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# Septin Ring Assembly Requires Concerted Action of Polarisome Components, a PAK Kinase Cla4p, and the Actin Cytoskeleton in *Saccharomyces cerevisiae*

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**Septins are filament-forming proteins that function in cytokinesis in a wide variety of organisms. In budding yeast, the small GTPase Cdc42p triggers the recruitment of septins to the incipient budding site and the assembly of septins into a ring. We herein report that Bni1p and Cla4p, effectors of Cdc42p, are required for the assembly of the septin ring during the initiation of budding but not for its maintenance after the ring converts to a septin collar. In *bni1Δ cla4-75-td* mutant, septins were recruited to the incipient budding site. However, the septin ring was not assembled, and septins remained at the polarized growing sites. Bni1p, a formin family protein, is a member of the polarisome complex with Spa2p, Bud6p, and Pea2p. All *spa2Δ cla4-75-td*, *bud6Δ cla4-75-td*, and *pea2Δ cla4-75-td* mutants showed defects in septin ring assembly. Bni1p stimulates actin polymerization for the formation of actin cables. Point mutants of *BNI1* that are specifically defective in actin cable formation also exhibited septin ring assembly defects in the absence of Cla4p. Consistently, treatment of *cla4Δ* mutant with the actin inhibitor latrunculin A inhibited septin ring assembly. Our results suggest that polarisome components and Cla4p are required for the initial assembly of the septin ring and that the actin cytoskeleton is involved in this process.**

## INTRODUCTION

The development of both unicellular and multicellular organisms requires cells to respond to intracellular and extracellular cues that direct growth and division. These signals regulate polarized cell growth, maintenance of cell shape, cell motility, and cytokinesis (Drubin and Nelson, 1996). Polarized cell growth is a complex process that requires the directed organization of the actin cytoskeleton and the coordinated function of many polarity proteins and signal transduction cascades. Cells of the yeast *Saccharomyces cerevisiae* grow by budding, a process in which a rigid cell wall is locally expanded as a result of polarized secretion (Pruyne and Bretscher, 2000a). Before bud emergence, cells polarize the actin cytoskeleton toward the future bud site and assemble a septin ring at that site.

The septins are a conserved family of filament-forming proteins that play important roles in a variety of cell functions in fungal and animal cells (Longtine *et al.*, 1996; Trimble, 1999; Gladfelter *et al.*, 2001; Longtine and Bi, 2003). Typical septins have a variable N-terminal region, a conserved core that includes the element of a GTP-binding site, and a variable C-terminal region. Septins were first identified as temperature-sensitive *cdc* mutants in *S. cerevisiae* (Hartwell, 1971). Septins are assembled into a ring before bud formation and remain as a collar subjacent to the plasma membrane at the mother-bud neck for most of the cell cycle. The septin ring is assembled by the copolymerization of the septins Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p into filaments (Frazier *et al.*, 1998; Field and Kellogg, 1999). Purified septins from yeast form filaments of 7–9-nm diameter and of various lengths (Frazier *et al.*, 1998).

One major role for the septins in various organisms is in cytokinesis (Hartwell, 1971; Neufeld and Rubin, 1994; Kinoshita *et al.*, 1997; Bi *et al.*, 1998; Lippincott and Li, 1998). The septins localize to the division site (cleavage furrow) during cytokinesis (Longtine *et al.*, 1996; Trimble, 1999; Nguyen *et al.*, 2000; Westfall and Momany, 2002). The septins recruit a variety of other proteins, whose correct localization to the neck is critical for the performance of their various functions, including chitin deposition (DeMarini *et al.*, 1997), bud site selection (Chant *et al.*, 1995; Sanders and Herskowitz, 1996), and cell cycle control (Barral *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000). Thus, the septins have been proposed to function as a scaffold at the bud neck for protein anchoring and organization (Longtine *et al.*, 1996, 2000; Field and Kellogg, 1999; Gladfelter *et al.*, 2001). Additionally, the septins function as a diffusion barrier at the mother-bud junction to prevent membrane-associated proteins from moving freely between the mother and bud compartments (Barral *et al.*, 2000; Takizawa *et al.*, 2000). In spite

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Abbreviations used: BBD, Bud6p-binding domain; DIC, differential interference contrast; FH, formin homology; GAP, GTPase-activating protein; GFP, green fluorescent protein; ts, temperature-sensitive; LAT-A, latrunculin A; RBD, Rho-binding domain; SBD, Spa2p-binding domain.

of our plentiful knowledge of the functions of septins, little is known as to how the septin ring is assembled during budding initiation.

Reorganization of both actin and septins requires the Rho type small GTPase Cdc42p (Pringle *et al.*, 1995). Conditional *cdc42* mutant cells are defective in assembly of the septin ring and polarized reorganization of the actin cytoskeleton. Therefore, these cells are defective in bud emergence and localized cell surface growth, and they become arrested as large, multinucleate, unbudded cells at the restrictive growth temperature. Isolation of *cdc42* mutants, which specifically display defects in septin ring assembly, and analysis of the effect of Cdc42p GAPs on septin ring assembly suggest that cycles of GTP loading and hydrolysis by Cdc42p play an important role in septin ring assembly (Gladfelter *et al.*, 2002; Smith *et al.*, 2002; Caviston *et al.*, 2003). Although significant effort has been dedicated to deciphering the Cdc42p effector pathways important for actin polarization, little is known about how Cdc42p mediates septin ring assembly. Several effectors of Cdc42p have been identified, including the p21-activated kinase (PAK)-like kinases Ste20p, Cla4p, and Skm1p, a formin family protein Bni1p, and Gic1p and Gic2p (Pruyne and Bretscher, 2000a). Among these effectors, Cla4p is required for normal septin function (Cvrckova *et al.*, 1995; Weiss *et al.*, 2000; Schmidt *et al.*, 2003). However, *cla4*Δ mutant can still assemble a septin ring. Bni1p and its related protein Bnr1p assemble actin cables, which play a pivotal role in the polarized transport of secretory vesicles as a track for type V myosin, Myo2p (Evangelista *et al.*, 2002; Sagot *et al.*, 2002a; Schott *et al.*, 2002). Bni1p can stimulate polymerization of actin in vitro independent of the Arp2/3 complex (Pruyne *et al.*, 2002; Sagot *et al.*, 2002b). Bni1p, together with Spa2p, Bud6p, and Pea2p, constitutes a large complex termed the 12S polarisome (Sheu *et al.*, 1998; Pruyne and Bretscher, 2000b). Polarisome components are required for apical growth (Sheu *et al.*, 2000); in their absence, vegetative buds grow as spheres rather than ellipsoids. In the course of our study on the genetic interactions between effectors of *CDC42*, we found that a *bni1*Δ mutation shows a synthetic lethal interaction with the *cla4*Δ mutation. The *bni1*Δ *cla4-75-td* mutant showed abnormal morphology, which was caused by a defect in the assembly of the septin ring during budding initiation. Mutations in other polarisome components also showed a similar synthetic defect with the *cla4*Δ mutation in septin ring assembly. Interestingly, actin cables were suggested to be involved in the septin ring assembly, downstream of Bni1p.

## MATERIALS AND METHODS

### *Media and Genetic Techniques*

Unless otherwise specified, strains were grown in rich medium YPDA (1% yeast extract [Difco Laboratories, Detroit, MI], 2% bacto-peptone [Difco], 2% glucose, and 0.01% adenine). Strains carrying plasmids were selected in synthetic medium (SD) containing the required nutritional supplements (Sherman, 1991). For induction of the *GAL1* promoter, 3% galactose and 0.2% sucrose were used as carbon sources, instead of glucose (YPGA). Sporulation was performed as previously described (Mochida *et al.*, 2002). Standard genetic manipulations of yeast were performed as described (Sherman and Hicks, 1991). Yeast transformations were performed by the lithium acetate method (Elble, 1992; Gietz and Woods, 2002). *Escherichia coli* strains DH5α and XL1-Blue were used for construction and amplification of plasmids.

### *Strains and Plasmids*

Yeast strains used in this study are listed in Table 1. The complete deletion of the ORFs of *BNI1, BUD6, SPA2, PEA2, CLA4,* and *BNR1* was performed with the use of a PCR-based procedure as described (Longtine *et al.*, 1998b; Goldstein and McCusker, 1999). The ts-degron–tagged *cla4-75* (*cla4-75-td*) construct was integrated at the *URA3* locus as previously described (Holly and Blumer, 1999). The enhanced green fluorescent protein-tagged *CDC12* (*CDC12-EGFP*) construct was integrated at the *LEU2* locus. To overexpress a green fluorescent protein (GFP)-tagged Cdc42p or $Cdc42^{G12V}$p, the $P_{GAL1}$-*GFP-CDC42* or $P_{GAL1}$-*GFP-CDC42*$^{G12V}$ construct was integrated at the *URA3* locus. The *tpm1-2 tpm2*Δ, *myo2-20*, and *arp2-2* mutants were backcrossed three times to YEF473 background strains. Plasmids used in this study are listed in Table 2. Schemes for the construction of plasmids and nucleotide sequences of PCR primers are available upon request.

### *Construction of the bni1-116 bnr1*Δ *Strain*

We randomly mutagenized the entire *BNI1* gene by a PCR-based method (Cadwell and Joyce, 1992; Caviston *et al.*, 2002). The mutagenized PCR products were mixed with an equal amount of linearized YCp50-LEU2-BNI1, in which a major portion of the *BNI1* gene was removed by a restriction digestion with *Pvu*II (sequences from 2329-base pairs downstream of the *BNI1* ATG to 337-base pairs downstream of the *BNI1* stop codon were removed). This DNA mixture was transformed into YEF2182 (**a** *bni1*Δ*::HIS3 bnr1*Δ*::HIS3*) containing YEp352-BNI1. Transformants were selected on SD-Leu plates at 24°C and replicated onto SD-Leu plates containing 5-fluoroorotic acid to select for the loss of the *URA3*-containing plasmid. These $Ura^-$ $Leu^+$ colonies were replicated onto two sets of SD-Leu plates that were incubated at 24 and 37°C, respectively, to allow the identification of the temperature-sensitive (ts) mutants. The plasmid carrying the *bni1*-ts allele was isolated and the *bni1*-ts ORF was sequenced by a standard protocol.

To integrate a *bni1*-ts mutation onto the chromosomal locus, the plasmid carrying the mutant allele was digested with *Sph*I and *Afl*II, which released the entire DNA insert carrying the *bni1*-ts allele from the plasmid backbone. The digestion mixture was transformed into YEF2632 (**a** *bni1*Δ*::TRP1 bnr1*Δ*::HIS3*) containing the pRS316-BNR1. The transformation mixture was plated onto YPDA plates at 23°C for 3 d and replicated onto synthetic complete (SC) plates containing 5-fluoroorotic acid to select for the loss of the *URA3*-containing plasmid. The $Ura^-$ $Trp^-$ colonies were streaked onto two sets of YPDA plates and incubated at 24 and 37°C, respectively, to confirm temperature sensitivity. The ts phenotype of each *bni1-ts bnr1*Δ strain was fully complemented by a centromere-based plasmid carrying either *BNI1* or *BNR1* (unpublished results). In this study, we used *bni1-116 bnr1*Δ strain, one of them. The characterization of this mutant strain will be described elsewhere.

### *Microscopic Observations*

Cells were observed using a Nikon ECRIPS E800 microscope (Nikon Instec, Tokyo, Japan) equipped with HB-10103AF super high-pressure mercury lamp and 1.4NA 100× Plan Apo oil immersion objective (Nikon Instec) with appropriate fluorescence-filter sets (Nikon Instec) or differential interference contrast (DIC) optics. Images were acquired using a cooled digital CCD camera (C4742–95-12NR; Hamamatsu Photonics K.K., Hamamatsu, Japan) and AQUACOSMOS software (Hamamatsu Photonics K.K.). To observe Cdc12p-GFP, cells were fixed for 5 min at room temperature by direct addition of commercial 37% formaldehyde stock (Wako Pure Chemical Industries, Osaka, Japan) to a final concentration of 3.7% in the medium and washed twice with phosphate-buffered saline before mounting on a glass microscope slide. Fixed cells were observed using a GFP bandpass filter set (excitation, 460–500 nm; dichroic mirror, 505 nm; emission, 510–560 nm).

