 2) in bacteria; septin complexes purified from *Drosophila* bound to GTP but GTP hydrolysis did not influence septin polymerization (Field et al., 1996); endogenously added guanine nucleotides had no effect on *in vitro* assembly of filaments from multi-septin complexes (Kinoshita et al., 2002; Sheffield et al., 2003). Future studies are necessary to resolve or explain these contradicting results.

Zhang et al., (1999), showed that mammalian septin H5 (Sept4) is associated with the plasma membrane and binds to the phospholipids phosphatidylinositol 4,5-bisphosphate (Ptdlns(4,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (Ptdlns(3,4,5)P3). A conserved polybasic region in Sept4, which was essential for the interaction with phospholipids, was found next to the GTP binding motif. GTP binding and hydrolysis by Sept4 significantly reduced its Ptdlns (4,5) P2-binding capability, indicating that septin-GTP binding regulates the interaction of septins with lipids thereby playing an important role in the binding of septins to membranes.

There are various models hypothesized regarding septin GTP-binding/ hydrolysis and septin function such as the "scaffold model" and the "diffusion barrier model". Complete knowledge about the architecture of the septin cortex and its regulation is necessary to understand septin function. Though septin localization is essential for its function, little is known about the mechanism of septin localization. There are many questions regarding septin organization and function that remain unanswered. Do GTP binding and hydrolysis play a role in septin-septin interaction or septin non-septin protein interaction? Is GTP-binding/hydrolysis essential for septin localization? Answers to above question are of great biological importance and will yield many insights in the near

future. The work presented here brings new insights about the role of nucleotide binding in septin-septin interaction, septin localization and septin dependent processes.

# CHAPTER 3

# MATERIALS AND METHODS

## Table 1: Yeast strains used in this study

| Strain | Genotype |
|---|---|
| M-2297 | a *cdc11-6* |
| M-2299 | a *cdc11Δ::TRP1 ura3-52*:YIp211*(URA3):cdc11R35E* |
| M-2301 | a/□ cdc11Δ::TRP1/cdc11Δ:: TRP1 ura3-52:YIp211*(URA3):cdc11R35E/ura3-52*:YIp211*(URA3):cdc11R35E* |
| M-2305 | a *cdc11Δ::TRP1 ura3-52*:YIp211*(URA3):cdc11S36N* |
| M-2311 | a *cdc11Δ::TRP1 ura3-52:*YIp211*(URA3):cdc11R35T* |
| M-2313 | a/□ *cdc11Δ::TRP1/cdc11Δ::TRP1 ura3-52:*YIp211*(URA3):cdc11R35T/ura3-52:*YIp211*(URA3):cdc11R35T* |
| M-2322 | a/□*cdc11Δ::TRP1/cdc11Δ::TRP1ura3-52:*YIp211*(URA3):cdc11G32V#1/ura3-52:*YIp211*(URA3):cdc11G32V swe1Δ::LEU2/swe1Δ::LEU2* |
| M-2323 | a *cdc11Δ::TRP1 ura3-52:*YIp211*(URA3):cdc11G32V* |
| M-2332 | a *cdc11Δ::TRP1 ura3-52:*YIp211*(URA3):CDC11WT* |
| M-2334 | a/□ *cdc11Δ::TRP1/cdc11Δ::TRP1ura3-52:*YIp211*(URA3):CDC11WT/ura3-52:*YIp211*(URA3):CDC11WT* |
| M-2344 | a/□ *cdc11-6/cdc11-6* |
| M-2453 | α *pep4Δ::HIS3MX6* w/pEGKT *(2μM URA3 LEU2d GALprom-GST*) |
| M-2454 | α *pep4Δ::HIS3MX6* w/YEp195/*GST-CDC11FL (2μM URA3 GALprom/GST-CDC11WTFL)* |
| M-2469 | α *pep4Δ::HIS3MX6* w/YEp195/*GST-cdc11G32V (2μM URA3 HIS3-GALprom/GST-cdc11G32V)* |
| M-2470 | α *pep4Δ::HIS3MX6* w/YEp195/*GST-cdc11R35E (2μM URA3 HIS3-GALprom/GST-cdc11R35E)* |
| M-2672 | a *cdc11Δ::TRP1 with* YCp111/*cdc113nt* |
| M-2840 | a *cdc12ΔPCR-2::TRP1* [w/ YCp111/*cdc12 D98A,G101A*] |
| M-2855 | a *cdc10Δ::HIS3* w/YCp111/*CDC10 S-X WTa TS+ w/(pRS316/GFP-CDC3*) |
| M-2857 | a *cdc10Δ::HIS3* w/YCp111/*cdc10K45Ta ts-w/(pRS316/GFP-CDC3)* |
| M-2895 | a *cdc10Δ::HIS3* w/YCp111/*cdc10G42V,K45E,S46N*c and w/pRS316/GFP-C3 |
| M-2908 | α *cdc3ΔPCR-5::TRP1 w/* YCp111/*CDC3ΔS-XWTa & pRS76/GFP-CDC12 (CEN)TS* |
| M-2915 | a *cdc11Δ::TRP1* w/ YCp111/*cdc11ASV* |
| M-2917 | a *cdc11Δ::TRP1* w/ YCp111/*cdc11ASIX* |
| M-2913 | α *cdc3ΔPCR-5::TRP1* w/ YCp111/*cdc3G129V,K132E,T133N*a & *pRS76/GFP-CDC12 (CEN HIS3)TS+* |
| YNT-81 | α *cdc3ΔPCR-5::TRP1* w/ YCp111/*CDC3ΔS-XWTa* |
| YNT-107 | a *cdc12Δ::TRP1* YCp111/*CDC12WTa* |
| YNT-129 | a *cdc10Δ::HIS3* w/ YCp111/*cdc10G42V,K45E,S46Nc* |
| YNT-131 | a *cdc12Δ::TRP1* w/ YCp111/*cdc12G44V,K47E,T48Na* |
| YNT-133 | a *ura3-52 lys2-801$^{amber}$leu2-Δ1 his3-Δ200 trp1-Δ63* w/ YCp111a |
| YNT-179 | α *cdc3ΔPCR-5::TRP1* w/ YCp111/*cdc3G129V,K132E,T133Na* |
| YNT-223 | a/□ *cdc10-1/cdc10-1* w/ YCp111/ *cdc10G42Va* |
| YNT-233 | a *cdc12Δ::TRP1* w/ YCp111/*cdc12G44Va* |

[a] *pSP64TT1R16HisN/CDC3FL* Amp$^r$ Vector for SP6 coupled transcription/translation, *TRP1 ARS1,* 6xHis, *URA3, CDC3*
[b] *pSP64TT1R16HisN/CDC10FL* Amp$^r$ Vector for SP6 coupled transcription/translation, *TRP1 ARS1,* 6xHis, *URA3, CDC10*
[c] *pSP64TT1R16HisN/CDC11FL* Amp$^r$ Vector for SP6 coupled transcription/translation, *TRP1 ARS1,* 6xHis, *URA3, CDC11*
[d] *pSP64TT1R16HisN/CDC12FL* Amp$^r$ Vector for SP6 coupled transcription/translation, *TRP1 ARS1,* 6xHis, *URA3, CDC12*
[e] *pSP64T(S)XBBSE/CDC3FL* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC3*
[f] *pSP64T(S)XBBSE/CDC10FL* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC10*
[g] *pSP64T(S)XBBSE/CDC11FL* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC11*
[h] *pSP64T(S)XBBSE/CDC12FL* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC12*
[i] *pSP64T(S)XBBSE/CDC11G32E* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC11G32E*
[j] *pSP64T(S)XBBSE/CDC11G32V* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC11G32V*
[k] *pSP64T(S)XBBSE/CDC11R35E* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC11R35E*
[l] *pSP64T(S)XBBSE/CDC11R35T* Amp$^r$ Vector for SP6 coupled transcription/translation with *CDC11R35T*

[a] *In vivo* Recombination of XhoI cut pSP64T-T1R16xHisURA3 with PCR product of ML677 & ML678.
[b] *In vivo* Recombination of XhoI cut pSP64T-T1R16xHisURA3 with PCR product of ML679 & ML680.
[c] *In vivo* Recombination of XhoI cut pSP64T-T1R16xHisURA3 with PCR product of ML669 & ML671
[d] *In vivo* Recombination of XhoI cut pSP64T-T1R16xHisURA3 with PCR product of ML672 & ML675.

[e]Subcloned BamHI/SmaI fragment containing *CDC3* from pGEX4T/CDC3 into BglII/SmaI digested pSP64T(S)XBSSE.
[f]Subcloned XbaI/SacI digested PCR product (~1.2 kb) of ML477 & ML478 on pALTER/CDC10 into XbaI/SacI digested pSP64T(S)XBSSE.
[g]Subcloned ~ 1.3kb SalI (blunted)/BglII fragment containing *CDC11* from pSL301/CDC11 into BglII/SmaI digested pSP64T(S)XBSSE.
[h]Subcloned ~ 1.3kb NotI (blunted)/BamHI fragment containing *CDC12* from pGEX4T/CDC12 into BglII/SmaI digested pSP64T(S)XBSSE.
[i]*In vivo* Recombination of BglII/MfeI cut pSP64T(S)XBSSE/CDC11 with the BglII/MfeI digested PCR product of ML621 & ML566 on YCp111/cdc11G32E.
[j]*In vivo* Recombination of BglII/MfeI cut pSP64T(S)XBSSE/CDC11 with the BglII/MfeI digested PCR product of ML621 & ML566 on YCp111/cdc11G32V.
[k]*In vivo* Recombination of BglII/MfeI cut pSP64T(S)XBSSE/CDC11 with the BglII/MfeI digested PCR product of ML621 & ML566 on YCp111/cdc11R35E.
[l]*In vivo* Recombination of BglII/MfeI cut pSP64T(S)XBSSE/CDC11 with the BglII/MfeI digested PCR product of ML621 & ML566 on YCp111/cdc11R35T.

