

# Interplay between septin organization, cell cycle and cell shape in yeast

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## Summary

Septins are conserved filament-forming proteins that assemble into cortical cytoskeletal structures in animal and fungal cells. Although rapid progress has been made into the functions of septins, the mechanisms governing their localization and organization remain mysterious. In *Saccharomyces cerevisiae*, Cdc42p organizes the septin cytoskeleton into a ring in preparation for bud formation, following which septins remain as a collar at the mother-bud neck. We have dissected the phenotype of *cdc42*<sup>V36T,K94E</sup> cells that display an aberrant cell shape correlated with the development of ectopic septin caps and rings within the bud. The results suggest that a well-assembled septin cortex plays a novel role in directing growth to shape the nascent bud, and that a disorganized septin cortex directs improper growth generating an

aberrant neck. Conversely, we found that the elongated bud shape arising as a result of the morphogenesis checkpoint cell cycle delay that accompanies septin perturbation can feed back to exacerbate minor defects in septin organization, by maintaining a bud-tip-localized septin assembly activity that competes with the neck-localized septin cortex. Using this exacerbation as a tool, we uncovered septin organization defects in many mutants not previously known to display such defects, expanding the cast of characters involved in proper assembly of the septin cortex to include *CLN1*, *CLN2*, *BNI1*, *BNI4*, *BUD3*, *BUD4* and *BUD5*.

Key words: *CDC42*, septin, *SWE1*, secretion

## Introduction

The cortex at the yeast mother-bud neck is organized by a family of evolutionarily conserved filament-forming proteins called septins (Gladfelter et al., 2001b; Longtine et al., 1996). Septins form a barrier at the mother-bud neck that prevents diffusion of integral and peripheral membrane proteins between the mother and the bud (Barral et al., 2000; Takizawa et al., 2000). In addition, septins are required for proper cell wall deposition, cytokinesis and bud site selection, presumably through their ability to recruit chitin synthases, actomyosin ring components, and bud-site-selection landmarks as well as other proteins to the neck (Gladfelter et al., 2001b). Studies with mutants that affect septin behavior have also revealed a strong correlation between defects in septin organization and aberrant cell shape (Gladfelter et al., 2004), suggesting that septins can influence the pattern of cell growth, or vice versa.

Septins were originally identified through temperature-sensitive *cdc* mutants that displayed a lethal defect in cytokinesis, an elongated bud morphology, and a cell cycle delay at restrictive temperature (Hartwell, 1971). The latter two phenotypes were traced to the action of Swe1p, a cell-cycle-regulatory kinase that inhibits mitosis-promoting complexes containing B-type cyclins (Clb) and the cyclin-dependent kinase Cdc28p (Barral et al., 1999; Booher et al., 1993; Longtine et al., 2000). As Clb/Cdc28p complexes promote both actin depolarization [the apical-isotropic switch (Lew and

Reed, 1993)] and nuclear division, delayed Clb/Cdc28p activation can account for both the bud elongation (prolonged apical growth) and the G2 delay in septin-mutant cells. Swe1p degradation requires factors that are localized to the septin cortex (McMillan et al., 1999a), and it has been proposed that Swe1p is part of a morphogenesis checkpoint pathway (Lew and Reed, 1995a) that monitors either septin defects (Barral et al., 1999; Sakchaisri et al., 2004; Shulewitz et al., 1999) or the change in septin organization that accompanies bud emergence (Longtine et al., 2000; Theesfeld et al., 2003).

Cytoplasmic septin complexes assemble into a ring at the presumptive bud site in late G1, and then expand to demarcate an hourglass-shaped cortical zone at the mother-bud neck as the bud forms (Gladfelter et al., 2001b; Longtine and Bi, 2003). At the end of the cell cycle, the septin hourglass splits into two rings that are then inherited by daughter cells, and disassemble during the following G1. It seems likely that cell cycle cues trigger at least some of the observed septin reorganizations during the cell cycle. However, spreading of the ring to an hourglass is correlated with bud emergence, and splitting of the hourglass into two rings is correlated with cytokinesis, raising the possibility that these septin changes arise as a result of changes in cell shape (Gladfelter et al., 2001b).

A number of mutants have been identified in which septins are mislocalized, and assemble as ectopic rings within the bud or as caps at bud tips (Blacketer et al., 1993; Bouquin et al.,

2000; Caviston et al., 2003; Gladfelter et al., 2002; Gladfelter et al., 2004; Thomas et al., 2003). The mutants exhibit Swe1p-dependent bud elongation phenotypes and additional abnormalities in cell shape, including unusually wide necks and bent or kinked buds. In no case do we yet understand the genesis of the mutant phenotype. For instance, do the cell shape abnormalities arise as the result of an underlying septin misorganization? Or do some mutants have a primary defect in morphogenesis, which indirectly impacts the septins? How do mislocalized septins reach their ectopic locations in mutant cells, and what does that tell us about how septins are tightly restricted to the neck in wild-type cells?