Time-lapse analyses of cell morphology and septin ring assembly were carried out as follows. Cells were grown to an early logarithmic phase in SC medium at 25°C, harvested by brief centrifugation, washed once with SC, and resuspended in SC. The cell suspension was spotted onto a thin layer of SC medium containing 1% agarose on a glass microscope slide, which was quickly covered with a coverslip. Around the edges of the coverslip, a small amount of vaseline (Wako) was applied for sealing. An image at each time point was acquired as described above. During observation, the sample was kept at 37°C by Thermo plate (Tokai HIT Co., Fujinomiya, Japan).

Initial septin ring assembly was monitored by observing Cdc12p-GFP in cells exiting from cell cycle arrest. Cells were synchronized in the $G_1$ phase of the cell cycle by the addition of α-factor and released from the block by removal thereof. In brief, cells were grown to a logarithmic phase, pelleted, and resuspended in YPDA containing 1 μg/ml α-factor (Sigma Chemical, St. Louis) at 0.3–0.5 × $10^7$ cells/ml. When cells were observed to have shmoos, cells were washed with 10 ml of YP (1% yeast extract, 2% bacto-peptone) three times and released back into fresh YPDA at 25 or 37°C. The septin ring assembly phenotypes were also examined in cells exiting from stationary phase. Stationary phase cells were collected as described by Ayscough *et al.* (1997) with minor modifications. An overnight culture was inoculated in YP containing 2% raffinose and 0.01% adenine and grown for 2 d at 25°C. Cells were pelleted and resuspended in YP containing 1 M sorbitol. Cells were spun at 500 × *g* for 1 min. Unbudded cells remained in the supernatant fraction. Cells were repeatedly centrifuged until a uniform population of unbudded cells was obtained. These cells were pelleted, resuspended in YPDA or YPGA at 1.0 × $10^7$ cells/ml, and released from stationary phase at 25 or 37°C. These two experiments gave essentially the same results. Therefore, the results of the α-factor arrest-release experiment were presented, unless otherwise mentioned. *cdc15-2* and *bni1*Δ *cdc15-2* mutant cells were synchronized as described (Fitch *et al.*, 1992). When the effect of latrunculin A (LAT-A; Wako) was

**Table 1.** *S. cerevisiae* strains used in this study

| Strain[a] | Genotype | Reference or source |
|---|---|---|
| ABY530 | *MATα lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2-3,112 ade2-101 myo2-20::HIS3* | Schott *et al.* (1999) |
| ABY944 | *MAT***a** *lys2-801 ura3-52 his3Δ-200 trp1-1 leu2-3,112 tpm1-2::LEU2 tpm2Δ::HIS3* | Pruyne *et al.* (1998) |
| K1993 | *MAT***a** *ura3 his3-11,15 trp1-1 leu2-3,112 ade2-1 can1-100 ssd1-Δ2 cdc15-2* | A gift from K. Nasmyth |
| YMW221U | *MAT***a** *lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 ade2-101 arp2-2(G19D)::URA3* | Madania *et al.* (1999) |
| YEF473 | *MAT***a**/α *lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1* | Bi and Pringle (1996) |
| YEF2182 | *MAT***a** *bni1Δ::HIS3 bnr1Δ::HIS3* [YEp352-BNI1] | This study |
| YEF2632 | *MAT***a** *bni1Δ::TRP1 bnr1Δ::HIS3* [pRS316-BNR1] | This study |
| YEF2669 | *MAT***a** *bni1-116 bnr1Δ::HIS3* | This study |
| YKT38 | *MAT***a** *lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1* | Mochida *et al.* (2002) |
| YKT91 | *MAT***a** *myo3Δ::TRP1 myo5-1::KanMX6* | Toi *et al.* (2003) |
| YKT382 | *MAT***a** *bni1Δ::HIS3MX6* | This study |
| YKT440 | *MAT***a** *bnr1Δ::HphMX4 HIS3::$P_{GAL1}$-3HA-BNI1* | This study |
| YKT483 | *MAT***a** *cla4Δ::KanMX6 HIS3::$P_{GAL1}$-3HA-BNI1* | This study |
| YKT541 | *MAT***a** *bni1Δ::HIS3MX6 cla4Δ::KanMX6 URA3::cla4-75-td* | This study |
| YKT618 | *MAT***a** *cla4Δ::KanMX6 URA3::cla4-75-td* | This study |
| YKT637 | *MAT***a** *LEU2::CDC12-EGFP* | This study |
| YKT638 | *MAT***a** *bni1-116 cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT649 | *MAT***a** *bni1Δ::HIS3MX6 cla4Δ::KanMX6 URA3::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT664 | *MAT***a** *bud6Δ::HphMX4 cla4Δ::KanMX6 URA3::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT666 | *MAT***a** *spa2Δ::HphMX4 cla4Δ::KanMX6 URA3::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT668 | *MAT***a** *pea2Δ::HphMX4 cla4Δ::KanMX6 URA3::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT676 | *MAT***a** *bni1Δ::HphMX4 LEU2::CDC12-EGFP* | This study |
| YKT678 | *MAT***a** *cla4Δ::KanMX6 URA3::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT717 | *MAT***a** *cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT743 | *MAT***a** *bni1-116 bnr1Δ::HphMX4 LEU2::CDC12-EGFP* | This study |
| YKT744 | *MAT***a** *bni1-116 bnr1Δ::HphMX4 cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT811 | *MAT***a** *bni1Δ::HIS3MX6 cla4Δ::KanMX6 TRP1::cla4-75-td LEU2::CDC12-EGFP* | This study |
| YKT812 | *MAT***a** *tpm1-2::LEU2 tpm2Δ::HIS3 LEU2::CDC12-EGFP* | This study |
| YKT813 | *MAT***a** *tpm1-2::LEU2 tpm2Δ::HIS3 cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT814 | *MAT***a** *myo2-20::HIS3 LEU2::CDC12-EGFP* | This study |
| YKT815 | *MAT***a** *myo2-20::HIS3 cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT816 | *MAT***a** *arp2-2(G19D)::URA3 LEU2::CDC12-EGFP* | This study |
| YKT817 | *MAT***a** *arp2-2(G19D)::URA3 cla4Δ::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT818 | *MAT***a** *myo3Δ::TRP1 myo5-1::KanMX6 LEU2::CDC12-EGFP* | This study |
| YKT819 | *MAT***a** *myo3Δ::TRP1 myo5-1::KanMX6 cla4Δ::CgHIS3 LEU2::CDC12-EGFP* | This study |
| YKT891 | *MAT***a** *LEU2::CDC12-EGFP URA3::$P_{GAL1}$-GFP-CDC42* | This study |
| YKT892 | *MAT***a** *LEU2::CDC12-EGFP URA3::$P_{GAL1}$-GFP-CDC42$^{G12V}$* | This study |
| YKT893 | *MAT***a** *bni1Δ::HphMX4 LEU2::CDC12-EGFP URA3::$P_{GAL1}$-GFP-CDC42* | This study |
| YKT894 | *MAT***a** *bni1Δ::HphMX4 LEU2::CDC12-EGFP URA3::$P_{GAL1}$-GFP-CDC42$^{G12V}$* | This study |
| YKT939[b] | *MAT***a** *cdc15-2 LEU2::CDC12-EGFP* | This study |
| YKT940[b] | *MAT***a** *bni1Δ::KanMX6 cdc15-2 LEU2::CDC12-EGFP* | This study |

[a] YEF and YKT strains, except for YKT939 and YKT940, are isogenic derivatives of YEF473. For YEF and YKT strains, only relevant genotypes are described.

[b] These strains are derivatives of K1993.

examined, $G_1$-arrested cells were treated with 100 μM LAT-A (added to the medium from a 20 mM stock in DMSO) as described by Ayscough *et al.* (1997). As a control, an equal volume of DMSO alone was added. At least 100 cells containing polarized Cdc12p-GFP were observed in each experiment, where >90% of them showed a uniform phenotype, unless otherwise mentioned. A representative image of the cells is shown in each figure.

To measure septin ring diameter, cells were released from $G_1$ arrest for 30 min and fixed before bud emergence. The diameter of the septin ring, which was defined as the maximum distance across the septin ring, was measured from the center between outer and inner edges of the septin ring to the opposite center between the edges using Adobe illustrator version 9.0 (Adobe Systems, San Jose, CA). For each determination of the average diameter of septin rings, 100 cells were chosen randomly and measured.

### *Isolation of Multicopy Suppressors of the bni1-116 cla4Δ mutant*

The *bni1-116 cla4*Δ strain (YKT530) was transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEp24. After transformation, cells were incubated on SD-Ura plates at 25°C for 40 h to allow recovery, replica-plated onto fresh YPDA plates, and then incubated at 35°C for 3 d. About 50,000 transformants were screened, and 12 transformants that reproducibly grew at 35°C were obtained. From each of the transformants, plasmids were recovered for further analysis. PCR revealed that five plasmids contained *BNI1* ORF, but no plasmid contained *CLA4* ORF. All of the remaining seven plasmids conferred temperature-resistant growth on YKT530. The genes present in the seven plasmids were identified by sequencing both ends of the inserts. The suppressor gene in those was identified by testing individual subcloned fragments for suppressing activity. Two of them were *BEM3Δ1-114* and *RGA1Δ1-632*. The other five genes will be described elsewhere.

## RESULTS

### *Loss of Bni1p Causes Defective Septin Organization in the Absence of Cla4p*

The effectors of Cdc42p are involved in cellular polarization through the cell cycle, but the details of their functions are unknown. To further explore these functions, we examined the genetic interactions between effectors of *CDC42*. Previous reports showed that a cell lacking both Cla4p and Ste20p

**Table 2.** Plasmids used in this study

| Plasmid | Characteristics | Reference or source |
|---|---|---|
| pFA6a-TRP1 | *TRP1* | Longtine *et al.* (1998b) |
| pFA6a-KanMX6 | $P_{TEF}$-*KanMX6*-$T_{TEF}$ | Longtine *et al.* (1998b) |
| pAG32 | $P_{TEF}$-*HphMX4*-$T_{TEF}$ | Goldstein and McCusker (1999) |
| pcla4-75-degron | *cla4-75-ts degron URA3* | Weiss *et al.* (2000) |
| YCp50-LEU2-BNI1 | *BNI1 LEU2 CEN4* | A gift from J. Pringle |
| YEp352-BNI1 | *BNI1 URA3 2 μm* | A gift from J. Pringle |
| YEp24 | *URA3 2 μm* | Rose and Broach (1990) |
| pRS316 | *URA3 CEN6* | Sikorski and Hieter (1989) |
| pRS316-BNR1 | *BNR1 URA3 CEN6* | This study |
| pKT1178 [pRS316-CDC12G] | *CDC12-EGFP URA3 CEN6* | Iwase and Toh-e (2001) |
| pKT1254 [pRS316-CLA4] | *CLA4 URA3 CEN6* | This study |
| pKT1364 [YEp24-BEM3(Δ1-114)] | *BEM3* (Δ1-114) *URA3 2 μm* | This study |
| YEplac195 | *URA3 2 μm* | Gietz and Sugino (1988) |
| pKT1365 [YEplac195-RGA1(Δ1-632)] | *RGA1* (Δ1-632) *URA3 2 μm* | This study |
| pRS314 | *TRP1 CEN6* | Sikorski and Hieter (1989) |
| pKT1226 [pRS314-BNI1] | *BNI1 TRP1 CEN6* | This study |
| pKT1420 [pRS314-BNI1(Δ6-808)] | *BNI1* (Δ6-808) *TRP1 CEN6* | This study |
| pKT1421 [pRS314-BNI1(Δ826-986)] | *BNI1* (Δ826-986) *TRP1 CEN6* | This study |
| pKT1422 [pRS314-BNI1(1-1750)] | *BNI1* (1-1750) *TRP1 CEN6* | This study |
| pKT1423 [pRS314-BNI1(Δ1228-1414)] | *BNI1* (Δ1228-1414) *TRP1 CEN6* | This study |
| pKT1424 [pRS314-BNI1(Δ1553-1646)] | *BNI1* (Δ1553-1646) *TRP1 CEN6* | This study |
| pRS305 | *LEU2* | Sikorski and Hieter (1989) |
| pKT1452 [pRS305-CDC12-EGFP] | *CDC12-EGFP LEU2* | This study |
| pRS306 | *URA3* | Sikorski and Hieter (1989) |
| pKT1474 [pRS306-$P_{GAL1}$-GFP-CDC42] | $P_{GAL1}$-*GFP-CDC42 URA3* | This study |
| pKT1475 [pRS306-$P_{GAL1}$-GFP-CDC42$^{G12V}$] | $P_{GAL1}$-*GFP-CDC42*$^{G12V}$ *URA3* | This study |

kinases was inviable (Cvrckova *et al.,* 1995; Holly and Blumer, 1999; Goehring *et al.,* 2003). We examined the genetic interaction of *BNI1* with *CLA4* or *STE20*. Interestingly, *cla4*Δ mutation led to a synthetic lethal interaction with the *bni1*Δ mutation, whereas there was no genetic interaction for cell growth between *ste20*Δ and *bni1*Δ (unpublished results).