### 3.1 Preparation of protein extract and immunoprecipitation:

Unless indicated otherwise, all steps were carried out at 4˚C. 500 µl of bead beating buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 10% glycerol, protease inhibitors, phosphatase inhibitors and PMSF) (BBF) was added to a cell pellet corresponding to 25 $OD_{600}$ of cells (~50 mg of cell pellet). 250 µl of glass beads were added, followed by vortexing at 4˚C for 5 min at high speed, incubation on ice for 2.5 min, then vortexing at high speed for an additional 5 min. The mixture was then centrifuged at 14000 rpm for 10 min and the supernatant was transferred to a fresh tube. Protein concentration was determined by Bio-Rad assay and ~5-25 mg of total protein was isolated.

25 µl of protein-G beads was added to ~12 mg of total protein extract and ~2 mg/ml of anti-Cdc3p (Sigma Genosys) antibody was added and the mixture was gently rocked for ~2.5 hrs at 4°C. The beads were then pelleted and washed four times with 500 µl of wash buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$ 10% glycerol, protease inhibitors, phosphatase inhibitors and PMSF). The washed beads were then incubated overnight in 1.6 volumes (40 µl) of elution buffer [20 mM

Tris-HCl, pH 7.8 1 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 4% sucrose with 0.8 mg/ml Cdc3 C-terminal peptide (Sigma Genosys)]. The beads were then pelleted at 500X g, and the supernatant containing the septin complexes was used for the UV cross-linking assay.

**3.2 High pressure liquid chromatography (HPLC):**

The samples for HPLC were obtained by over-expressing the septins using galactose induction, and purifying them over GA-beads (Glutathione agarose). After washing the beads with BBF the protein was then denatured by the addition of 30 µl 8 M urea, 100 mM Tris-HCl, pH7.2, followed by heating to 100ºC for 1min. 30 µl of water was added and the mixture was transferred to 10 kDa cut-off Microcon filter and was centrifuged at 13000 RPM for 15 minutes. This was followed by two more washes with water (30 µl each). The total volume (120 µl) was then assayed by HPLC through a mono-Q column (Amersham Biosciences) of 1ml bed volume with a loading capacity of 50mg. The column was prepared by equilibration with 100 mM ammonium bicarbonate and the sample was eluted using a gradient of 100-500 mM ammonium bicarbonate over 30 min at a flow rate of 1 ml/min, was used for the sample elution. Standard (a mixture of 100pmol of ATP, GDP and GTP) was run separately using the identical conditions.

**3.3 UV-crosslinking:**

Septin complexes purified as described above were incubated with [$\alpha$-$^{32}$P] GTP (2µM GTP SA, 800 Ci/mmol) and 300 µM non-radioactive ATP at 30°C with or without competing non-radioactive GTP at 300 µM. Also [$\alpha$-$^{32}$P] GTP with no competitor nucleotide and [$\alpha$-$^{32}$P] GTP with 1 mM EDTA were assayed. 5 µl of sample was taken

for SDS-PAGE analysis to assay for complex purification. 2-mercaptoethanol was added to the remaining portion of the sample to 1%, followed by incubation on ice for 1 min and then exposure to UV light on ice for five periods of 3 min each with 1min pause between each UV exposure. UV-crosslinking was carried out using a Stratagene UV Stratalinker 1800 with 254 nm bulbs. The samples were placed 7 cm from the UV lamps and the power of the instrument was approximately1140μW/$cm^2$. After UV exposure, 8 μl of 6X SDS-PAGE sample buffer was added and the sample heated for 30 s at 100°C. 200μl of cross-linking buffer (20 mM Tris-HCl, pH7.8, 100 mM KCl, 0.5 mM EGTA, and 2 mM $MgCl_2$) was added to each sample. The mixture was filtered through a 10-kDa cut off microcon filter until 98% of the liquid went through (13000 rpm, 20 min). The top of the filter was resuspended in 30μl of 1X sample buffer, heated for 1min at 100°C and subjected to 10% SDS PAGE. The proteins from gel were then transferred to a PVDF membrane by electroblotting and the membrane was used for autoradiography and phosphoimaging.

**3.4 Co-immunoprecipitation:**

Yeast cells grown exponentially in appropriate media at room temperature were shifted to 37ºC for 2 hours. The cells were then disrupted with glass beads by vortexing at 4º C in (25 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 10% glycerol, protease inhibitors, phosphatase inhibitors and PMSF). After centrifugation to remove cell debris, the supernatant was used for immunoprecipitation. 30 μl of beads, ~2 mg of the total protein and 3-4 μg of antibody (anti-Cdc11p and anti-Cdc12p) were mixed and incubated at 4ºC for 2 hrs. The beads were then washed four times, each with

350 µl BBF. Proteins bound to the beads were eluted with SDS sample buffer, analyzed by SDS-PAGE and western blotting using anti-Cdc3p, anti-Cdc10p (Santa Cruz Biotechnology), anti-Cdc11p and anti-Cdc12p (Sigma Genosys) antibodies.

**3.5 In vitro transcription and translation (TNT) and metal affinity purification:**

Fifty micro liters of TNT mixture in our laboratory contains 2.92 µl water, 9.58 µl buffer (10 mM creatine phosphatase, 1 mM DTT, 3 mM magnesium acetate, 110 mM potassium acetate, and 0.6 mM each amino acid except for methionine. 0.8 mM each rNTPspH7.3) 1 µl plasmid (1 µg/µl), 0.5 µl of $^{35}$S-methionine (Perkin Elmer, SA, 1175.0 Ci/mmol), 35 µl of rabbit reticulocyte lysate and 1 µl of SP6 RNA polymerase. The above reagents were aliquoted into a 1.5 ml microfuge tube and mixed well avoiding air bubbles. After incubating for 30 min at 37ºC, the mixture was clarified by centrifugation. The supernatants were then mixed and allowed to interact for 1 hr at 23ºC, followed by affinity purification using 25 µl of Talon beads (BD Biosciences) at 23ºC for 1 hr. 5 µl of the supernatant was taken as the unbound (UB) sample. The beads were washed four times, with 350 µl of wash buffer (BBF). 30 µl of 1X sample buffer was added and heated for 5 min at 100ºC. Bound (B) and unbound (UB) fractions were separated by SDS-PAGE, electroblotted to PVDF membrane and detected by autoradiography.

# CHAPTER 4

# RESULTS

## 4.1 Co-immunoprecipitation

Purified septin complexes from *S. cerevisiae* form filaments in vitro (Frazier et al., 1998; Versele et al., 2004). In addition to septin-septin interactions, septins also interact with non-septin proteins (reviewed in the Introduction). Septins contain a putative GTP binding domain and it has been reported that septins bind GTP (Vrabioiu et al., 2003). Though septins bind GTP, the role of GTP binding is not yet clear.

To study the role of GTP binding in yeast septins, we constructed several septin mutants by mutating residues at the P-loop using site directed mutagenesis. We then asked if these mutations affected septin-septin interactions by carrying out a series of co-immunoprecipitation experiments. The mutant alleles analyzed in these studies were *cdc3*$^{G129V,K132E,T133N}$,*cdc10*$^{K45T}$, *cdc 11-6* (*cdc11*$^{G32E}$), *cdc11*$^{R35T}$ and *cdc11*$^{R35E}$. and*cdc12*$^{G44V}$, *cdc12*$^{G44V, K47E, T48N}$ and *cdc12*$^{D98A, G101A}$.

Cells were grown to mid-log phase at 23ºC and then shifted to 37ºC for 2 hr. Protein extracts were then made from the cells grown at 23ºC and 37ºC (for Cdc3p and Cdc10p experiments) or after shift to 37ºC (for Cdc11p and Cdc12p experiments) to assay for septin-septin interactions. Septin proteins were then immunoprecipitated using anti-Cdc11p or anti-Cdc12p antibodies and protein-G beads. Immunoprecipitated septin

complexes were separated by SDS-PAGE and analyzed by Western blotting, using four different antibodies which recognize Cdc3p, Cdc10p, Cdc11p, and Cdc12p.

The results of these experiments for wild-type Cdc3p and cdc3pG129V, K132E, T133N strains are shown in Figure 1. First, after a two hour shift to 37˚C, all four septins are stable and present in wild-type amounts (Fig 1A), indicating no effect on protein stability. As shown in Fig 1B, cdc3pG129V, K132E, T133N immunoprecipitated less efficiently with Cdc11p than did wild-type Cdc3p . In contrast, in extracts from wild-type cells Cdc3p efficiently immunoprecipitates with Cdc11p. Also note, that when cdc3p levels are reduced, there is a concomitant reduction in the amount of immunoprecipitated Cdc10p. Similar results were obtained with immunoprecipitation using anti-Cdc12p antibodies (Fig. 1C): cdc3pG129V, K132E, T133N associated less efficiently with Cdc12p than did wild-type Cdc3p, and there was a concomitant reduction in the amount of Cdc10p in the complex. Thus, the triple-P-loop mutation of Cdc3p reduces its interaction with Cdc11p and Cdc12p, consistent with the observed temperature-sensitive viability of this strain. In addition, these results indicate that Cdc10p association with Cdc11p and Cdc12p depends upon the presence of Cdc3p, suggesting that Cdc10p association in the complex may be mediated by a direct interaction with Cdc3p.