In this report, we have addressed these questions in detail for the *cdc42*<sup>V36T,K94E</sup> mutant (Gladfelter et al., 2002), providing a coherent description of the mutant phenotype. Our results indicate that the mutant phenotype stems from several parallel pathways affected by an initial loss of septin integrity, which interact combinatorially to produce the final phenotype. These include effects on localized secretion and cell wall deposition, cell cycle and cell polarity. The analysis revealed two novel functions for the septin hourglass in wild-type cells: directing secretion towards the base of the forming bud, and inhibiting the assembly of septin rings at ectopic locations. In addition, the interactions between septin organization, cell cycle and cell polarity allowed us to devise a simple assay for subtle septin defects, using which we identified novel roles of several genes in septin organization.

## Materials and Methods

### Yeast strains

*S. cerevisiae* strains are listed in Table 1. The generation of the following alleles has been described previously: *cln1::TRP1* and *cln2::LEU2* (Hadwiger et al., 1989), unmarked *cln1*, *cln2* and *cln3* alleles (Cross and Tinkelenberg, 1991), *bni1*, *bud2*, *bud3* and *bud5* alleles (Zahner et al., 1996), *GAL1p-CLB1<sup>Δ152</sup>::LEU2* (Ghiara et al., 1991), *GAL1p-SWE1::LEU2* (Booher et al., 1993), *GAL1p-SWE1-myc::URA3* (Sia et al., 1998), *cdc12-6::LEU2* (Longtine et al., 2000), *cdc42::GAL1p-CDC42::LEU2* (Gladfelter et al., 2001a), *cdc42*<sup>V36T,K94E</sup> and *rga2::URA3* (Gladfelter et al., 2002), *rga1::TRP1* and *bem3::LEU2* (Bi et al., 2000), *cla4::TRP1* (Benton et al., 1997), *gin4-Δ9* (Longtine et al., 1998), *nap1::kan<sup>R</sup>* (Longtine et al., 2000) and *elm1::URA3* (Blacketer et al., 1995). Standard media and methods were used for yeast manipulations (Guthrie and Fink, 1991), and multiple mutant combinations were generated by crosses between mutants in isogenic strain backgrounds. Plasmids pMOSB55 and 57 are CEN URA3 vectors containing *CDC42* and *cdc42*<sup>V36T,K94E</sup>, respectively. Expression is from the *CDC42* promoter (Moskow et al., 2000).

### Growth conditions, galactose induction and microscopy

Fixation, septin staining by immunofluorescence with anti-Cdc11p antibodies, and microscopic analysis was carried out as previously described (Gladfelter et al., 2001a). FITC-Con A staining was performed as described previously (Lew and Reed, 1993). For the experiments summarized in Table 2, cells were grown at 30°C overnight in sucrose-containing medium and *SWE1* expression was induced with the addition of galactose (2% final concentration) for 4 hours before fixation. *cdc12-6* and *cdc12-6 GAL1p-SWE1* cells were grown and induced at 24°C (permissive temperature) because cells failed to proliferate well at 30°C, except where a shift to restrictive temperature is indicated. Panels in several figures bring together individual cells from separate fields.

## Results

A note on terminology: in what follows we make several distinctions regarding defects in septin localization patterns. ‘Failure to assemble’ denotes that no septin structures were detectable by immunofluorescence: septins were distributed diffusely throughout the cell, as occurs in temperature-sensitive *cdc3*, *cdc10*, *cdc11* and *cdc12* mutants at restrictive temperature (Ford and Pringle, 1991; Haarer and Pringle, 1987; Kim et al., 1991). ‘Mislocalization’ indicates that cortical septin structures were detected at sites other than the mother-bud neck (usually as a ring in the bud or a cap at the bud tip). ‘Misorganization’ refers to septin structures at the mother-bud neck that have an aberrant appearance (fuzzy, irregular, extended or asymmetric staining as opposed to the crisp wild-type hourglass).