To examine the terminal phenotype of *cla4*Δ mutant lacking Bni1p, we constructed a series of strains expressing an integrated *cla4-75*-ts degron construct (*cla4-75-td*). This version of Cla4p was degraded rapidly after a shift to the restrictive temperature (Holly and Blumer, 1999), and thus the *bni1*Δ *cla4-75-td* mutant showed a ts growth (Figure 1A). At the restrictive temperature, *bni1*Δ *cla4-75-td* mutant cells exhibited a wide bud neck and abnormally elongated bud morphology (Figure 1B). We examined the organization of septin, a constituent of bud neck filaments, by observing GFP-tagged Cdc12p (Figure 1B). In wild-type and single mutant cells, the septins localized at the bud neck. In contrast, the septins localized as a cap at the polarized growing site in the *bni1*Δ *cla4-75-td* mutant, suggesting that Bni1p and Cla4p are redundantly involved in septin organization.

Whereas our study was in progress, Sprague and colleagues reported a similar genetic interaction between *BNI1* and *CLA4* (Goehring *et al.,* 2003). Our results are consistent with their data, in which they also showed that loss of Bni1p or its interacting proteins (Spa2p, Bud6p, and Pea2p; see below) in a *cla4*Δ mutant resulted in lethality and caused cells to form elongated buds with mislocalized septin rings. Furthermore, they showed that Bni1p is a Ste20p-dependent phosphoprotein and may be directly regulated by Ste20p. We extended this study and found that, as described below, Bni1p and Cla4p are required for the assembly of the septin ring during budding initiation.

### *Bni1p and Cla4p Are Required for the Initial Septin Ring Assembly, but Not for the Maintenance of Septin Collar*

We performed time-lapse observations of a *bni1*Δ *cla4-75-td* mutant expressing Cdc12p-GFP. When unbudded *bni1*Δ *cla4-75-td* mutant cells that had not yet assembled a septin ring were shifted to 37°C, these cells could not assemble normal septin ring like that of wild-type cells (Figure 2A). Their septins, instead, accumulated as a cap at the incipient budding site and remained at the polarized growing site. In contrast, when budding *bni1*Δ *cla4-75-td* mutant cells that had formed a septin collar were shifted to 37°C, these cells could accomplish cytokinesis normally without disorganizing their septin collar (Figure 2B). However, the resulting mother and daughter cells showed the defect in septin ring assembly in the next cell cycle. To observe the initial septin assembly in detail, we performed time-lapse analyses with the 5-min time points. We observed 16 individual *bni1*Δ *cla4-75-td* mutant cells and found that none of them could assemble a septin ring at 37°C, suggesting that this mutant cannot assemble a septin ring even with a transient manner (Figure 2C). These results indicate that the *bni1 cla4* double mutation causes a defect in the initial assembly of septin ring but not in the maintenance of the formed septin collar.

The *cla4*Δ mutant shows defects in septin functions (Cvrckova *et al.,* 1995; Weiss *et al.,* 2000; Schmidt *et al.,* 2003). Because the septin defects have not been described in the *bni1*Δ mutant in detail, we carefully observed the septin rings assembled during the initiation of budding in cells exiting from the α-factor–induced $G_1$ arrest. The $G_1$-arrested wild-type cells made a very polarized shmoo, whereas the *bni1*Δ mutant did not and instead formed a broad blunt projection, as described (Evangelista *et al.,* 1997). Septins formed an array of bars at the base of the projection in $G_1$-arrested cells (Figure 3A), as described (Longtine *et al.,*

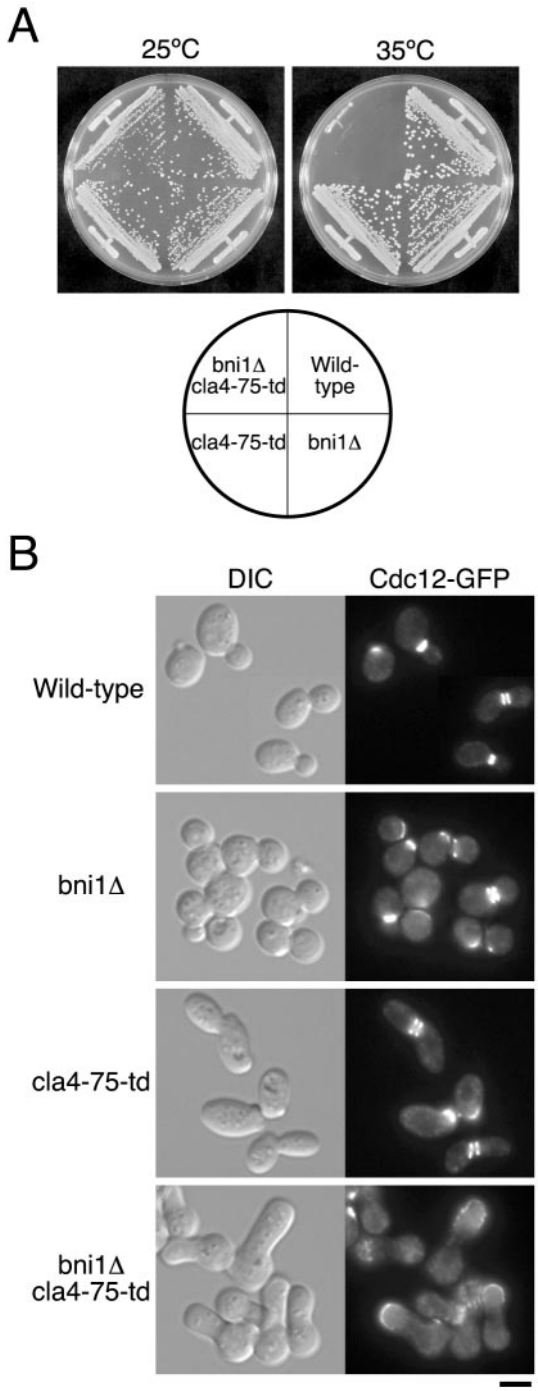



**Figure 1.** Loss of *BNI1* causes defects in cell growth, cell morphology, and septin organization in the absence of Cla4p. (A) YKT38 (wild-type), YKT382 (*bni1*Δ), YKT618 (*cla4-75-td*), and YKT541 (*bni1*Δ *cla4-75-td*) were streaked on a YPDA plate, which was incubated at 25 or 35°C for 2 d. (B) YKT637 (*CDC12-GFP*), YKT676 (*bni1*Δ *CDC12-GFP*), YKT678 (*cla4-75-td CDC12-GFP*), and YKT649 (*bni1*Δ *cla4-75-td CDC12-GFP*) were grown in YPDA medium at 25°C and then shifted to 37°C for 2 h. Cells were fixed and observed by DIC and fluorescence microscopy. Bar, 5 μm.

1998a). After release from the arrest, we could observe the formation of a septin ring that is clearly bright compared with the shmoo-based septin array (Figure 3A). We found that the septin ring in *bni1*Δ mutant exiting from $G_1$ arrest displays an irregular and jagged morphology (Figure 3B). This abnormal morphology was found in >95% of *bni1*Δ mutant cells. We furthermore found that the diameter of the septin ring was greater in the *bni1*Δ mutant (1.27 ± 0.13 μm) than in wild-type (0.98 ± 0.08 μm) cells (Figure 3C). To confirm the septin ring abnormalities observed in *bni1*Δ mutant more solidly, we performed other cell synchronization methods. We observed septin rings with a larger diameter in *bni1*Δ cells released from nutritional arrest, although the jagged morphology was less severe than that seen in the α-factor–arrested cells (unpublished results). However, it was difficult to obtain the statistic data of the initial septin ring morphology because the degree of synchrony, when released from nutritional arrest, was not sufficiently high compared with that achieved with an α-factor block and release. To obtain a better synchrony, we also used a *cdc15-2* mutation, which causes a temperature-sensitive arrest in mitotic exit (Fitch *et al.*, 1992). We examined the initial septin ring assembly in *cdc15-2* or *bni1*Δ *cdc15-2* mutant exiting from telophase. We found that the initial septin ring in *bni1*Δ *cdc15-2* mutant had a larger diameter (1.25 ± 0.15 μm) than in *cdc15-2* mutant (0.99 ± 0.10 μm) and displayed an aberrant morphology as that in *bni1*Δ mutant exiting from $G_1$ arrest (Figure 3, B and C). These results suggest that the *bni1*Δ mutation affects the integrity of the septin ring and thus shows a synthetic defect with *cla4*Δ mutation in septin ring assembly.

### *The Effects of Bni1p Truncation Mutants on Septin Ring Assembly in the Absence of Cla4p*

Bni1p is a member of a protein family that is characterized by formin homology (FH) domains, FH1 and FH2. The proline-rich FH1 domain binds to profilin as well as to peptide-recognition modules such as SH3 and WW domains (Wasserman, 1998; Ridley, 1999; Zeller *et al.*, 1999). The FH2 domain is highly conserved, and both FH1 and FH2 are involved in actin filament assembly (Evangelista *et al.*, 2002; Sagot *et al.*, 2002a). Bni1p also has a Rho-binding domain (RBD), which interacts with Rho1p and Cdc42p, in its amino-terminal region (Kohno *et al.*, 1996; Evangelista *et al.*, 1997), a Spa2p-binding domain (SBD) in the middle region (Fujiwara *et al.*, 1998), and a Bud6p-binding domain (BBD) at the carboxyl-terminal region (Evangelista *et al.*, 1997). The *bni1*Δ mutation shows a synthetic lethal interaction with the *bnr1*Δ mutation due to defects in actin cable assembly (Evangelista *et al.*, 2002; Sagot *et al.*, 2002a). To examine the effects of Bni1p truncation mutants on cell growth in the absence of Cla4p, we constructed five truncation mutations of *BNI1* and tested these for the ability to complement the $P_{GAL1}$-*BNI1 bnr1*Δ or $P_{GAL1}$-*BNI1 cla4*Δ mutation in glucose medium, in which the activity of *GAL1* promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the $P_{GAL1}$-*BNI1 bnr1*Δ or $P_{GAL1}$-*BNI1 cla4*Δ mutant at all temperatures tested (Figure 4B). We also examined assembly of the septin ring upon exit from $G_1$ arrest induced by α-factor (Figure 4C). We observed Cdc12p-GFP in *bni1*Δ *cla4-75-td* mutants expressing truncated Bni1p after 30- and 60-min incubations at 37°C. Typically, >70% and >90% of *bni1*Δ *cla4-75-td* mutant expressing full-length Bni1p assembled a normal septin ring after 30- and 60-min incubations, respectively (Figure 4C, unpublished results). In *bni1*Δ *cla4-75-td* mutant expressing either ΔFH1 or ΔFH2, septins formed a cap-like structure at the incipient budding site after a 30-min incubation; this was reminiscent of the phenomena observed in *bni1*Δ *cla4-75-td* mutant at 37°C. Less than 5% of cells expressing ΔFH2 could form a septin ring-like struc-