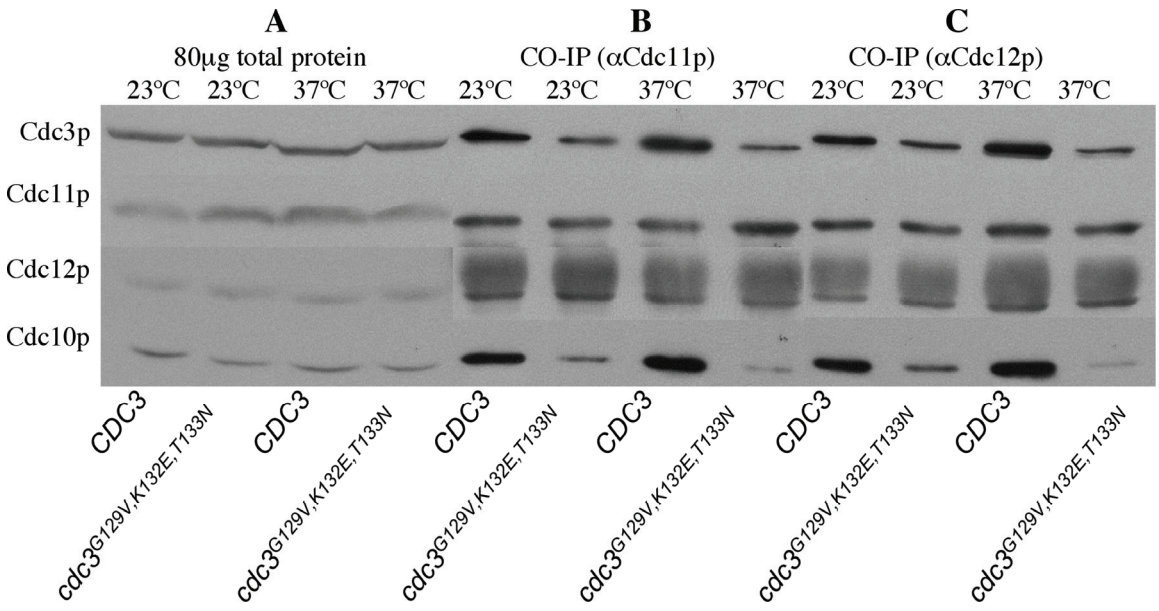


Figure 1**. Co-immunoprecipitation of septin complexes from *CDC3* and *cdc3pG129V, K132E, T133N* mutant strains.** Yeast cells grown exponentially in SDC–His medium at 23˚C were shifted to 37ºC for 2 hr and protein extracts prepared. 80μg of total protein was taken (A) and ~2mg of total protein was used for immunoprecipitation with IgG beads bound to (B) anti-Cdc11p antibody or (C) anti-Cdc12p antibody. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and septins detected by immuno-blotting using anti-Cdc3p, anti-Cdc10p anti-Cdc11p and anti-Cdc12p antibodies, as indicated**.**

Investigation of the effect of cdc10K45T on its incorporation into a septin complex is shown in Fig. 2. After a two hour shift to 37˚C, all four septins are stable and present in wild-type amounts (Fig 2A), indicating no effect on protein stability. Consistent with its full, or nearly full function in viability at 23˚C (Satish Nagaraj, personal communication), cdc10pK45T efficiently interacts with both Cdc11p- and Cdc12p-containing septin complexes at 23˚C (Fig. 2A-B). In agreement with the temperature-sensitive viability of cdc10pK45T strains (Satish Nagaraj, personal communication), at 37˚C cdc10p K45T is no longer associated with septin complexes containing Cdc11p or Cdc12p (Fig. 2B,C). However, in contrast to observation that Cdc10p association with Cdc11p or Cdc12p depends upon the presence of Cdc3p (Fig. 1), Cdc3p association with Cdc11p and Cdc12p occurs independently of the presence of Cdc10p (Fig. 2B, C).

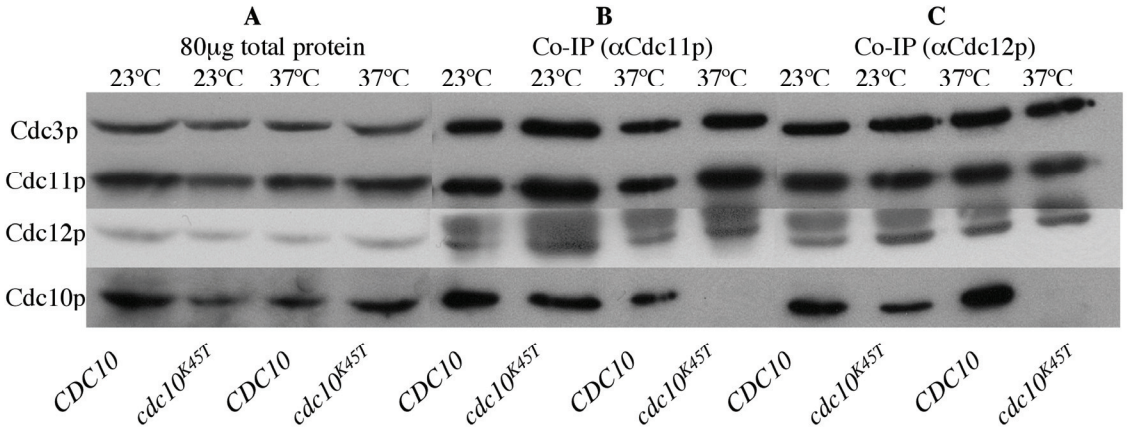


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Figure 2. **Co-immunoprecipitation of septin complexes from *CDC10* and *cdc10K45T* mutant strains**. Yeast cells grown exponentially in SDC–Leu-Ura medium at 23˚C were shifted to 37ºC for 2 hr and protein extracts prepared. 80μg of total protein was taken (A) and ~2mg of total protein was used for immunoprecipitation with IgG beads bound to (B) anti-Cdc11p antibody or (C) anti-Cdc12p antibody. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and septins detected by immuno-blotting using anti-Cdc3p, anti-Cdc10p anti-Cdc11p and anti-Cdc12p antibodies, as indicated.

The effects of mutations on the P-loop of Cdc11p on its participation in septin-complex formation are shown in Fig. 3. After a two hour shift to 37˚C, all septins are stable and present in wild-type amounts (Fig 3A) in the mutant strains analyzed (*cdc11R35T*, *cdc11-6*, and *cdc11*$^{R35E}$) , indicating no affect on protein stability. *cdc11-6* (*cdc11*$^{G32E}$) strains show strong temperature-sensitive viability defects, being inviable at 32˚C and above (Satish Nagaraj, personal communication). cdc11pG32E does not interact with cdc11p or Cdc12p-containing septin complexes at 37˚C (Fig. 3A, B), suggesting that defects of cdc11pG32E in septin-septin interactions may cause the observed inviability. I also assayed the defects in septin complex formation of cdc11pR35T and cdc11pR35E. cdc11pR35E is expected to show a more dramatic defect in nucleotide binding than cdc11pR35T, due to the presence of charge-charge repulsion of the glutamate with the phosphates of the bound nucleotide. Consistent with this idea, cdc11pR35E shows temperature sensitive viability while cdc11pR35T is wild-type for viability at all temperatures (Satish Nagaraj, personal communication). As expected, cdc11pR35T interacts well with the other septins, while cdc11pR35E shows clearly reduced incorporation into a septin heteromeric complex (Fig. 3B, C). Interestingly, in the absence of cdc11p (Fig. 3C, *cdc11-6* lane) the remaining three septins interact efficiently (though with reduced levels of Cdc10p). Also, we note that in the intermediate *cdc11* allele (*cdc11R35E*), cdc11p and Cdc12p can be found as a co-immunoprecipitating sub-complex (Fig. 3B, *cdc11R35E* lane), suggesting a strong interaction between Cdc11p and Cdc12p. Thus, these data suggest that a complex of Cdc3p, Cdc10p, and Cdc12p forms in the absence of Cdc11p, and that Cdc11p and Cdc12p may show a strong, and likely direct, interaction.,

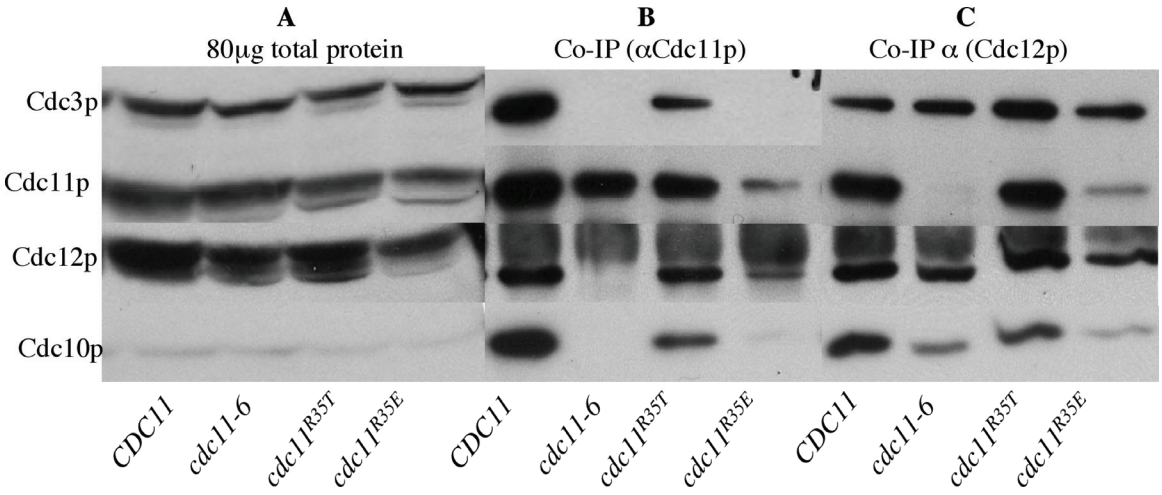


Figure 3. **Co-immunoprecipitation of septin complexes from *CDC11* and *cdc11* mutant strains**. Yeast cells grown exponentially in YM-1 media medium at 23˚C were shifted to 37ºC for 2 hr and protein extracts prepared. 80μg of total protein was taken (A) and ~2mg of total protein was used for immunoprecipitation with IgG beads bound to (B) anti-Cdc11p antibody or (C) anti-Cdc12p antibody. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and septins detected by immuno-blotting using anti-Cdc3p, anti-Cdc10p anti-Cdc11p and anti-Cdc12p antibodies, as indicated.

Finally, I assayed the effects of disrupting Cdc12p GTP binding or GTP hydrolysis on its participation in septin-septin complexes. As expected from their full viability at all temperatures (Satish Nagaraj, personal communication), both Cdc12p and cdc12pD98A, G101A, efficiently formed a complex containing Cdc3p, Cdc11p, Cdc12p (or cdc12pD98A, G101A), and Cdc10p (Fig. 4 B, C). cdc12G44V strains show only a slight defect in viability at 37˚C (Satish Nagaraj, personal communication), consistent with ability of cdc12pG44V to participate nearly as effectively as Cdc12p in septin complex formation. However, in this strain there does appear to be slightly reduced levels of Cdc11p and Cdc10p in the complex (Fig. 4C, *cdc12*$^{G44V}$ lane). In contrast, a cdc12$^{G44V, LK47E, T48N}$ strain that shows clear viability defects at 37˚C (Satish Nagaraj) is unable to participate in septin complex formation (Fig. 4B, C). Notably, if cdc12p is not associated with the other septins (Fig. 4C, cdc12$^{G44V, LK47E, T48N}$ lane), Cdc11p is also unable to associate with Cdc3p or Cdc10p (Fig. 4B cdc12$^{G44V, LK47E, T48N}$ lane).