### Influence of septins on cell morphology

Conditional septin-mutant strains that completely fail to assemble cortical septin-containing structures nevertheless form buds that have a relatively narrow neck diameter (Longtine et al., 1996). In contrast, *cdc42*<sup>V36T,K94E</sup> mutants have very wide necks, as well as misorganized septins at the neck and mislocalized septins at the bud tip or part way into the bud (Gladfelter et al., 2002). In these mutants, Cdc42p interacts poorly with the effector kinases Cla4p and Ste20p as well as other effectors (Gladfelter et al., 2001a), and previous studies showed that *ste20Δ cla4-75* mutants similarly displayed wide necks and mislocalized septins (Cvrckova et al., 1995). Because assembled septins are dispensable for generating narrow necks, it was argued that *ste20Δ cla4-75* mutants had a primary defect in targeting secretion and cell wall deposition to a small patch of cortex, independent of any additional septin defects (Cvrckova et al., 1995). Alternatively, however, it could be that the misorganized septins at the necks of these mutants actively contributed to the mistargeting of cell growth, in which case the wide-neck phenotype should be septin dependent.

To distinguish between these possibilities, we generated a *cdc42*<sup>V36T,K94E</sup> *cdc12-6* double-mutant strain. In the temperature-sensitive *cdc12-6* septin mutant, all septins are dispersed from the neck to the cytoplasm at restrictive temperature (Ford and Pringle, 1991; Haarer and Pringle, 1987; Kim et al., 1991). Nevertheless, mutant cells do focus secretion and cell wall deposition to the bud tip, generating buds with narrow necks. We reasoned that if the *cdc42*<sup>V36T,K94E</sup> mutant had a septin-independent defect in focusing growth, then the *cdc42*<sup>V36T,K94E</sup> *cdc12-6* double-mutant should display wide necks just like the *cdc42*<sup>V36T,K94E</sup> single mutant. However, if improperly assembled septins were responsible for the wide-neck phenotype, then this defect should be rectified in the *cdc42*<sup>V36T,K94E</sup> *cdc12-6* double-mutant at the restrictive temperature.

Not surprisingly, we found that many *cdc42*<sup>V36T,K94E</sup> *cdc12-6* double-mutant cells had a severely aberrant morphology even at the permissive temperature, with some very large, elongated and wide-necked cells (Fig. 1A). However, some normal-shaped cells were present in the population, and following growth to stationary phase it was easy to find unbudded cells of relatively normal size and shape. We inoculated cells from stationary phase populations into fresh medium at 37°C, the restrictive temperature for *cdc12-6*. Following a 3 hour

incubation, we examined the neck morphology of the first buds to emerge. As shown in Fig. 1B, most *cdc42*<sup>V36T,K94E</sup> *cdc12-6* cells (>75%, *n*=400) displayed a narrow neck morphology that was nearly indistinguishable from that of the *cdc12-6* single mutant. In contrast, narrow necks were rare in the

*cdc42*<sup>V36T,K94E</sup> single-mutant cells (<25% *n*=400). Even in the minority of *cdc42*<sup>V36T,K94E</sup> *cdc12-6* cells with wider necks, the neck was still thinner than that observed in most *cdc42*<sup>V36T,K94E</sup> single-mutant cells. Thus, the wide-neck phenotype of the *cdc42*<sup>V36T,K94E</sup> mutant is largely septin-dependent, implying