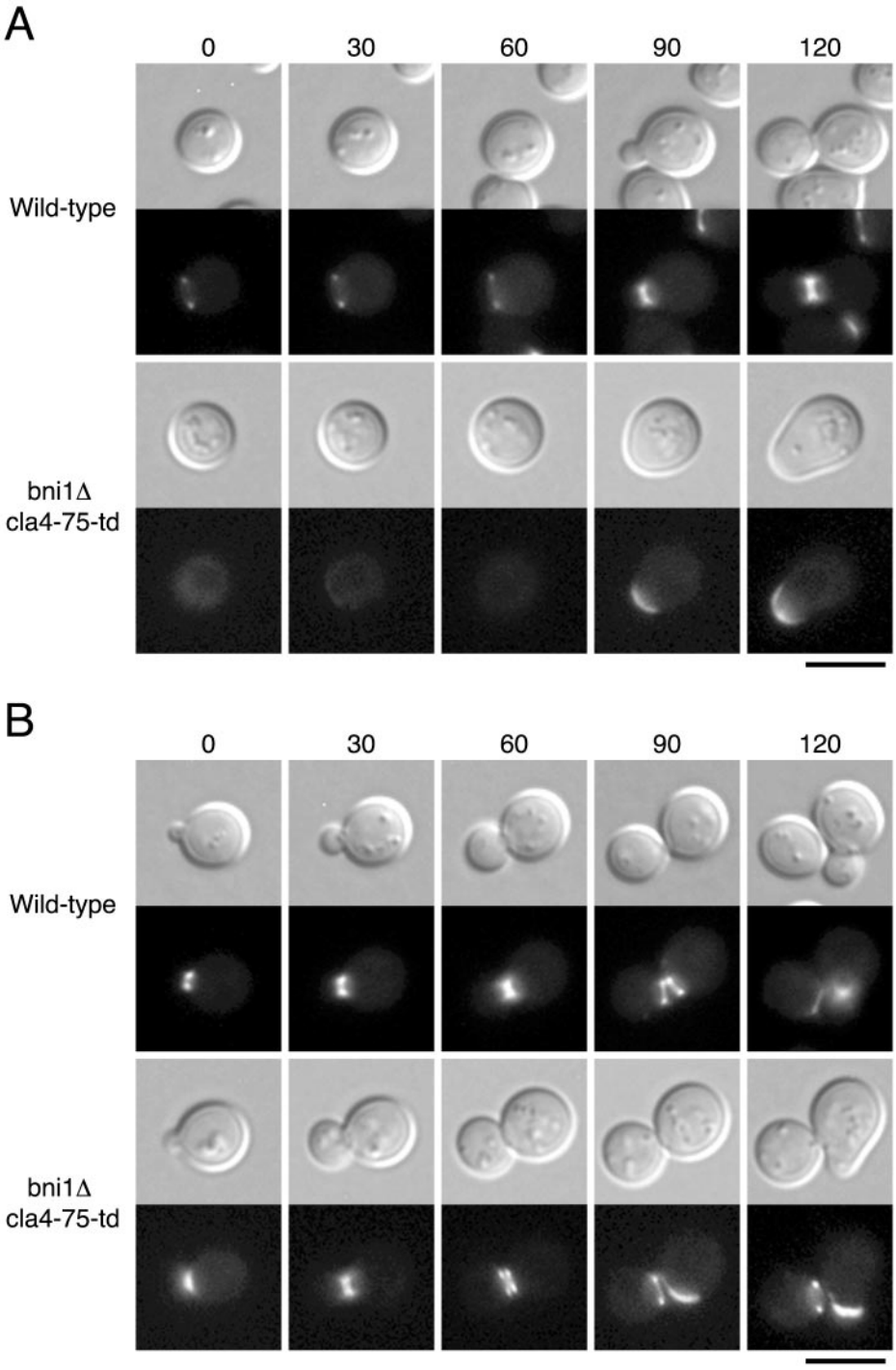



**Figure 2.**

ture, which was much fainter and thinner than the septin ring in wild-type cells, but the septins became diffused and formed a cap-like structure on the bud tip by 60 min (unpublished results). Our results suggest that actin cables, which are formed by the action of Bni1p, are required for septin ring assembly in the absence of Cla4p.

ΔSBD and ΔBBD restored the growth of the $P_{GAL1}$-*BNI1 bnr1*Δ mutant at all temperatures tested, suggesting that the interaction of Bni1p with either Spa2p or Bud6p is not required for formation of the actin cables that are sufficient for polarized cell growth. Consistently, a *bnr1*Δ mutation did not show a synthetic lethal interaction with either a *spa2*Δ or *bud6*Δ mutation (unpublished results). Interestingly, neither ΔSBD nor ΔBBD restored the growth of $P_{GAL1}$-*BNI1 cla4*Δ mutant in glucose medium at high temperatures, suggesting that the interaction of Bni1p with either Spa2p or Bud6p plays an important role for growth in the absence of Cla4p (Figure 4B). Assembly of the septin ring was examined in the *bni1*Δ *cla4-75-td* mutant expressing either ΔSBD or ΔBBD upon exit from $G_1$ arrest at 37°C. When grown for 30 min,

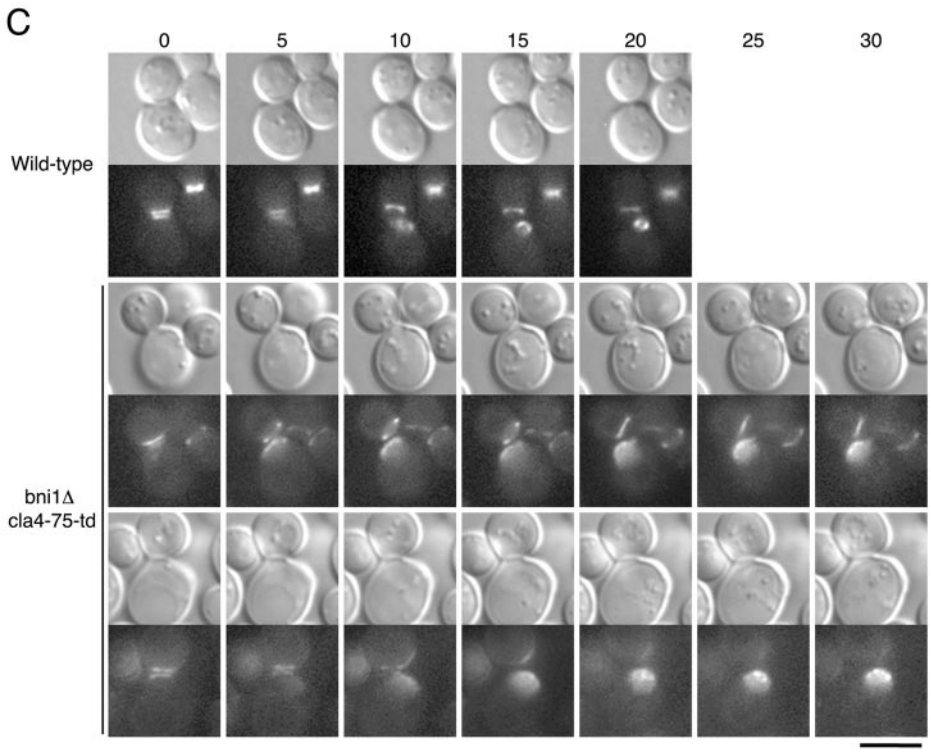



**Figure 2 (facing page).** *bni1*Δ *cla4-75-td* mutant is defective in initial septin ring assembly. Time-lapse microscopy was performed at 37°C on YKT637 (*CDC12-GFP*) and YKT649 (*bni1*Δ *cla4-75-td CDC12-GFP*). (A) Unbudded cells that had not yet assembled a septin ring were shifted to 37°C. (B) Budding cells that had formed a septin collar were shifted to 37°C. (C) Cells in cytokinesis were shifted to 37°C to observe the initial septin assembly. Images were recorded at the indicated time points (minutes). Bars, 5 $\mu$m.

>80% of cells expressing ΔBBD and <10% of cells expressing ΔSBD could form a septin ring-like structure at the incipient budding site. This septin ring-like structure appeared fainter and thinner than a normal septin ring in wild-type cells. However, the septins in both mutants became diffused and formed a cap-like structure on the bud tip after a 60-min incubation (unpublished results). Therefore, the interaction of Bni1p with either Spa2p or Bud6p seems to be required for normal septin ring assembly in the absence of Cla4p.

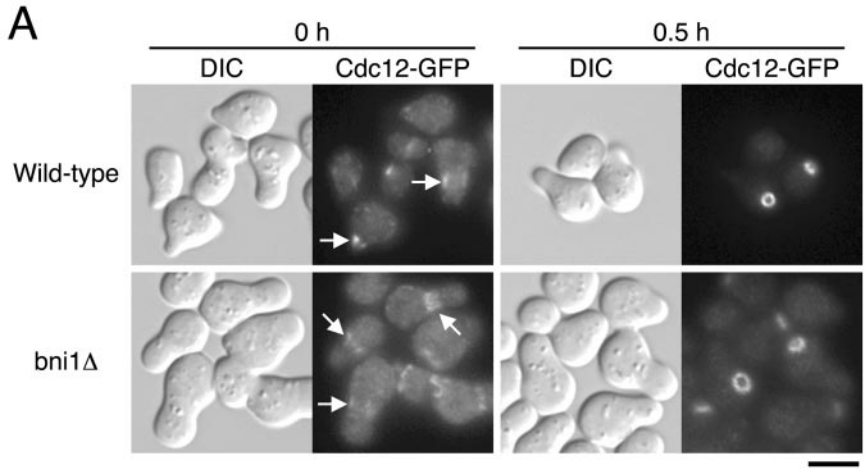
ΔRBD restored the growth of $P_{GAL1}$-*BNI1 bnr1*Δ mutant in glucose medium at all temperatures tested. Interestingly, ΔRBD could not restore the growth of $P_{GAL1}$-*BNI1 cla4*Δ mutant in glucose medium at high temperatures, suggesting that the interaction between Bni1p and Rho-GTPase is also important for growth in the absence of Cla4p (Figure 4B). The *bni1*Δ *cla4-75-td* mutant expressing ΔRBD could not assemble a septin ring upon exit from $G_1$ arrest (Figure 4C). These results suggest that all of the functional domains of Bni1p, which have been identified so far, are required for septin ring assembly during budding initiation, especially at high temperatures in the absence of Cla4p.

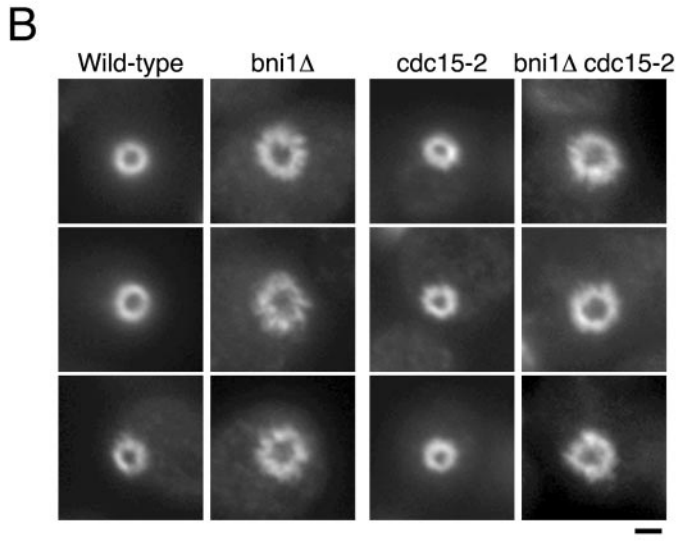
### *Loss of Polarisome Components Causes Defective Septin Ring Assembly in the Absence of Cla4p*

If the interaction between Bni1p and a polarisome component is important for the growth in the absence of Cla4p, polarisome genes would also show the genetic interaction with *CLA4*. The *cla4*Δ mutant was crossed with *bud6*Δ, *spa2*Δ, or *pea2*Δ mutant, and the resulting diploid cells were sporulated and dissected for tetrad analysis. The *bud6*Δ *cla4*Δ and *spa2*Δ *cla4*Δ double mutants exhibited a synthetic lethality at 25°C, and the *pea2*Δ *cla4*Δ double mutant exhibited a poor growth phenotype at 25°C (unpublished results). We also examined the terminal phenotype of *cla4-75-td* mutants lacking a polarisome component. The cell morphological phenotypes of *bud6*Δ *cla4-75-td*, *spa2*Δ *cla4-75-td*, and *pea2*Δ *cla4-75-td* mutants at the restrictive temperature mostly resembled that of the *bni1*Δ *cla4-75-td* mutant (Figure 5A). In each strain, Cdc12p-GFP localized as a cap at the polarized growing site or as a patch around the wide bud neck. These results are consistent with the recent report by Sprague and colleagues (Goehring *et al.*, 2003). To further examine whether a polarisome component is required for initial septin ring assembly in the absence of Cla4p, we analyzed the assembly of the septin ring upon exit from $G_1$ arrest at 37°C (Figure 5B). When grown for 30 min, both *spa2*Δ *cla4-75-td* and *pea2*Δ *cla4-75-td* mutants could not assemble a normal septin ring as well as *bni1*Δ *cla4-75-td* mutant. *bud6*Δ *cla4-75-td* mutant could form a septin ring-like structure, but an additional 30 min later, this structure became diffused and formed a cap-like structure on the bud tip as well as *bni1*Δ *cla4-75-td* mutant carrying ΔBBD (unpublished results). These results suggest that the presence of a polarisome component and its interaction with Bni1p is required for initial septin ring assembly in the absence of Cla4p.