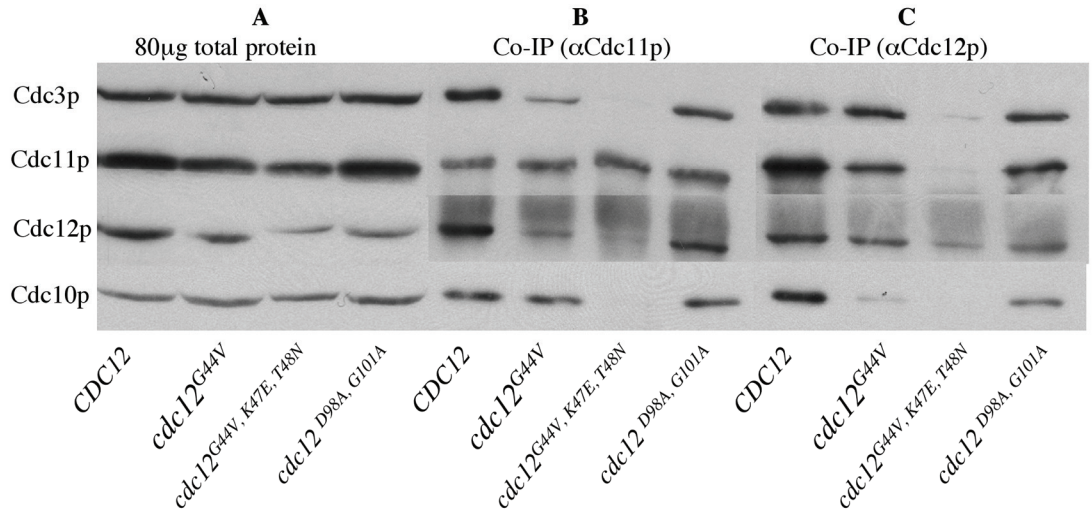


Figure 4**. Co-immunoprecipitation of septin complexes from Cdc12p and its mutants.** Yeast cells grown exponentially in SDC-LEU media medium at 23˚C were shifted to 37ºC for 2 hr and protein extracts prepared. 80μg of total protein was taken (A) and ~2mg of total protein was used for immunoprecipitation with IgG beads bound to (B) anti-Cdc11p antibody or (C) anti-Cdc12p antibody. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and septins detected by immuno-blotting using anti-Cdc3p, anti-Cdc10p anti-Cdc11p and anti-Cdc12p antibodies, as indicated.

**4.2 Affinity of septin towards the complex:**

The above co-immunoprecipitation analyses suggested that *cdc11* P-loop mutant proteins assemble into heteromeric septin complexes at 23˚C as well as wild-type Cdc11p. However, this conclusion seemed unlikely for several reasons. First, work by our collaborators (Chris Field and Alina Vriabiou) suggested that septin filament formation *in vitro* was defective in extracts from cdc11pR35T cells with a more dramatic defect in cdc11pR35E cells. In addition, quantitation of coomassie-blue stained bands of septin complexes purified from these strains suggested a mild reduction in the stoichiometry of the mutant cdc11p proteins compared to that of wild-type Cdc11p. Finally, two-hybrid and in vitro studies (Satish Nagaraj, personal communication) indicate that there are detectable defects in septin-septin interactions in most P-loop mutants even at 23˚C.

To ask if there may be subtle, but reproducible, defects in septin-septin interactions at 23˚C upon disruption of nucleotide-binding, I carefully analyzed the levels of wild-type and P-loop-mutant cdc11p that co-immunoprecipated with Cdc3p. Cdc11p and its cdc11p P-loop-mutant strains were grown at 23˚C, septins were immunoprecipitated using anti-Cdc3p antibody, and the ratio of immunoprecipitated wild-type and mutant Cdc11p to the immunoprecipitated Cdc3p was compared. The experiment was done four times and the average value was taken for further experimental analysis. An example of one of these experiments is shown in Figure 5B. These data suggest that there is no detectable defect in the association of cdc11pR35T with Cdc3p. However, at RT, there are clear defects in the interaction of other P-loop mutant cdc11p with Cdc3p. Notably, the defect in interaction (cdc11pG32V, R35E,

S36N>cdc11pG32V≥cdc11pG32E, cdc11pR35E >cdc11pS36N>cdc11pR35T**)** closely parallels the defects in temperature-sensitive viability of these strains (Satish Nagaraj, personal communication). Together, these data suggest that there are slight, but quantifiable, defects in interactions of cdc11p P-loop mutant septins with Cdc3p at 23˚C.

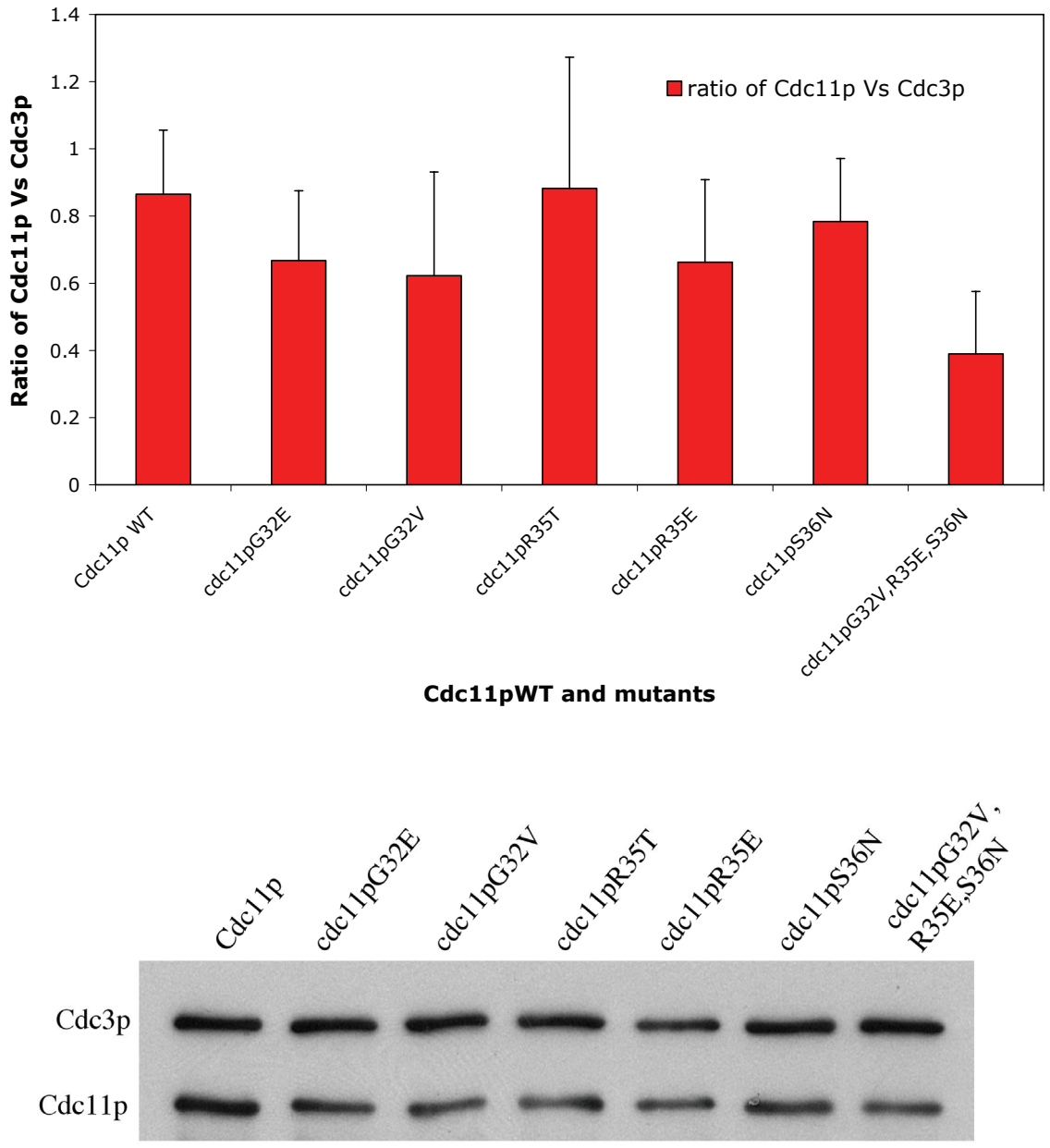


Figure 5. **Effect of mutation on the affinity of Cdc11p towards the complex.** *CDC11* and *cdc11* P-loop mutant strains were grown to log phase in SDC–Ura media at 23˚C. Protein extracts were prepared, and septin complexes immunoprecipitated using anti-Cdc3p antibody and protein-G beads. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and Cdc3p and Cdc11p were detected by immunoblotting using anti-Cdc3p and anti-Cdc11p antibodies, respectively (A) Ratio of Cdc11p and cdc11p mutant proteins to Cdc3p, taken from the average of four separate experiments. (B) One of the experiments used to generate the graph shown in (A).

**4.3 Septin-septin interaction and septin complex formation in vitro:**

Septins are present as multi-septin complexes *in vivo*. My co-immunoprecipitation studies gave some clue on septin-septin interactions. However, I wanted to further dissect septin-septin interactions. In this work, I used rabbit reticulolysate (RRL) coupled *in vitro* transcription and translation (TnT) assays to investigate septin-septin interactions. For purification, one septin was tagged with six N-terminal histidine residues, with the remaining septins untagged. The $His_6$-tagged protein and any associated septins were then purified by metal (cobalt) affinity chromatography and subsequently analyzed by SDS-PAGE and autoradiography.

First, I assayed for direct interactions of septins in pair wise assays. The results of these assays are shown in Figure 6. First, Cdc11p and Cdc12p interact directly and efficiently, forming an ~1:1 complex (Fig. 6A, B, and C, 6HisCdc11p+Cdc12p lanes and Fig. 6B 6HisCdc12p+Cdc11p lanes). In addition, these assays detected an interaction between 6His-Cdc3p and Cdc12p (Fig. C, 6HisCdc3p+Cdc12p lane). It is unclear why no detection was detected between 6HisCdc12p+Cdc3p (Fig. 6B).

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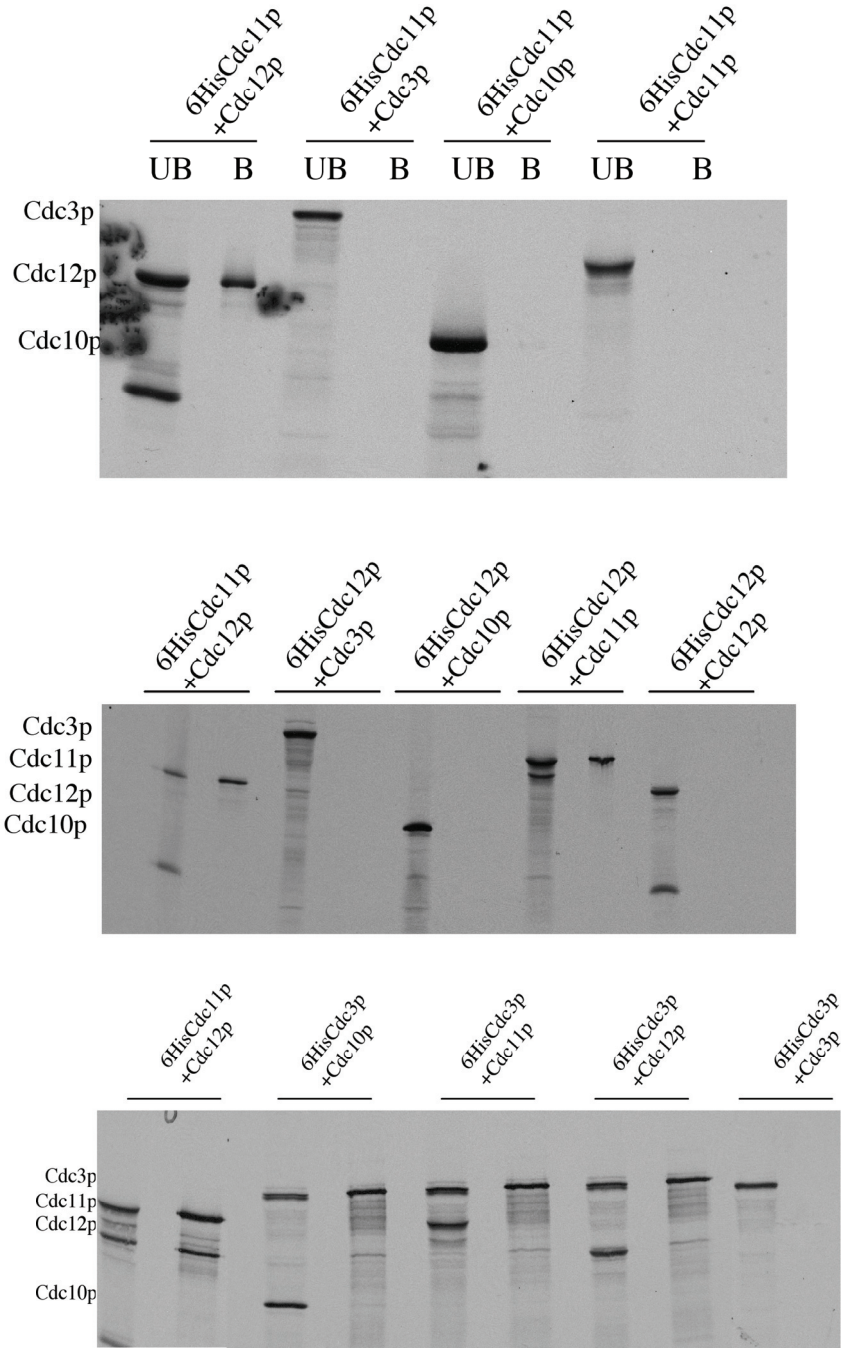


Figure 6**. Pair wise *in vitro* septin-septin interactions.**
Cdc3p, Cdc10p, Cdc11p and Cdc12p were synthesized using RRL/TNT and labeled with $^{35}S$. These proteins were then mixed and allowed to interact for 1 hr at 23ºC,followed by affinity purification using Talon beads. Unbound (UB) and bound (B) fractions were separated by SDS PAGE, transferred to PVDF membrane, and detected by autoradiography. In panel A and B 6HisCdc11p and 6HisCdc12p were not labeled with $^{35}S$-Met, and thus are not undetected by autoradiography.

Next, I used the *in vitro* RRL/TnT assay to investigate formation of ternary and quaternary septin-septin complexes. First, all four septins associate efficiently, forming an apparently stoichiometric complex (Fig. 7 A,B 6HisCdc11p+Cdc3p+Cdc10p+Cdc12p lanes). There was a decrease in the stoichiometry of purified Cdc3p, Cdc11p, and Cdc12p when using 6His-tagged Cdc10p, suggesting the 6His tag may disrupt the ability of Cdc10p to participate in complex formation. The detection of this complex is based upon a specific interaction of the 6His-tagged protein with the talon resin, as a mixture of the four untagged septins does not associate with the Talon beads (Fig. 7, Cdc11p+Cdc12p+Cdc10p+Cdc3p lanes). This assay also indicates that N-terminal 6His-tagged Cdc10p is defective, as 6HisCdc10p does not interact with the other septins (Fig. 8) while untagged Cdc10p interacts well. In contrast, N-terminally 6His-tagged Cdc11p, Cdc12p, and Cdc3p all efficiently form the quaternary complex, indicating that the tags do not affect protein-protein interactions. As the septins in this assay efficiently interact, it appears to be a valid method to investigate ternary septin-septin interactions.

Interactions of three septins are investigated in Figure 7. As shown previously, Cdc11p and Cdc12p interact stoichiometrically in pair wise interactions, but neither alone interacts with Cdc3p (Fig. 6). In the presence of both Cdc11p and Cdc12p, however, a sub-stoichiometric (but reproducible) amount of Cdc3p interacts to form a ternary complex (Fig. 7, compare 6HisCdc11p+Cdc12p+Cdc3p bound lane with Fig. 6A 6 HisCdc11p+Cdc3p lanes and with Fig. 6B 6HisCdc12p+Cdc3p lanes). Thus, Cdc3p interacts more efficiently with a complex of Cdc11p and Cdc12p than to either single septin. Surprisingly, no other ternary complex was detected. Thus, these data suggest

while Cdc11p and Cdc12p may be involved in direct interaction and are required for the efficient interaction of Cdc3p which may have a direct interaction with Cdc10p.

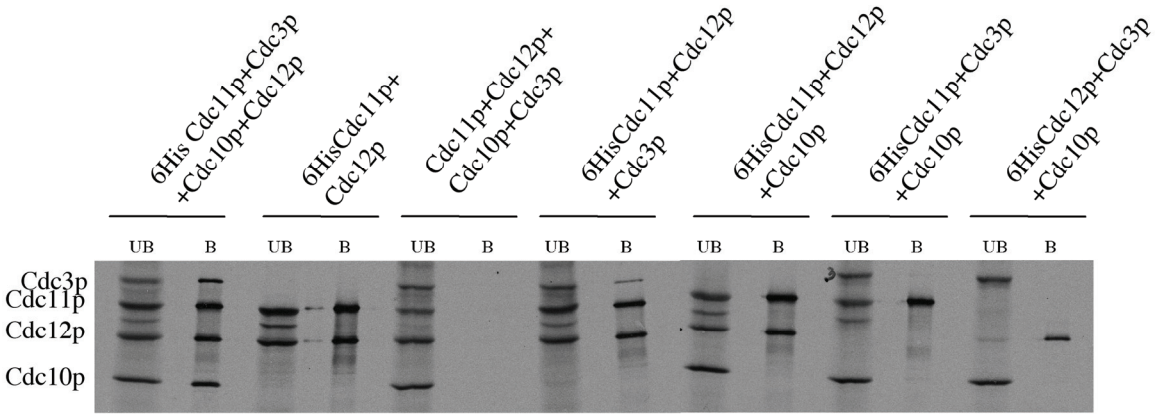


Figure 7. **Ternary *in vitro* septin-septin interactions.** Cdc3p, Cdc10p, Cdc11p and Cdc12p were synthesized using RRL/TNT and labeled with $^{35}S$. These proteins were then mixed and allowed to interact for 1 hr at 23ºC followed by affinity purification using Talon beads. Unbound (UB) and bound (B) fractions were separated by SDS PAGE, transferred to PVDF membrane, and detected by autoradiography.

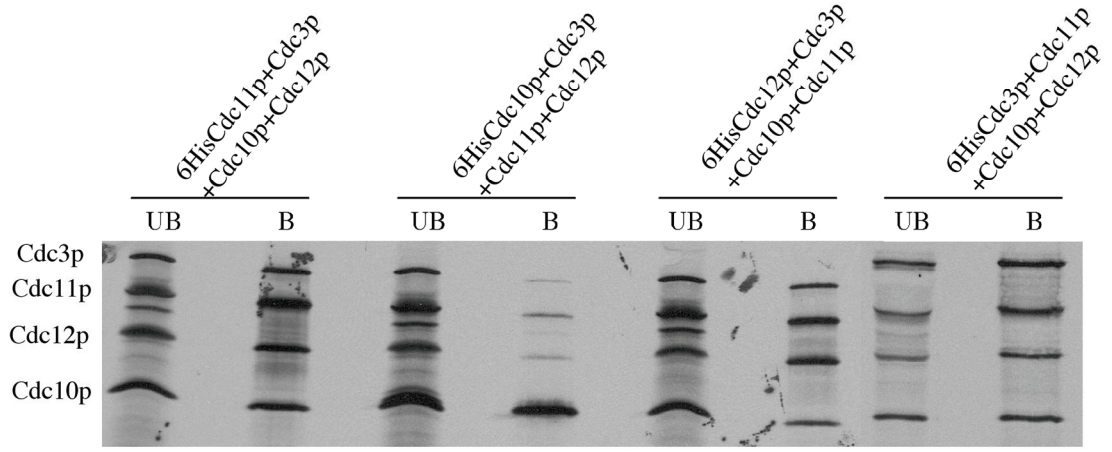


Figure 8. **Quaternary *in vitro* septin-septin interactions.** Cdc3p, Cdc10p, Cdc11p and Cdc12p were synthesized using RRL/TNT and labeled with $^{35}S$. These proteins were then mixed and allowed to interact for 1 hr at 23ºC followed by affinity purification using Talon beads. Unbound (UB) and bound (B) fractions were separated by SDS PAGE, transferred to PVDF membrane, and detected by autoradiography.