### *Actin Polymerization Is Required for Septin Ring Assembly in the Absence of Cla4p*

The FH2 domain of Bni1p is required for actin filament assembly in vivo (Evangelista *et al.*, 2002; Sagot *et al.*, 2002a) and can stimulate actin polymerization in vitro (Pruyne *et al.*, 2002; Sagot *et al.*, 2002b). As described above, ΔFH2 could not restore the growth of the *bni1*Δ *cla4*Δ double mutant. This result suggests that the actin-nucleating activity of Bni1p may be required for septin ring assembly in the absence of Cla4p. However, it is also possible that the FH2 domain possesses a distinct function in septin ring assembly. To discriminate these possibilities, we isolated a ts-allele of *BNI1*, *bni1-116*, which causes amino acid substitutions




C

The diameter of septin ring

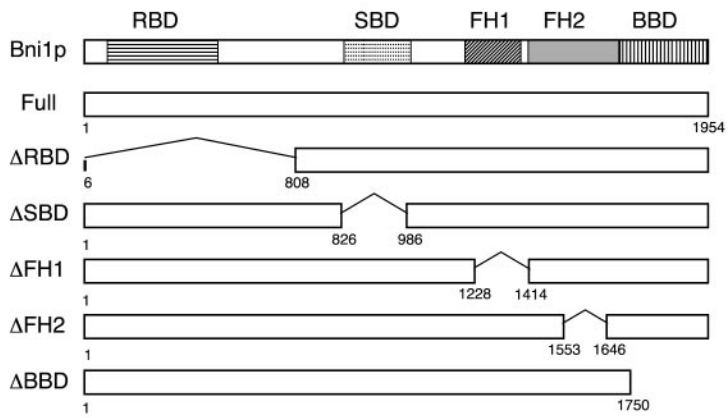
| Strains | mean ± SD (n=100) |
|---|---|
| Wild-type | 0.98 ± 0.08 μm |
| *bni1Δ* | 1.27 ± 0.13 μm |
| *cdc15-2* | 0.99 ± 0.10 μm |
| *bni1Δ cdc15-2* | 1.25 ± 0.15 μm |

**Figure 3.** Loss of *BNI1* causes defects in the integrity of the septin ring. (A) The septin ring in YKT637 (*CDC12-GFP*) and YKT676 (*bni1*Δ *CDC12-GFP*). Cells were $G_1$-arrested with α-factor and released into fresh medium at 25°C. After 0- and 30-min incubations, cells were fixed and observed by fluorescence microscopy. At 0 min, septins formed an array of bars at the base of the projection (arrows). At 30 min, the initial septin rings were assembled. Bar, 5 μm. (B) The morphology of the septin ring in YKT637 (*CDC12-GFP*), YKT676 (*bni1*Δ *CDC12-GFP*), YKT939 (*cdc15-2 CDC12-GFP*), and YKT940 (*bni1*Δ *cdc15-2 CDC12-GFP*). YKT637 and YKT676 were $G_1$-arrested with α-factor and released for 30 min as in A. YKT939 and YKT940 were arrested at telophase by 2-h incubation at 37°C and then released for 50 and 60 min, respectively, after a shift to 25°C. Cells were fixed and observed by fluorescence microscopy. Bar, 1 μm. (C) The average diameter of the septin ring in YKT637, YKT676, YKT939, and YKT940. To measure the average septin ring diameter, 100 cells of each strain were chosen randomly. The values are means with SD.

(V1475A, K1498E, and D1511N) in the FH2 domain. In cells carrying *bni1-116 bnr1*Δ mutations, actin cables visualized by rhodamine-phalloidin disappear in 2 min after upshift to 37°C (unpublished results). This effect on actin cables was not due to degradation of Bni1-116p, because Bni1-116p-GFP was localized to a bud tip in cells grown at 37°C (unpublished results). The *bni1-116 cla4*Δ mutant showed growth defects above 35°C and abnormally elongated buds with wide necks, results similar to *bni1*Δ *cla4-75-td* mutant cells (unpublished results). We examined assembly of the septin ring upon exit from $G_1$ arrest at 37°C in the *bni1-116 cla4*Δ mutant. When grown for 30 min, *bni1-116 cla4*Δ mutant as well as *bni1*Δ *cla4-75-td* mutant could not assemble normal septin ring (Figure 6A). *bni1-11* (amino acid substitutions: D1511G and K1601R; Evangelista *et al.*, 2002) and *bni1-FH2#1* (amino acid substitutions: R1528A and R1530A; Sagot *et al.*, 2002a) are previously characterized mutations in the FH2 domain. Both mutations also cause rapid disassembly of actin cables at 37°C. We confirmed that *bni1-11 cla4*Δ and *bni1-FH2#1 cla4*Δ mutants showed the defects in cell growth and septin ring assembly as *bni1*Δ *cla4-75-td* mutant (unpublished results). These results suggest that actin polymerization mediated by the FH2 domain of Bni1p is required for the assembly of the septin ring during budding initiation.

A previous study suggested that assembly of the septin ring is independent of the actin cytoskeleton (Ayscough *et al.*, 1997). They showed that the actin assembly inhibitor

A




B

| | $P_{GAL1}$-*BNI1 cla4*Δ | | | $P_{GAL1}$-*BNI1 bnr1*Δ | | |
|---|---|---|---|---|---|---|
| | 30°C | 35°C | 37°C | 30°C | 35°C | 37°C |
| Full | +++ | +++ | +++ | +++ | +++ | +++ |
| ΔRBD | ++ | +/- | - | +++ | +++ | ++ |
| ΔSBD | ++ | + | - | +++ | +++ | +++ |
| ΔFH1 | - | - | - | - | - | - |
| ΔFH2 | - | - | - | - | - | - |
| ΔBBD | ++ | +/- | - | +++ | +++ | ++ |

C

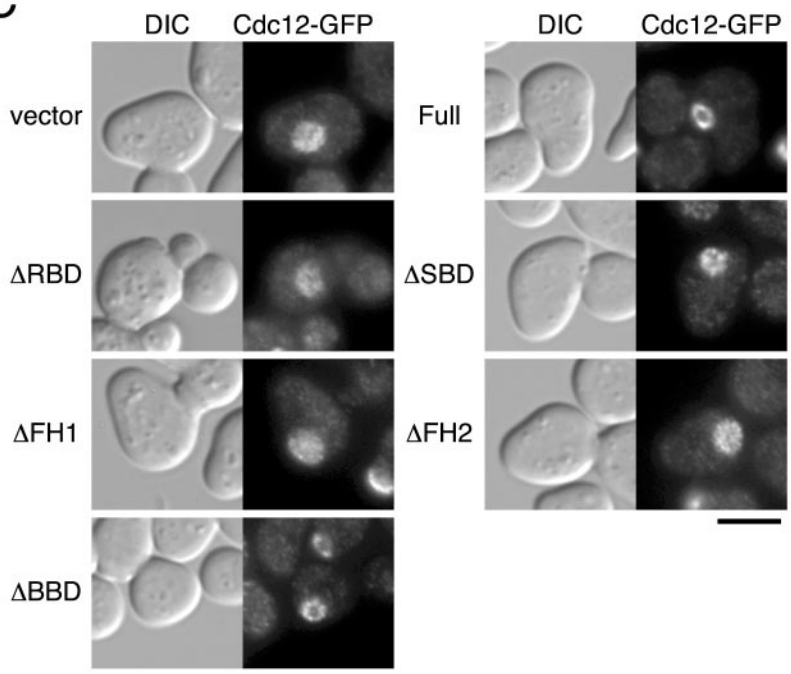



**Figure 4.** The effects of Bni1p truncation on cell growth and septin ring assembly in the absence of Cla4p. (A) Structure of Bni1p truncation proteins. Each number indicates the amino acid residue. Domains include Rho-binding domain (RBD), Spa2p-binding domain (SBD), formin-homology domain (FH1 and FH2), and Bud6p-binding domain (BBD). Each horizontal bar represents a segment of Bni1p: Full, full-length Bni1p; ΔRBD, Bni1(Δ6–808)p; ΔSBD, Bni1(Δ826-986)p; ΔFH1, Bni1(Δ1228-1414)p; ΔFH2, Bni1(Δ1553-1646)p; and ΔBBD, Bni1(1-1750)p. (B) The growth of either *bni1*Δ *bnr1*Δ or *bni1*Δ *cla4*Δ mutants expressing a truncated Bni1p. Five truncation mutants of *BNI1* were cloned into pRS314, and the resultant plasmids were introduced into YKT440 ($P_{GAL1}$-*BNI1 bnr1*Δ) or YKT483 ($P_{GAL1}$-*BNI1 cla4*Δ). Growth was examined on YPDA plates at the temperature indicated. Symbols indicate relative growth rate from wild-type (+++) to no growth (−). (C) Initial assembly of septin ring in *bni1*Δ *cla4-75-td* mutants expressing truncated Bni1p. The plasmids used in B were introduced into YKT649 (*bni1*Δ *cla4-75-td CDC12-GFP*). The transformants were $G_1$-arrested with α-factor at 25°C and released into fresh medium at 37°C. After a 30-min incubation, cells were fixed and observed by DIC and fluorescence microscopy. Bar, 5 μm.

LAT-A did not affect septin ring assembly in wild-type cells. The results described above suggest that LAT-A may inhibit septin ring assembly in the *cla4*Δ mutant. We analyzed septin ring assembly upon exit from $G_1$ arrest at 25°C in the presence or absence of LAT-A in the wild-type, *bni1*Δ, and *cla4*Δ mutants. When grown for 30 min after release from $G_1$ arrest in the absence of LAT-A, each strain assembled a septin ring. An extended diameter and jagged morphology of the septin ring were observed in *bni1*Δ mutant as shown in Figure 3A, and the periphery of the septin ring in *cla4*Δ

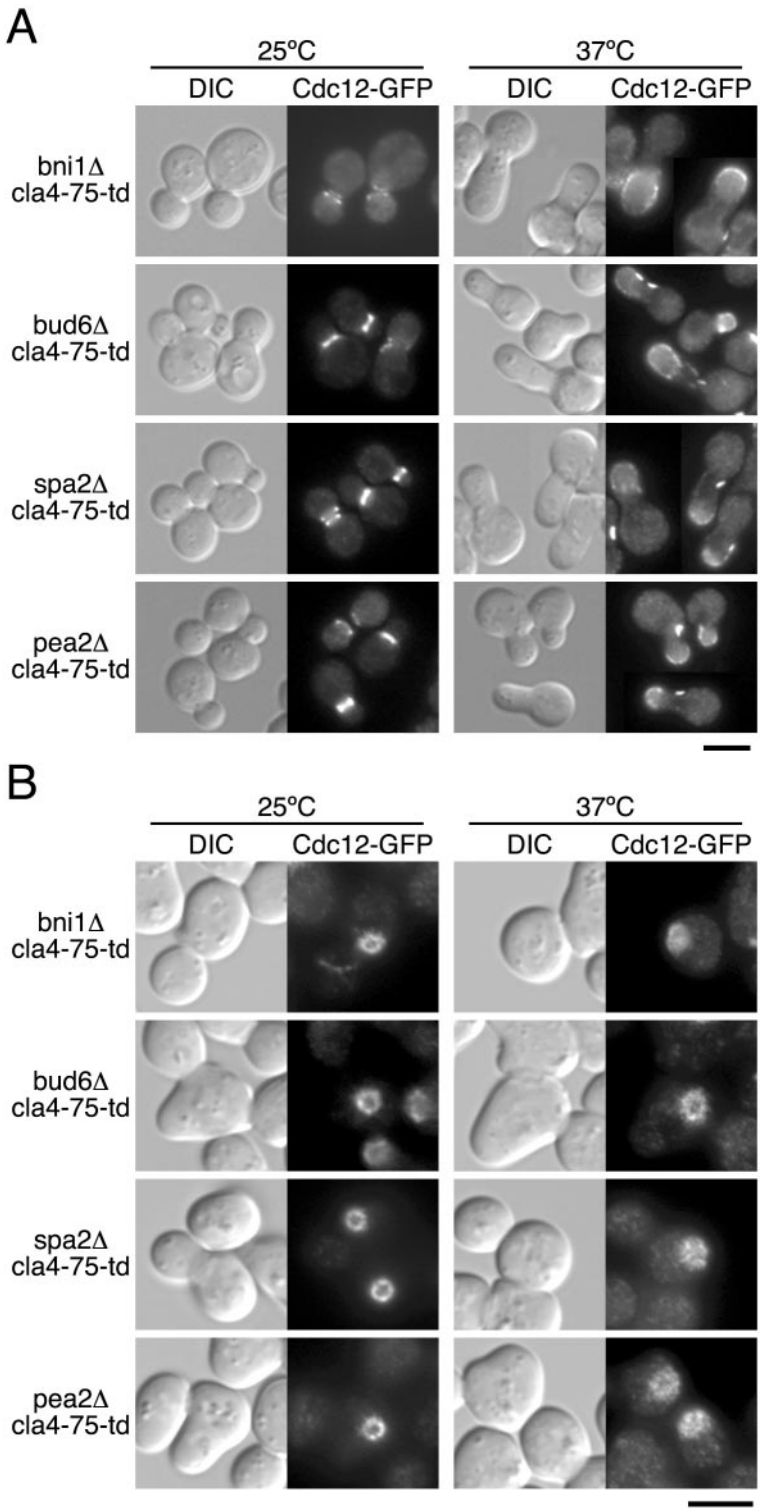



**Figure 5.** Defective septin ring assembly in *cla4-75-td* mutants lacking a polarisome component. (A) YKT649 (*bni1*Δ *cla4-75-td CDC12-GFP*), YKT664 (*bud6*Δ *cla4-75-td CDC12-GFP*), YKT666 (*spa2*Δ *cla4-75-td CDC12-GFP*), and YKT668 (*pea2*Δ *cla4-75-td CDC12-GFP*) were grown in YPDA medium at 25°C and then shifted to 37°C for 2 h. Cells were fixed and observed by DIC and fluorescence microscopy. (B) Cells of each strain were $G_1$-arrested with α-factor at 25°C and released into fresh medium at 25°C or 37°C. After a 30-min incubation, cells were fixed and observed by DIC or fluorescence microscopy. Bars, 5 μm.

mutant looked fainter compared with wild-type (Figure 6B). After an additional 30 min, each strain formed a small bud and the septin collar localized to the bud neck (unpublished results). The diameter of the septin collar in the *bni1*Δ mutant was slightly greater than that in wild-type. Less than 10% of the *cla4*Δ mutant cells localized the septins on the bud tip (unpublished results). When grown for 30 min in the presence of LAT-A, each strain localized the septins at the incipient budding site, but did not assemble a septin ring (unpublished results). An additional 30 min later, >70% of the wild-type and *bni1*Δ mutant cells assembled a septin ring (Figure 6B), suggesting that LAT-A treatment caused a delay in septin ring assembly. However, the *cla4*Δ mutant could not assemble a septin ring (Figure 6B), even after a 6-h

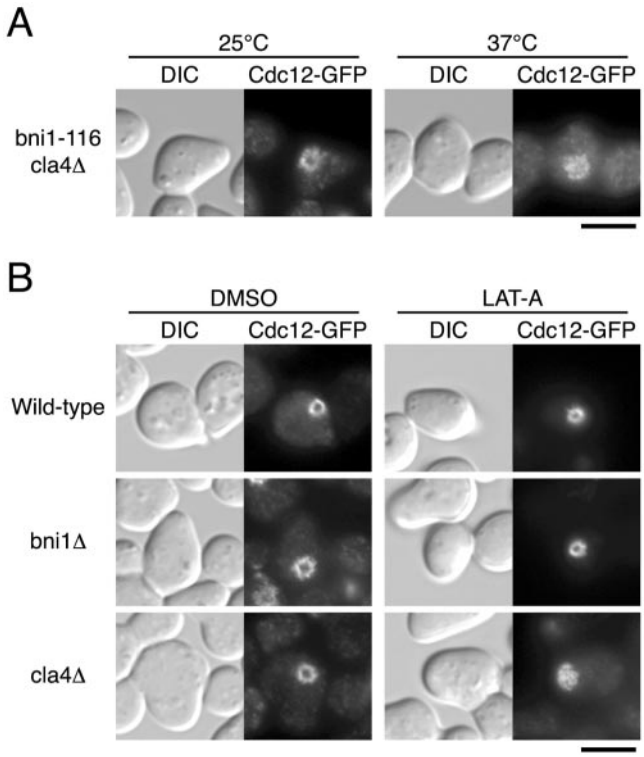



**Figure 6.** Perturbation of actin polymerization causes defective septin ring assembly in the absence of Cla4p. (A) Point mutant in the FH2 domain of *BNI1,* characterized as that defective in actin cable assembly, causes the defects in septin ring assembly in the absence of Cla4p. YKT638 (*bni1-116 cla4*Δ *CDC12-GFP*) was $G_1$-arrested with α-factor at 25°C and released into fresh medium at 25 or 37°C. After a 30-min incubation, cells were fixed and observed by DIC and fluorescence microscopy. (B) LAT-A inhibits septin ring assembly in *cla4*Δ mutant. YKT637 (*CDC12-GFP*), YKT676 (*bni1*Δ *CDC12-GFP*), and YKT717 (*cla4*Δ *CDC12-GFP*) were $G_1$-arrested with α-factor and released into fresh medium at 25°C in the absence (DMSO only) or presence of LAT-A. After 30- (DMSO) and 60 (LAT-A)-min incubations, cells were fixed and observed by DIC or fluorescence microscopy. Bars, 5 μm.

incubation (unpublished results). Previous study reported that actin perturbation by LAT-A triggers a morphogenesis checkpoint and induces a delay of nuclear division (McMillan *et al.,* 1998). To assess some aspect of cell cycle progression in the LAT-A–treated cells, we monitored the mitotic spindle assembly in the wild-type, *bni1*Δ, and *cla4*Δ mutant cells expressing GFP-Tub1p. When grown for 60 min after release from $G_1$ arrest in the presence of LAT-A, the fraction of the cells that assembled a mitotic spindle decreased from 70 to 40% compared with the untreated cells in each strain (unpublished results), suggesting that the LAT-A treatment induces the cell cycle delay. The extent of the cell cycle delay, however, was similar between the wild-type, *bni1*Δ, and *cla4*Δ mutants, suggesting that the *cla4*Δ mutant cannot assemble a septin ring in the presence of LAT-A independent of the cell cycle delay. These results are consistent with our observations that Bni1p-dependent actin polymerization is required for initial septin ring assembly in the absence of Cla4p.

### *Actin Cable-dependent Transport May Be Required for Septin Ring Assembly in the Absence of Cla4p*

We examined actin cable-related factors for their involvement in septin ring assembly. We constructed *cla4*Δ mutants combined with actin cable-related ts mutations such as *bni1-116 bnr1*Δ, *tpm1-2 tpm2*Δ, and *myo2-20* (Pruyne *et al.,* 1998; Schott *et al.,* 1999). Tropomyosins (Tpm1p and Tpm2p) are actin cable-stabilizing proteins. Myo2p, a type V myosin, has a direct role in secretory vesicle targeting using actin cables as tracks for transport. We analyzed septin ring assembly upon exit from $G_1$ arrest at 37°C. When grown for 60 min, >80% of *bni1-116 bnr1*Δ, *tpm1-2 tpm2*Δ, and *myo2-20* mutants assembled a septin ring (Figure 7). In contrast, *bni1-116 bnr1*Δ *cla4*Δ, *tpm1-2 tpm2*Δ *cla4*Δ, and *myo2-20 cla4*Δ mutant cells could not assemble septin rings (Figure 7). Deletion of only *BNR1* from *cla4*Δ mutant affected neither growth nor morphology of the cells (unpublished results). Because loss of Bni1p is known to cause depolarization of cortical actin patches (Kohno *et al.,* 1996; Evangelista *et al.,* 1997), we examined the involvement of an actin patch-related protein in septin ring assembly. We constructed the *cla4*Δ mutants combined with the *arp2-2* and *myo3*Δ *myo5-1* ts mutations (Geli and Riezman, 1996; Madania *et al.,* 1999). Arp2p is a subunit of the Arp2/3 complex, which is the major known contributor to actin nucleation in vivo in yeast (Winter *et al.,* 1999). Myo3/5p, type I myosins, are known as regulators of the Arp2/3 complex (Evangelista *et al.,* 2000; Lechler *et al.,* 2000). Both *arp2-2* and *myo3*Δ *myo5-1* mutants showed normal septin ring assembly, and depletion of Cla4p in these mutants did not affect this assembly (Figure 7). These results suggest that actin cable-dependent transport, but not actin patch function, is required for the assembly of a normal septin ring.

### *Cdc42p and Its GAPs May Be Involved in the Regulation of Septin Ring Assembly by Bni1p and Cla4p*

To identify genes involved in septin ring assembly mediated by Bni1p and Cla4p, we isolated multicopy suppressors of the ts growth phenotype of *bni1-116 cla4*Δ mutant. We isolated truncated fragments of *BEM3* and *RGA1* that encode GTPase-activating protein (GAP) for Cdc42p (Zheng *et al.,* 1993; Stevenson *et al.,* 1995; Johnson, 1999). Both of the isolated fragments, *BEM3*Δ*1-114* and *RGA1*Δ*1-632*, contained a region encoding a GAP domain (Figure 8A). Multicopy *BEM3*Δ*1-114* and *RGA1*Δ*1-632* also suppressed the growth defect of the *bni1*Δ *cla4-75-td* mutant (Figure 8B). The full-length *BEM3* suppressed the growth defect of the *bni1*Δ *cla4-75-td* mutant, but to a lesser extent than that of *BEM3*Δ*1-114*, whereas the full-length *RGA1* did not, suggesting that an N-terminal region of Rga1p and Bem3p may possess a negative regulatory role for GAP activity (unpublished results). We also examined the assembly of the septin ring upon exit from $G_1$ arrest at 35°C in *bni1*Δ *cla4-75-td* mutants carrying multicopy *BEM3*Δ*1-114* or *RGA1*Δ*1-632* (Figure 8C). The cells carrying *BEM3*Δ*1-114* or *RGA1*Δ*1-632* did not assemble septin rings either after 30-min (Figure 8C) or 60-min growth (unpublished results). Surprisingly, after 3 h, they often formed ring-like structures comprised of septins around the bud neck and eventually accomplished cytokinesis (Figure 8C). These results suggest that the defects in septin assembly in *bni1*Δ *cla4-75-td* mutant can be alleviated during polarized growth after budding has occurred.