The RRL/TnT assay was then used to determine if cdc11p P-loop-mutant proteins showed defects in septin-septin interactions. Consistent with the co-immunoprecipitation results described earlier (Figs. 4 and 5), cdc11pR35T, cdc11pR35E, cdc11pG32V and cdc11pG32E all displayed defects in assembly into septin complexes (Fig. 9). As expected, cdc11R35T retained the ability to interact with the other septins (Fig. 9, compare 6HisCdc3p+Cdc11p+Cdc3p+cdc11pR35T lanes with 6HisCdc3p+Cdc11p+Cdc10p+Cdc12p). However, this interaction was reproducibly slightly reduced compared to wild-type Cdc11p. Similar reduction was observed in pair-wise interactions of cdc11pR35T with Cdc12p (Satish Nagaraj, personal communication). Consistent with their more dramatic defects in function in vivo (Satish Nagaraj, personal communication), cdc11pR35E, cdc11pG32V and cdc11pG32E were all unable to interact with the septin complex (Fig. 9) or with Cdc12p in pair wise interactions (Satish Nagaraj, personal communication). Together, these data suggest that disruption of nucleotide binding by Cdc11p disrupts its interaction with the septin complex *in vivo* (Figs. 4 and 5) and *in vitro* (Fig. 9).

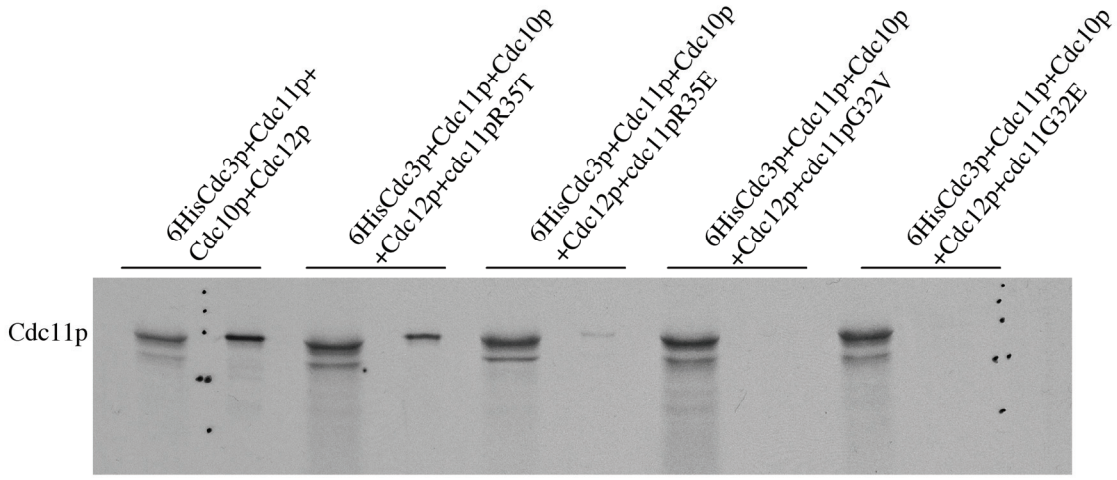


Figure 9. **Interaction of Cdc11p and its P-loop mutants with the complex**. Cdc11p and its P-loop mutants were synthesized using RRL/TNT. $S^{35}$ methionine was used to label the proteins. These proteins were then mixed and allowed to interact with other septins synthesized by TnT, for 1hr at 23ºC followed by affinity purification using talon beads (metal affinity). Bound (B) and unbound (UB) fractions were separated by SDS PAGE, electroblotted to PVDF membrane and detected by autoradiography.

**4.4 High pressure liquid chromatography (HPLC):**

Co-immunoprecipitation and in vitro binding studies indicate that mutations in the P-loop of septins disrupt septin-septin interactions. However, these studies do not directly demonstrate that the introduced mutations affect nucleotide binding. As an attempt to address this question, we over-expressed N-terminally GST-tagged (glutathione-S-transferase) wild-type and P-loop-mutant Cdc11p under the control of the galactose-inducible, *GAL1* promoter. Cells were grown to mid-log phase at 23˚C in raffinose containing medium and expression of the GST-tagged induced by the addition of galactose to 2% final concentration, followed by a 12 hr period of induction at 23˚C. Protein extracts were prepared, and ~5 mg of total protein was incubated with 30 µl bed volume glutathione-agarose resin for 1 hr at 23˚C. Following 4X washes of 350 µl for 2.5 min each, the bound proteins and nucleotides were eluted by the addition of 30µl of 8M urea and heating at 100˚C for 1min. Typically, this resulted in the purification of ~25 µg of the GST-tagged septin. The supernatant was filtered through a 10 Kd microcon filter by centrifuging for 15 min. After washing the filter thrice the filtrate was pooled and was loaded on a 1 ml MonoQ column that was equilibrated in buffer containing 100 mM ammonium bicarbonate. The sample was assayed by running a 100 mM to 500 mM gradient of ammonium bicarbonate, with control runs performed by using different concentrations of GTP, GDP, and/or ATP (Fig 10A) to determine elution times of these nucleotides and that the amount of nucleotide was directly correlated with the peak area for the bound nucleotide, using absorbance at 260 nm. An example of a control run is shown in Fig. 10A. In sample runs with purified septins, ATP was used as an internal control. First, we assayed a positive control, expressing GST-tagged wild-type Cdc42p

with a C188S mutation so it is not tightly associated with membranes (Mol. Biol. Cell. (1993) 4:1307–1316. Subcellular localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. M. Ziman, D. Preuss, J. Mulholland, J. M. O'Brien, D Botstein, and D. I. Johnson). As expected, Cdc42p was bound predominantly to GDP (Fig. 10C), which elutes just before the added ATP (Fig. 10A) under these conditions. Quantitation of the amount of purified proteins (not shown) and the amount of purified GDP was in ~1:1 stoichiometry. In contrast, GST-Cdc11p was almost entirely nucleotide free, with only ~5% of the protein containing bound GDP and no detectable levels of GTP (Fig. 10D). Similar results were obtained for GST-control and cdc11pR35E, with the exception of even lower levels of bound GDP. These data are consistent with reduced GDP binding to GST-cdc11pR35E compared to GST-Cdc11p wild-type protein. However, the most dramatic finding is that overexpressed GST-tagged septins are largely nucleotide free. This contrasts with data from the Field lab indicating that septins purified from yeast as a complex containing stoichiometric amounts of bound guanine nucleotide |(Vrabioiu et al., 2003). Thus, we conclude that grossly overexpressed Cdc11p or P-loop mutant cdc11p proteins display dramatic reductions in nucleotide binding. It is possible that these overexpressed septins are not folded properly (for example, the overexpression level overwhelms some aspect of the protein folding machinery involved in septin folding), and thus are non-functional or only poorly functional. Indeed, overexpressed GST-tagged Cdc11p does not interact with Cdc12p at the expected levels (Angie Thomure, personal communication), suggesting it is not folded properly and abnormaly nucleotide-free. Thus, it seems as if the overexpression strategy is not useful for analyzing septin nucleotide binding.

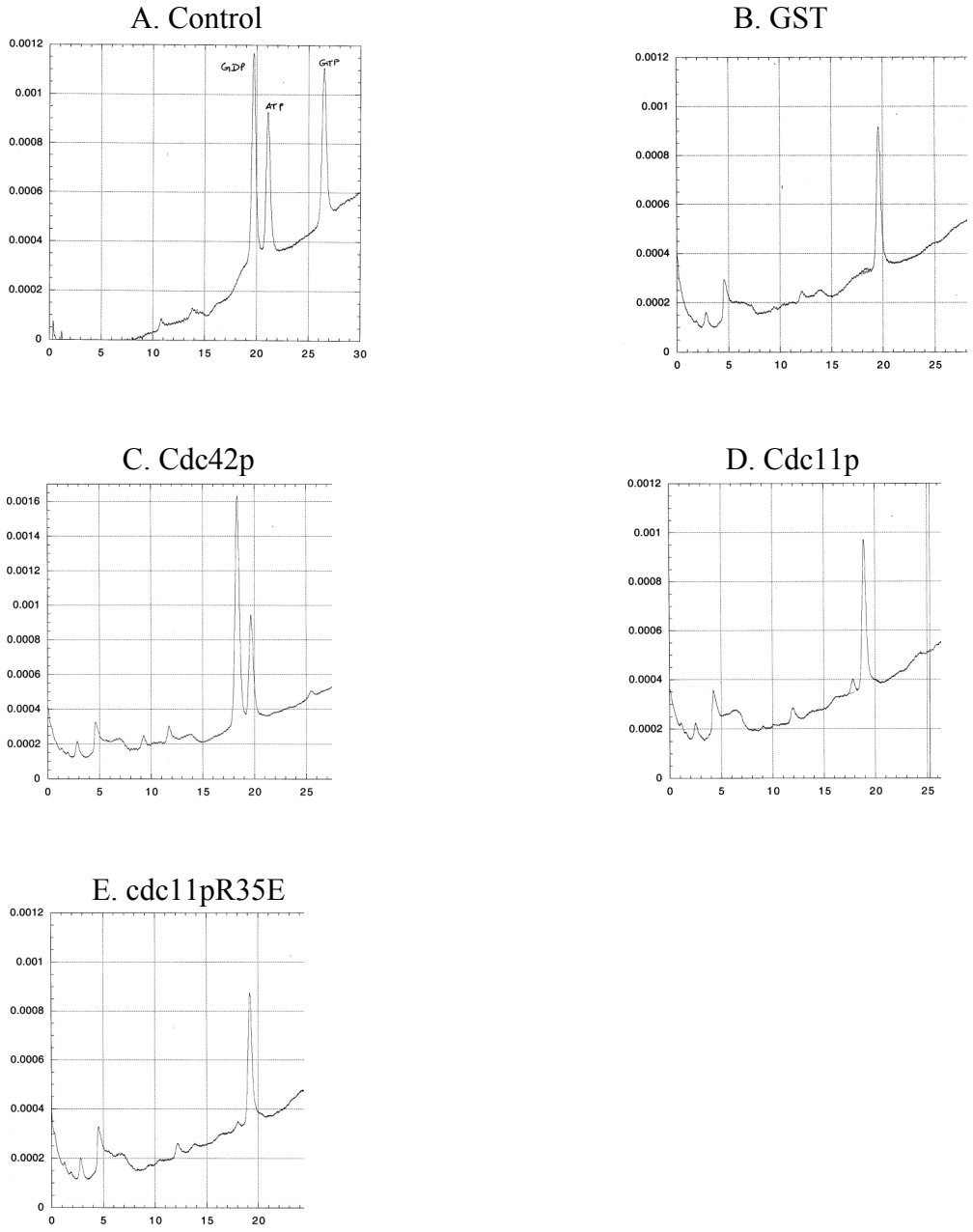


Figure 10. **Analysis of nucleotides released from over expressed septin**. Cdc11p and its mutants were over-expressed using GAL promoter. These over-expressed septins were purified using glutathione agarose beads and anti-Cdc11p anti-body as described in the materials and methods. After washing the beads with B3 buffer 30µl of urea was added and heated for 1 min. 30µl of water was added and the mixture was transferred to 10kDa cutoff microcon filter followed by 2 more washes of 30µ each. The eluent was then passed through HPLC Column.

**4.5 UV cross-linking of septins:**

UV cross-linking was next used to test the GTP-binding activity of wild-type and putative nucleotide-binding mutant septins. Septin complexes were purified using antibodies directed against Cdc3p, and were eluted using the peptide to which the antibody was raised. The soluble septin proteins (presumably largely as a complex) were then incubated with [$\alpha^{32}$-p] GTP for 45 min at 30ºC, followed by UV treatment to covalently crosslink the nucleotide to the septins (|(Vrabioiu et al., 2003). After UV exposure, the free nucleotide was removed by centrifugation, and the proteins separated by SDS-PAGE, and transferred to PVDF membrane for autoradiography and/or immuno-blotting. In some cases, 10% of the pre-UV irradiated, soluble septin complex was analyzed by SDS-PAGE and silver staining.

Control experiments showed that all four septins (Cdc3p, Cdc11p, Cdc12p, and Cdc10p) bound GTP. This binding was specific as it was efficiently competed by GTP but not ATP (Fig 11) and the interaction was reduced by the addition of EDTA, consistent with the expected requirement of $Mg^{++}$ for nucleotide binding.

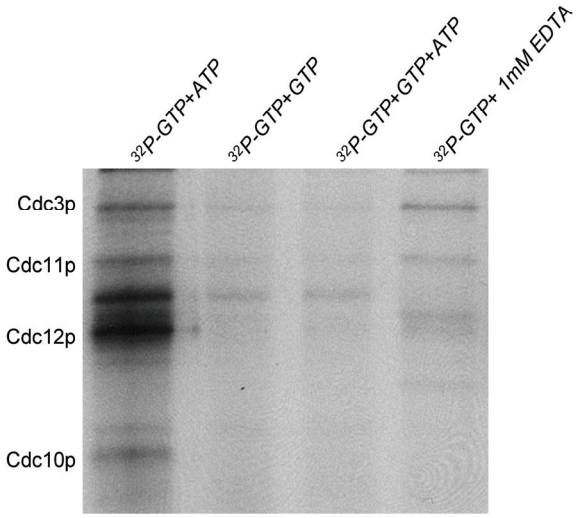


Figure 11. **Septins specifically bind GTP.** Purified septin complexes were incubated at 30ºC in the presence of 2µM [α-$^{32}$P] GTP (SA, 800 Ci/mmol) with 300µM ATP (1), 300µM GTP (2), 300µM ATP and 300µM GTP (3), 1mM EDTA (4). The mixture was UV cross-linked and separated on a 10% SDS-PAGE, transferred to PVDF membrane and subjected to autoradiography.

Figure 12 shows that wild-type Cdc11p and cdc11pR35T efficiently bind GTP while cdc11pG32V has a dramatic defect in GTP binding. This decrease in GTP binding by cdc11pG32V is much greater than the reduction in the levels of Cdc11p (Fig 12B, C) indicating that cdc11pG32V has a reduced affinity for GTP. Similar results were obtained for the cdc11pASIX protein, which contains a mutation in the G4 domain (Satish Nagaraj, personal communication) and is thus predicted to also have reduced GTP binding. In contrast, cdc11pASV, which contains a mutation in the G4 domain and is predicted to efficiently bind GTP, shows efficient crosslinking to GTP (Fig. 13A) Consistent with the results obtained with Cdc11p, mutations in the Cdc3p P-loop (*cdc3*$^{G129V,K132E,T133N}$) or Cdc12p P-loop (*cdc12* $^{G44V,K47E,T48N}$) also showed reduced GTP binding relative to their respective wild-type proteins (Fig14).

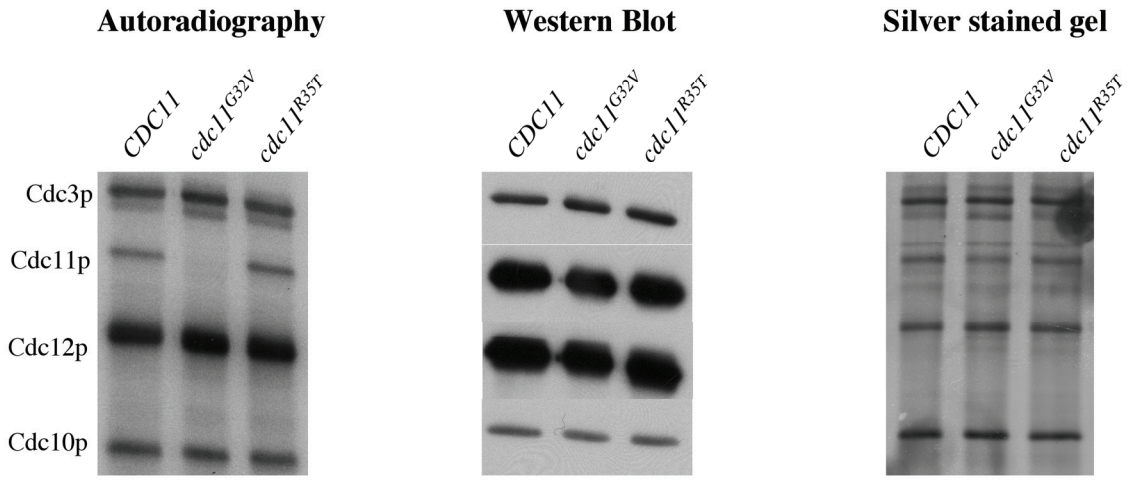


Figure 12. **UV cross-linking of Cdc11p, cdc11pG32V and cdc11pR35T to GTP.** Purified septin complexes were incubated at 30ºC in the presence of 2µM [$\alpha$-$^{32}$P] GTP (SA, 800 Ci/mmol) and 300µM ATP. Additional ATP and 2-mercaptoethanol were added to final concentrations of 300µM and 1%, respectively. The mixture was UV cross-linked on ice, heated in SDS-sample buffer, separated by SDS-PAGE and transferred to PVDF membrane and subjected to (A) autoradiography and then (B) immunoblotted with antibodies directed against Cdc3p, Cdc11p, Cdc12p, and Cdc10p. (C) Silver stained gel of 10% of the sample analyzed in (A) and (B).

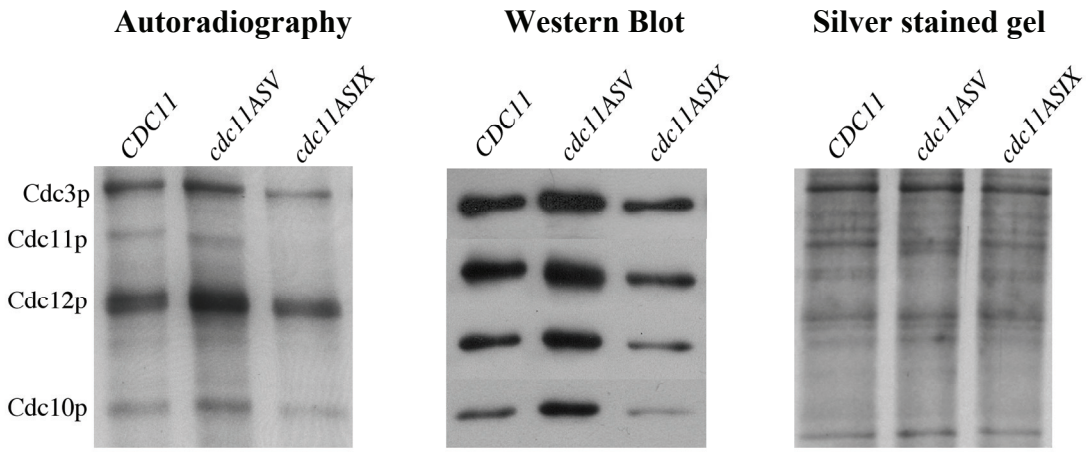


Figure 13. **UV cross-linking of Cdc11p, cdc11pASV and Cdc11pASIX to GTP.** Purified septin complexes were incubated at 30ºC in the presence of 2µM [α-$^{32}$P] GTP (SA, 800 Ci/mmol) and 300µM ATP. Additional ATP and 2-mercaptoethanol were added to final concentrations of 300µM and 1%, respectively. The mixture was UV cross-linked on ice, heated in SDS-sample buffer, separated by SDS-PAGE and transferred to PVDF membrane and subjected to (A) autoradiography and then (B) immunoblotted with antibodies directed against Cdc3p, Cdc11p, Cdc12p, and Cdc10p. (C) Silver stained gel of 10% of the sample analyzed in (A) and (B).