Multicopy *BEM3*Δ*1-114* and *RGA1*Δ*1-632* also suppressed the growth defects of *bud6*Δ *cla4-75-td*, *spa2*Δ *cla4-75-td*, and *pea2*Δ *cla4-75-td* mutants, but not that of *bni1-116 bnr1*Δ mutant (unpublished results), suggesting that they suppressed the defects caused by the *cla4*Δ mutation. Because the Cdc42p GAPs seem to down-regulate Cdc42p, expression of a dominant active version of Cdc42p may cause lethality in *bni1*Δ mutant. However, expression of

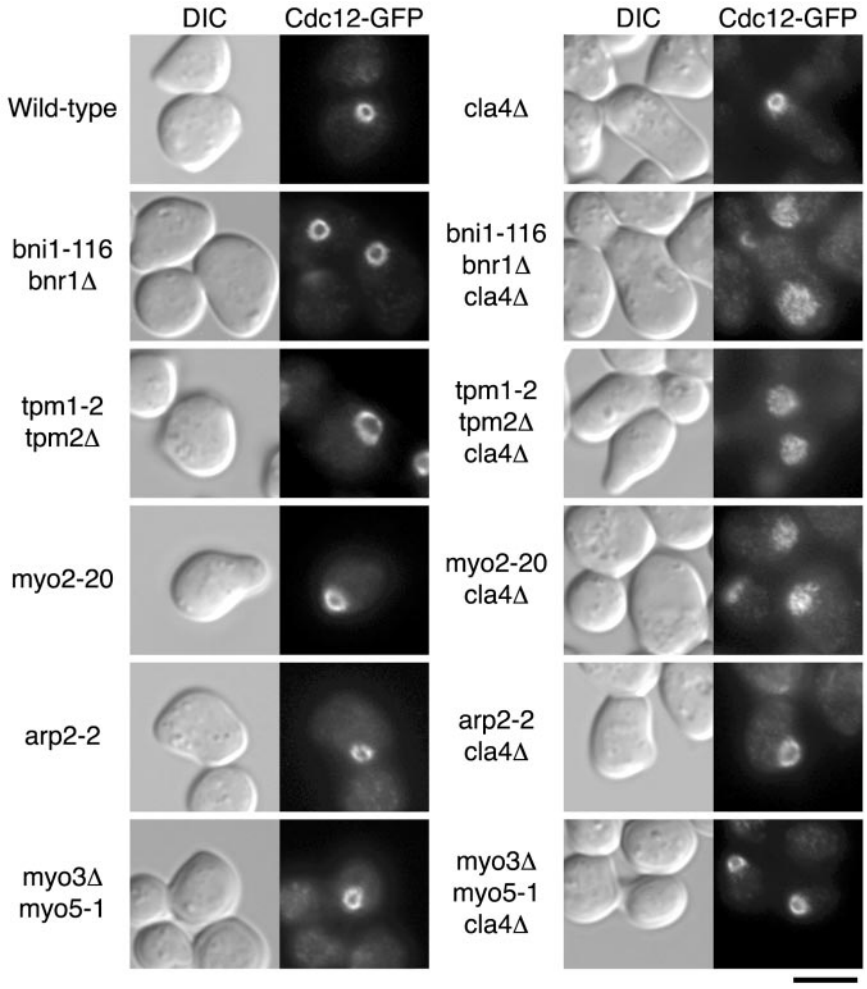



**Figure 7.** Synthetic effects of *cla4*Δ mutation and actin-related mutations on septin ring assembly. YKT637 (*CDC12-GFP*), YKT717 (*cla4*Δ *CDC12-GFP*), YKT743 (*bni1-116 bnr1*Δ *CDC12-GFP*), YKT744 (*bni1-116 bnr1*Δ *cla4*Δ *CDC12-GFP*), YKT812 (*tpm1-2 tpm2*Δ *CDC12-GFP*), YKT813 (*tpm1-2 tpm2*Δ *cla4*Δ *CDC12-GFP*), YKT814 (*myo2-20 CDC12-GFP*), and YKT815 (*myo2-20 cla4*Δ *CDC12-GFP*), YKT816 (*arp2-2 CDC12-GFP*), YKT817 (*arp2-2 cla4*Δ *CDC12-GFP*), YKT818 (*myo3*Δ *myo5-1 CDC12-GFP*), and YKT819 (*myo3*Δ *myo5-1 cla4*Δ *CDC12-GFP*) were $G_1$-arrested with α-factor at 25°C and then released into fresh medium at 37°C. After a 60-min incubation, cells were fixed and observed by DIC and fluorescence microscopy. Bar, 5 μm.

$Cdc42^{G12V}$p caused lethality in wild-type cells (unpublished results). Overexpression of a GFP-tagged dominant active version of Cdc42p, GFP-$Cdc42^{G12V}$p, did not inhibit the growth of wild-type cells (Figure 9A), suggesting that its activity is relatively weaker than that of $Cdc42^{G12V}$p. GFP-$Cdc42^{G12V}$p inhibited the growth of the *bni1*Δ mutant (Figure 9A), but not the wild-type (Figure 9A) or *cla4*Δ mutant (unpublished data). This growth inhibition appears to be due to GTP-bound Cdc42p, because GFP-Cdc42p did not inhibit the growth of the *bni1*Δ mutant. The *bni1*Δ mutant overexpressing GFP-$Cdc42^{G12V}$p also displayed a wide bud neck, although hyperpolarized growth was not remarkable compared with *bni1*Δ *cla4-75-td* mutant (Figure 9B). In these cells, the septins formed a broad ring around the bud neck, leading to defects in cytokinesis. These results suggest that the *cla4*Δ mutation induces effects similar to those caused by accumulation of Cdc42p-GTP and that this results in the synthetic defects in septin ring assembly with the *bni1*Δ mutation.

## DISCUSSION

### *The Role of Polarisome Components and Actin Cytoskeleton in Septin Ring Assembly*

We showed that the polarisome components (Bni1p, Spa2p, Bud6p, and Pea2p) are required for septin ring assembly in the absence of Cla4p. In double mutants carrying mutations in either of polarisome components and *CLA4*, Cdc12p-GFP was recruited to the budding site, but a septin ring was not assembled and Cdc12p-GFP was instead localized to the polarized site. Time-lapse analyses indicated that a polarisome component and Cla4p are not required for the stable maintenance of the septin collar, once a septin collar has been formed at the permissive temperature, but that they are specifically required for the initial assembly step of the septin ring. On the contrary, Goehring *et al.* (2003) reported that the *bni1*Δ *cla4*Δ mutant carrying the YCp-*cla4-75* plasmid, which contains the original *cla4-75* allele, not the *cla4-75-ts* degron allele, formed a normal initial septin ring, with septin abnormalities only appearing later. We also observed that ~30% of the *bni1*Δ *cla4*Δ mutant cells carrying YCp-*cla4-75* initially assembled a septin ring-like structure with deformed morphology, which decayed to a cap as the bud began to grow (unpublished results). The residual activity of Cla4-75p, caused by the lack of rapid degradation and the increased expression from YCp plasmid, may partially promote the initial septin ring assembly in the *bni1*Δ *cla4*Δ mutant.

Deletion analysis of Bni1p revealed the importance of Rho-, Spa2p-, and Bud6p-binding domains for septin ring assembly, a clear contrast to the results that these domains are not required for the suppression of growth defects of *bni1*Δ *bnr1*Δ mutant. These results suggest that the integrity

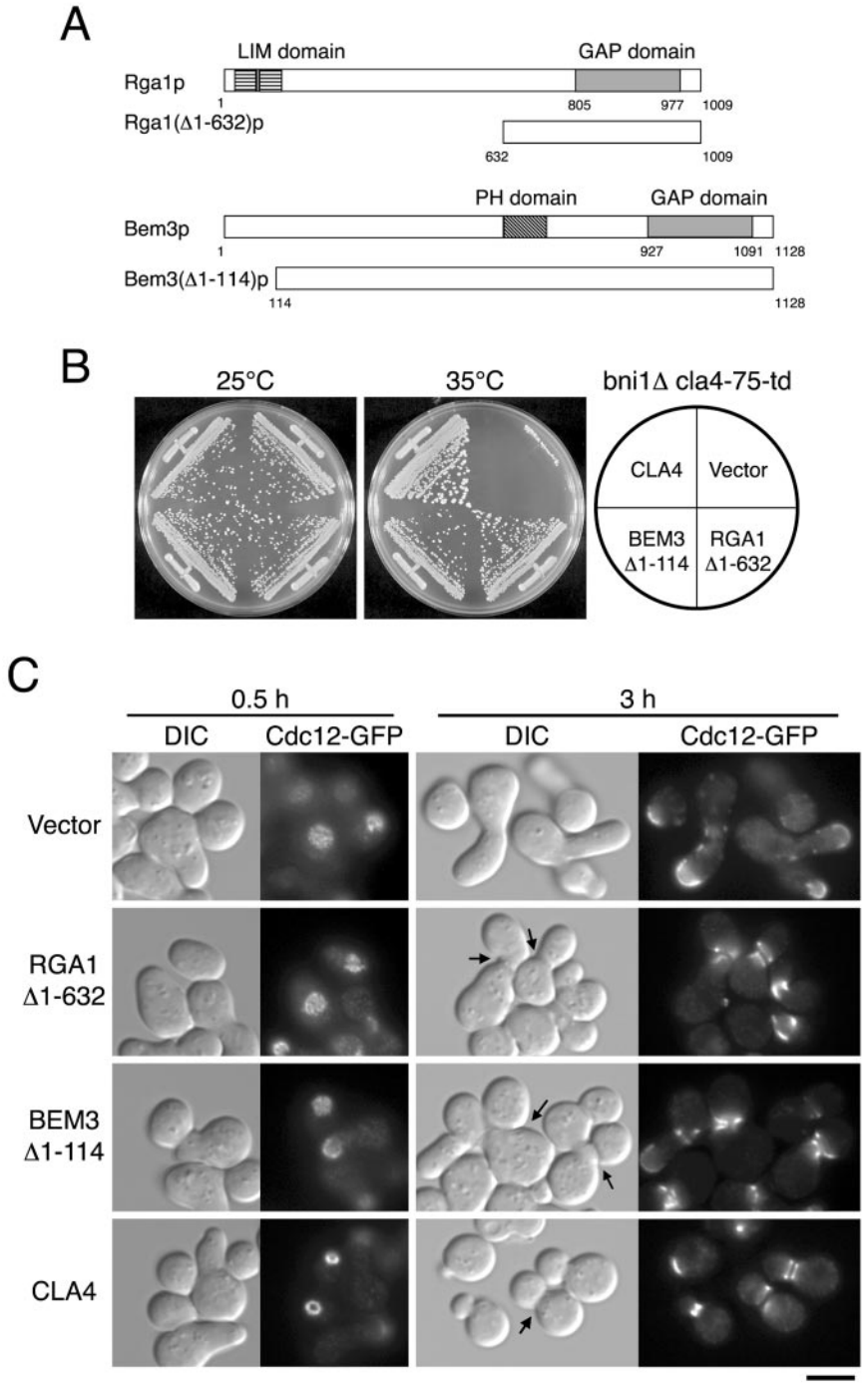



**Figure 8.** Suppression of the growth, morphological and septin organization defects of *bni1*Δ *cla4-75-td* mutants by multicopy *RGA1*Δ*1-632* or *BEM3*Δ*1-114*. (A) Structures of Rga1(Δ1-632)p and Bem3(Δ1-114)p. Each number indicates the amino acid residue. Both of the isolated fragments contained a region encoding a GAP domain. (B) Suppression of the growth defect. YKT811 (*bni1*Δ *cla4-75-td CDC12-GFP*) was transformed with plasmid YEp24 (vector), pKT1364 (YEp24-BEM3Δ1-114), pKT1365 (YEplac195-RGA1Δ1-632), or pKT1254 (pRS316-CLA4). The transformants were streaked on a YPDA plate, which was incubated at 25 or 35°C for 2 d. (C) Suppression of the septin assembly defect. Each transformant was $G_1$-arrested with α-factor at 25°C and released into fresh medium at 35°C. After 30-min and 3-h incubations, cells were fixed and observed by DIC and fluorescence microscopy. Arrows indicate the cells in cytokinesis. Bar, 5 μm.

of the polarisome complex is required for septin ring assembly, in addition to the presence of each component.