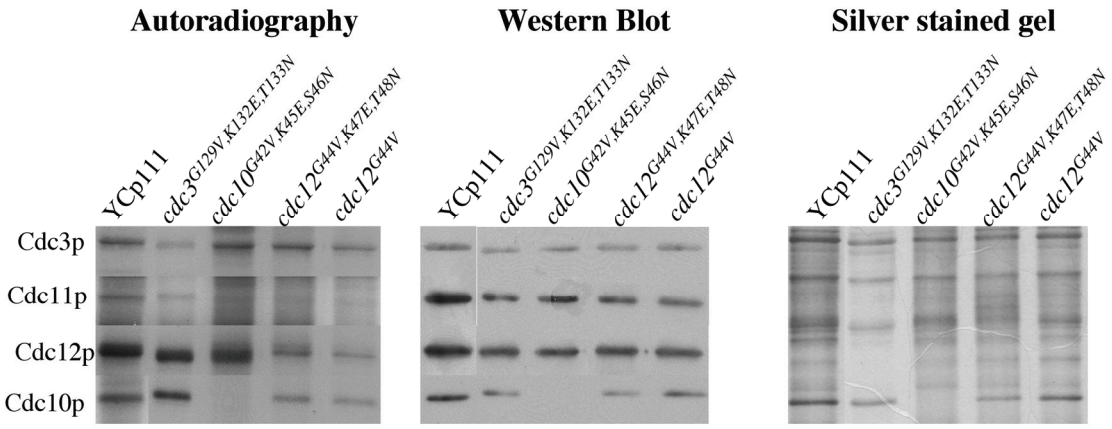


Figure 14. **UV cross-linking of Cdc3p, Cdc10p, Cdc12p P-loop mutants to GTP**. Purified septin complexes were incubated at 30ºC in the presence of 2µM [α-$^{32}$P] GTP (SA, 800 Ci/mmol) and 300µM ATP. Additional ATP and 2-mercaptoethanol were added to final concentrations of 300µM and 1%, respectively. The mixture was UV cross-linked on ice, heated in SDS-sample buffer, separated by SDS-PAGE and transferred to PVDF membrane and subjected to (A) autoradiography and then (B) immunoblotted with antibodies directed against Cdc3p, Cdc11p, Cdc12p, and Cdc10p. (C) Silver stained gel of 10% of the sample analyzed in (A) and (B).

# CHAPTER 5

# DISCUSSION

## GTP binding is necessary for septin-septin interactions:

To test the hypothesis that GTP binding is necessary for septin-septin interactions, we made a series of mutations in septin P-loop domains. The P-loop is a motif (GxxGxGKS/T), which interacts with bound nucleotide and is essential for ATP or GTP binding in a wide variety of proteins. The P-loop mutations commonly resulted in temperature sensitive viability and defects in septin localization and corresponding defects in bud morphology and cell division **(**Satish Nagaraj, personal communication). However, at 23˚C where septins localized normally to the mother-bud neck, septin dependent processes were not affected (Satish Nagaraj, personal communication), suggesting that GTP binding is not required for septin dependent processes.

In my work, I focused on the effect of GTP binding on septin-septin interactions. Coupled *in vitro* transcription and translation (TnT) assays revealed a strong and direct interaction of Cdc11p with Cdc12p. In contrast, cdc11p P-loop mutant proteins showed defects in interaction with Cdc12p *in vitro*, suggesting that GTP binding by Cdc11p is important in its interaction with Cdc12p. In these assays, cdc11pR35T, which has only a slight defected in septin function *in vivo*, showed a slight, but detectable defect in interaction with Cdc12p. Alleles with stronger defects in vivo, including cdc11pR35E

and cdc11pG32E, showed dramatic defects in interaction with Cdc12p in vitro. The correlation between the defects in function in vivo and defects in septin-septin interactions, suggest that the primary defect in cdc11p GTP-binding mutant cells is a defect in interaction of cdc11p with Cdc12p. It seems likely that this defect then results in the observed septin localization defects, and consequent disruption of septin function, *in vivo*.

To verify that disruption of septin GTP binding disrupts septin-septin interactions in vivo, septin co-immunoprecipitation assays were carried out with extracts from septin P-loop mutant strains grown at 23˚C or following a 2 hr shift to 37˚C. The level of interaction detected in these assays correlates to the severity of the mutation on septin function in vivo and on septin-septin interactions *in vitro.* In these assays, cdc11pR35T interacted well with the Cdc3p, Cdc10p, and Cdc12p septins at 37˚C, while a clear, but not complete, interaction defect was observed with cdc11pR35E. Consistent with its relatively low restrictive temperature for viability, cdc11pG32E (encoded by *cdc11-6*) shows a clear defect in association with the septin complex. In addition, we noted that in the absence of associated cdc11p, Cdc3p, Cdc10p, and Cdc12p still efficiently associate in a heteromeric complex, indicating that Cdc11p is not required for formation of a complex containing these three other septins.

Similar results were obtained with co-immunoprecipitation experiments using extracts from *cdc12*, *cdc10*, and *cdc3* septin P-loop-mutant strains. After shift of a *cdc12* P-loop mutant strain to 37˚C, cdc12p was no longer present in a complex with Cdc11p, and, in the absence of cdc12p, Cdc11p was unable to associate with Cdc10p or Cdc3p. Thus, the presence of Cdc11p in a heteromeric septin-septin complex likely depends on

the presence of Cdc12p. cdc3p P-loop mutant proteins only poorly formed a complex with the other septins. In the absence of Cdc3p, Cdc10p complex association was absent suggesting that association of Cdc10p with a heteromeric septin-septin complex is dependent upon the presence of Cdc3p. Finally, a P-loop mutant cdc10p no longer associated with the other septins after a shift to 37˚C. However, all three other septins (Cdc3p, Cdc11p, and Cdc12p) remained associated.

Together, these data confirm that defects in GTP binding affect septin-septin interactions *in vivo*. In addition, my data may explain why cells lacking Cdc11p or Cdc10p are viable while cells lacking Cdc3p or Cdc12p are inviable. I found that in the absence of Cdc10p or Cdc11p, a trimeric complex is nonetheless efficiently formed by the remaining septins. In contrast, in the absence of Cdc3p or Cdc12p, a trimeric complex of the remaining septins is not formed. Thus, I suggest that a complex of three, but not two, septins is required for viability. Versele et al (2004) reported the *in vitro* interaction of Cdc11p with Cdc12p and of Cdc12p with Cdc3p. They also report that Cdc10p has a higher affinity for the Cdc3p-Cdc12p complex, whereas our data suggest that Cdc10p interacts preferably with Cdc3p and does not interact with Cdc12p. This discrepancy may be due to the fact that septins in their study were expressed in *E. coli* and also in their assay one of the septins was N-terminally tagged with GST and the interacting partner had a 6XHis tag (N/C- terminal).

At 23˚C, septin P-loop mutants do not have any observable phenotype (Satish, Nagaraj, personal communication). They are completely viable, do not have any defect in morphology and the mutant septins localize normally. They show abnormalities only at elevated temperature. However, analysis of double-mutant strains (Satish Nagaraj

personal communication) suggests that these mutants may not be fully wild type at 23˚C. Consistent with this possibility, I found that there was reproducibly reduced cdc11p to Cdc3p ratios obtained after immunoprecipitation using anti-Cdc3p antibodies in extracts from cells grown at 23˚C. This ratio varied in proportion to the severity of mutation in the P-loop; e.g. $cdc11^{G32V,R35E,S36N}$ had a lower ratio compared to $cdc11^{R35T}$. This result indicates that septin P-loop mutants do have defects in GTP binding, even at 23˚C, but the compromised GTP binding and slight reduction in septin-septin interactions is not sufficient to yield a visible phenotype.

Our hypothesis that bound GTP affects septin-septin interactions was partly addressed by the biochemical analysis I have described. However, it is important that we demonstrate that the P-loop mutations have the expected biochemical effects on GTP binding. As one way to address this I carried out HPLC experiments to identify the nucleotide bound to over expressed Cdc11p wild type and P-loop-mutant proteins. I tagged *CDC11* and its P-loop mutants with GST and over-expressed these fusion proteins using GAL promoter. However, in contrast to the Cdc42p small GTPase control, most of the over expressed wild-type Cdc11p was nucleotide free. As work by others has indicated that yeast septins (|Vrabioiu et al., 2003) are bound in an ~1:1 stoichiometry with guanine nucleotides, we suspect that there was perhaps a folding defect in the over expressed Cdc11p protein. Thus, we concluded that this assay was not going to be useful for its desired purpose. Since the HPLC analysis was inconclusive, I did a UV cross-linking analysis, where I used UV light to covalently cross-link $\alpha^{32}$P-GTP to septin complexes purified from yeast. Cdc11p in the septin complex bound to GTP quite firmly where as cdc11pG32V and cdc11pASIX, both of which are predicted to disrupt GTP

binding, showed greatly reduced GTP binding in this assay. This was not due to reduced amounts of cdc11p in the immunopurified complex, as the reduction in nucleotide binding was very large, while very similar levels of cdc11p and wild-type Cdc11p were present in the isolated complexes. Furthermore, *cdc11AS-V*, a mutation predicted to reduce GTP hydrolysis but to not affect GTP binding, retained the ability to bind GTP, indicates that the reduced GTP binding was not due to reduced protein levels, suggesting a biochemical defect in GTP binding by the cdc11p P-loop mutant septin proteins. This assay also indicated the expected GTP-binding defects in cdc3p, cdc10p, and cdc12p P-loop mutant septin proteins. Thus, these results indicate that the P-loop mutations resulted in the expected defects in GTP binding.

These results support the “filament formation" model for septin GTP binding and hydrolysis. According to this model, the status of bound nucleotide regulates septin-septin interactions. We do not have any concrete data to prove that GTP binding and hydrolysis does not have a regulatory role in *S.cerevisiae* but because of the slow rate of GTP hydrolysis in *S.cerevisiae* septin reported by |Vrabioiu et al., 2000 , we speculate that GTP binding may play an structural role in yeast septins. However, the function of GTP hydrolysis is of interest, and it is important to note that the conserved biochemical ability to hydrolyze GTP and the purification of septins as complexes containing GTP and GDP suggest that there is still likely to be a (as yet undiscovered) role for GTP hydrolysis. In future work, it will be important to characterize the effect of GTP binding (and hydrolysis) on septin protein structure and how this structure affects septin-septin interactions.

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