We showed that a *bni1*Δ mutant assembles a septin ring with a wider diameter than that of wild-type cells. The polarisome component mutants display defects in apical growth because they fail to confine the polarized growth site to a small region at the bud tip (Sheu *et al.*, 2000). If polarisome mutants also display this defect during bud site assembly and if the area of bud site assembly determines the width of septin ring, depletion of a polarisome component would result in the assembly of a wider septin ring.

Interestingly, LAT-A treatment restored a septin ring with normal diameter and morphology in *bni1*Δ mutant. In *bni1*Δ mutant, Bnr1p assembles actin cables, and thus polarized growth still occurs. The LAT-A treatment completely abolishes polarized growth by inhibiting the actin polymerization. This may allow the *bni1*Δ mutant to have sufficient time to assemble a septin ring with normal morphology. Consistently, *bni1-116 bnr1*Δ mutant displayed a septin ring with relatively normal morphology (Figure 7). However, in the absence of *CLA4*, the *bni1-116 bnr1*Δ mutant did not assemble a septin ring, suggesting that the septin ring in *bni1*Δ mutant is altered not only morphologically, but also functionally. This is consistent with previous observations that *bni1* and *spa2* mutations show synthetic growth defects with *cdc12* (Zahner *et al.*, 1996) and *cdc10* (Flescher *et al.*, 1993) mutations, respectively.

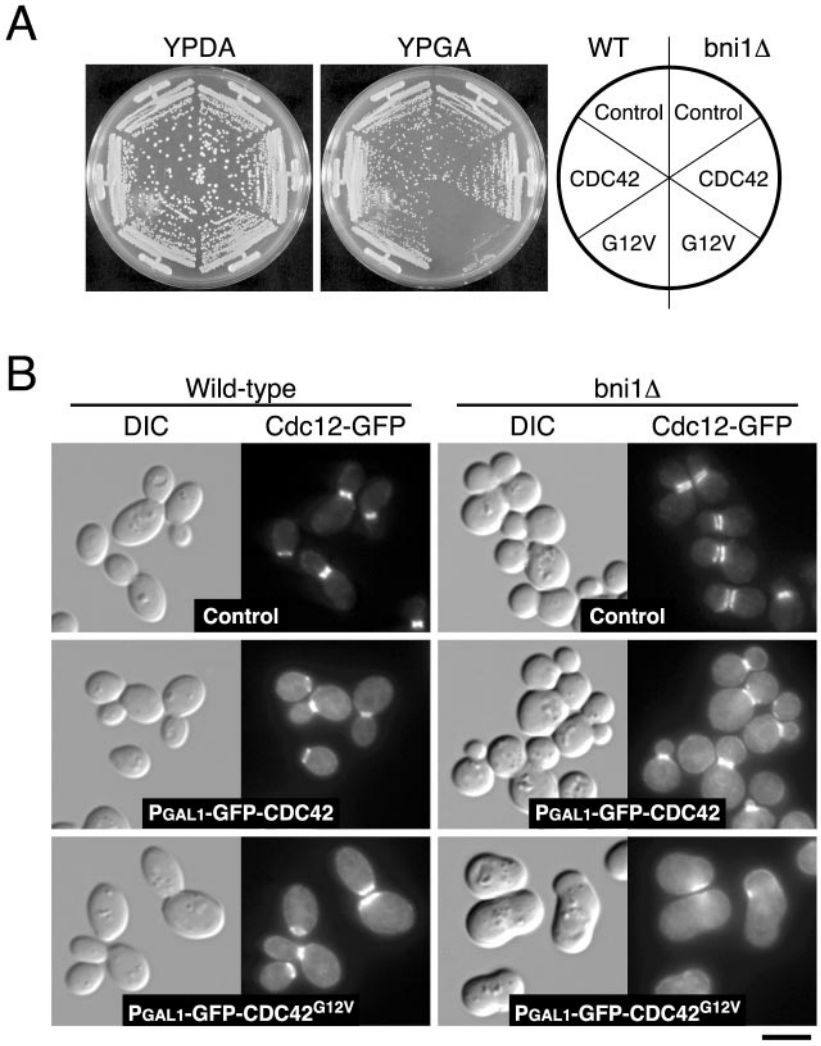



**Figure 9.** Constitutive activation of Cdc42p is sufficient to cause defective septin organization in the absence of Bni1p. (A) YKT637 (*CDC12-GFP*), YKT676 (*bni1*Δ *CDC12-GFP*), YKT891 ($P_{GAL1}$-*GFP-CDC42 CDC12-GFP*), YKT892 ($P_{GAL1}$-*GFP-CDC42*$^{G12V}$ *CDC12-GFP*), YKT893 ($P_{GAL1}$-*GFP-CDC42 bni1*Δ *CDC12-GFP*), and YKT894 ($P_{GAL1}$-*GFP-CDC42*$^{G12V}$ *bni1*Δ *CDC12-GFP*) were streaked on a YPDA or a YPGA plate, which was incubated at 25°C for 3 d. (B) The strains used in A were released from stationary phase into YPGA medium at 25°C. After a 6-h incubation, cells were fixed and observed by DIC and fluorescence microscopy. Cytosolic and plasma membrane staining in GFP-Cdc42p–expressing cells appear to be due to fluorescence of GFP-Cdc42ps. Bar, 5 μm.

A Bni1 mutant protein, which is defective in actin cable formation, also shows a defect in septin ring assembly in the absence of Cla4p, suggesting that the actin cytoskeleton is involved in septin ring assembly. Consistently, LAT-A treatment inhibited septin ring assembly in the *cla4*Δ mutant. Because LAT-A does not inhibit septin ring assembly in wild-type cells, the Cla4p functions in septin assembly appear to be independent of the actin cytoskeleton. Actin cables serve as a track for a type V myosin, Myo2p. Our results that *myo2-20 cla4*Δ, *tpm1-2 tpm2*Δ *cla4*Δ, and *bni1-116 bnr1*Δ *cla4*Δ mutants showed a defect in septin ring assembly suggest that Myo2p may be required for transport of a factor specifically required for septin ring assembly to the bud emergence site. Consistent with this, even in the presence of *CLA4*, the assembly of septin ring was delayed by LAT-A treatment (Figure 6) and in the *bni1-116 bnr1*Δ, *tpm1-2 tpm2*Δ, and *myo2-20* mutants (unpublished results), although there is another possibility that this delay may be attributed to a cell cycle delay triggered by LAT-A treatment or formin deficiency. However, it is more likely that the Bni1p-catalyzed formation of actin filaments is specifically required for septin ring assembly. In the *bni1*Δ *cla4-75-td* mutant, Myo2p is transported to a polarized site and thus polarized growth occurs, because Bnr1p can form actin cables. The Bni1p-specific actin cables may guide polarized growth to a focused region to assemble a septin ring with normal diameter and morphology. Another interesting possibility is that Bni1p provides an actin-based structure, which is different from the actin cables used for polarized transport. Interestingly, in mammalian systems, actin bundles can serve as a template for septin assembly via an actin-binding protein, anillin (Kinoshita *et al.*, 2002). It is an intriguing possibility that the actin cytoskeleton, formed by the action of Bni1p, plays a more direct role in septin assembly in conjunction with polarisome components.

### *Functional Interactions between Polarisome Components, Cla4p and Cdc42p GAPs for Septin Ring Assembly*

Cla4p has also been implicated in the initial assembly of the septin ring (Cvrckova *et al.*, 1995; Weiss *et al.*, 2000). Fluorescence-recovery-after-photobleaching (FRAP) studies have shown that septin ring is labile during budding initiation and mitotic exit and are stable during S, $G_2$, and M phases (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). Cla4p is required for this immobilization of the septin ring (Dobbelaere *et al.*, 2003). The defects in septins in the *cla4*Δ mutant, in conjunction with the spatial defects in polarisome mutants as to where septin rings are assembled, may result in severe defects in the assembly of the septin ring. Deletion of *SWE1*, which encodes a protein kinase thought to be part

of a morphogenesis checkpoint that negatively regulates Clb1, 2p-Cdc28p activity, restores normal bud morphology in *cla4* mutant (Longtine *et al.,* 2000; Weiss *et al.,* 2000; Mitchell and Sprague, 2001). Goehring *et al.* (2003) reported that deletion of *SWE1* restores the localization of septins to the mother-bud neck in unsynchronized cultures of *bni1*Δ *cla4-75* mutant. However, we observed septin ring assembly defects in *bni1*Δ *cla4-75-td swe1*Δ cells upon release from $G_1$ arrest (unpublished results), suggesting that Cla4p possesses specific functions for septin ring assembly.

We showed that overexpression of *BEM3*Δ*1-114* and *RGA1*Δ*1-632*, which encode truncated versions of Cdc42p GAPs, suppressed the growth defects of the *bni1*Δ *cla4-75-td* mutant. It was recently reported that a mutant in Cdc42p GAPs (*bem3*Δ *rga1*Δ *rga2*Δ) showed severe defects in the assembly of the septin ring (Caviston *et al.,* 2003). Therefore, it seems that a similar molecular defect underlies the *bni1*Δ *cla4-75-td* and *bem3*Δ *rga1*Δ *rga2*Δ mutants. Our results suggest that Cdc42p GAPs are involved in the Cla4p-related pathway for septin ring assembly. Because one of the plausible functions of Cdc42p GAPs is to down-regulate Cdc42p-GTP, Cdc42p-GTP may accumulate in the *cla4*Δ mutant. Consistently, we showed that a dominant active version of Cdc42p caused a septin ring assembly defect in *bni1*Δ mutant. Cla4p phosphorylates Cdc24p, resulting in the dissociation of Bem1p from Cdc24p and the subsequent down-regulation of Cdc42p-GTP formation (Gulli *et al.,* 2000), although the other group concluded that the Cla4p-dependent phosphorylation of Cdc24p does not affect the Bem1p-Cdc24p interaction (Bose *et al.,* 2001). Based on the observation that hyper activation of Cdc42p and depletion of Cdc42p GAPs caused defects in septin organization, Gladfelter *et al.* (2002) proposed that Cdc42p cycling between a GTP-bound "ON" state and GDP-bound "OFF" state is required for normal septin ring assembly. Septin ring assembly defects in the *cla4*Δ mutant may be partly due to hyper activation of Cdc42p.

We showed that overexpression of Cdc42p GAPs restored septin ring assembly in the *bni1*Δ *cla4-75-td* mutant. This suppression appeared to occur during polarized growth, rather than at bud emergence. Similar observations were made in the Cdc42p GAP triple mutant and in $cdc42^{V36G}$ mutant (Caviston *et al.,* 2003). They showed that, by a time-lapse analysis, the septin cap could be converted to a normal ring structure next to bud tip at some point during bud growth and that a bud of normal shape began to form distal to the septin ring. Therefore, a septin collar can be constructed, not only at the initiation of budding, but also during polarized growth. Caviston *et al.* (2003) proposed that septin ring formation consists of at least two steps: recruitment of septin proteins and their assembly into the septin ring. In this model, both steps depend on Cdc42p, whereas the Cdc42p GAPs and other "assembly factor," such as Cla4p, Gin4p, and Elm1p, function in the assembly step. How do the truncated Cdc42p GAPs induce the septin collar formation in the *bni1*Δ *cla4-75-td* mutant after budding has occurred? The truncated Cdc42p GAPs may partially suppress the defects in the initial septin assembly, to an extent that the effects on the septins cannot be visualized under the microscope, resulting in the formation of a septin collar later on. It is also possible that, due to deregulation by truncation, they could induce septin assembly even during polarized growth. Caviston *et al.* (2003) proposed that Cdc42p GAPs function as effectors of Cdc42p for septin ring assembly. According to this model, the Cdc42p GAPs-related function of Cla4p may be to interact more directly with septins, rather than to regulate the nucleotide-binding state of Cdc42p. It was recently shown that Cla4p interacts directly with and phosphorylates septins in vitro and in vivo (Versele and Thorner, 2004). Currently, we favor a hypothesis that Cla4p regulates the septin assembly through both direct interactions with septins and regulation of the nucleotide binding state of Cdc42p.

Our observations suggest that polarisome components also function in the assembly step together with Cdc42p GAPs and Cla4p. Further identification of a protein, which directs septin ring assembly through direct interactions with septins under the control of polarisome, Cla4p and Cdc42p GAPs, is an important step toward understanding of the molecular mechanisms of septin ring assembly.

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