**Table 1. Yeast strains used in this study**

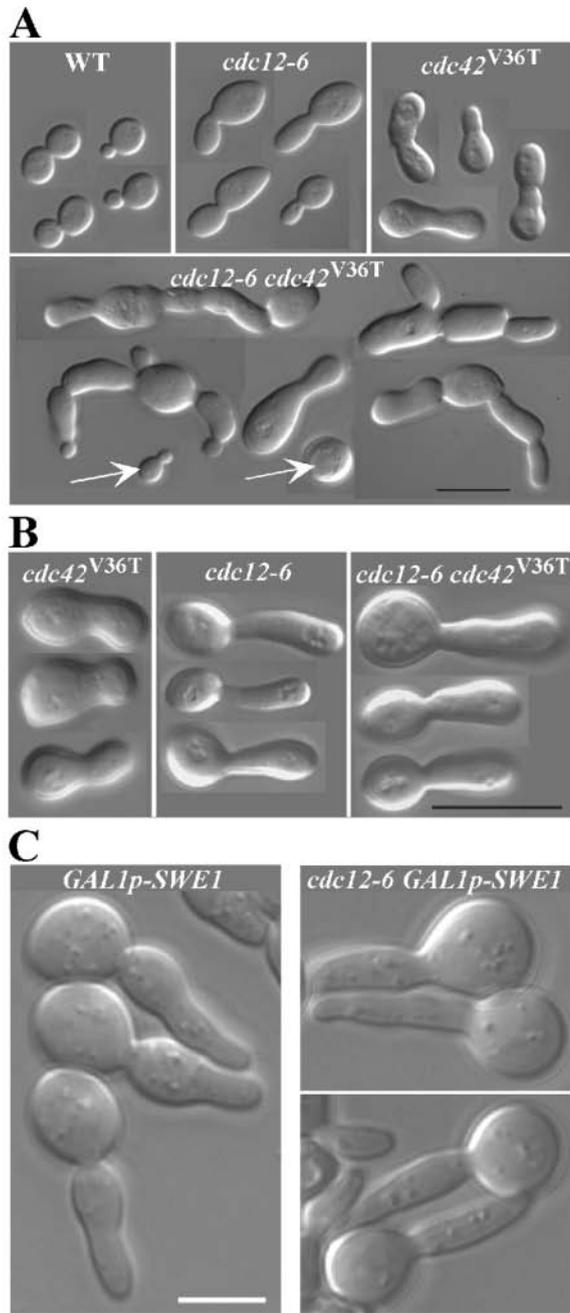
Strain	Relevant genotype*	Source
DLY5	<b>a</b> /α	Lew and Reed, 1993
DLY212	α <i>cln2::LEU2 cln3</i>	C. Wittenberg <sup>§</sup>
DLY213	α <i>cln1::TRP1 cln2::LEU2</i>	C. Wittenberg <sup>§</sup>
DLY1028	<b>a</b> <i>bar1 swe1::LEU2</i>	Sia et al., 1996
DLY2626	<b>a</b> <i>bar1 GAL1p-SWE1::LEU2</i>	McMillan et al., 1999b
DLY3067	<b>a</b> <i>bar1 cdc42::LEU2::GAL1p-CDC42</i>	Moskow et al., 2000
DLY3347	<b>a</b> <i>rgal1::TRP1 rga2::URA3</i>	Gladfelter et al., 2002
DLY3459 <sup>†</sup>	<b>a</b> <i>bnr1::HIS3</i>	E. Bi <sup>¶</sup>
DLY3463 <sup>†</sup>	<b>a</b> <i>bni1::HIS3</i>	E. Bi <sup>¶</sup>
DLY3485 <sup>†</sup>	<b>a</b> <i>bni1::HIS3 GAL1p-SWE1::LEU2</i>	This study
DLY4037	<b>a</b> <i>cdc42</i> <sup>V36T,K94E</sup>	This study
DLY4035 <sup>†</sup>	<b>a</b> <i>GAL1p-SWE1-myc::URA3</i>	This study
DLY4039	α <i>cdc42</i> <sup>V36T,K94E</sup>	This study
DLY4136	<b>a</b> <i>swe1::LEU2 cdc42</i> <sup>V36T,K94E</sup>	This study
DLY4141	α <i>GAL1p-SWE1::LEU2 cdc42</i> <sup>V36T,K94E</sup>	This study
DLY4410	α <i>gin4::kan<sup>R</sup></i>	Gladfelter et al., 2004
DLY4412	<b>a</b> <i>cdc42::LEU2::GAL1p-CDC42 cdc12-6::LEU2</i>	This study
DLY4686	<b>a</b> <i>gin4::kan<sup>R</sup> cla4::TRP1</i>	Gladfelter et al., 2004
DLY4687	<b>a</b> <i>elm1::URA3</i>	Gladfelter et al., 2004
DLY4790	<b>a</b> <i>bar1 nap1::kan<sup>R</sup></i>	Gladfelter et al., 2004
DLY4838	<b>a</b> <i>GAL1p-SWE1-myc::URA3 cdc12-6::LEU2</i>	This study
DLY4848	<b>a</b> <i>gin4::kan<sup>R</sup> nap1::kan<sup>R</sup></i>	Gladfelter et al., 2004
DLY4854	α <i>nap1::kan<sup>R</sup> cla4::TRP1</i>	Gladfelter et al., 2004
DLY5071 <sup>†</sup>	α <i>bud2::HIS3</i>	J. Pringle**
DLY5073 <sup>†</sup>	α <i>bud5::HIS3</i>	J. Pringle**
DLY5080	<b>a</b> /α <i>cdc42</i> <sup>V36T,K94E</sup> / <i>cdc42</i> <sup>V36T,K94E</sup>	Gladfelter et al., 2002
DLY5102	<b>a</b> <i>bar1 cln1 cln2</i>	F. Cross <sup>††</sup>
DLY5103	<b>a</b> <i>bar1</i>	F. Cross <sup>††</sup>
DLY5104	<b>a</b> <i>bar1 cln1 cln2 swe1::GAL1p-SWE1::URA3</i>	F. Cross <sup>††</sup>
DLY5106	<b>a</b> <i>bar1 swe1::GAL1p-SWE1::URA3</i>	F. Cross <sup>††</sup>
DLY5419	α <i>GAL1p-SWE1::LEU2 gin4::kan<sup>R</sup></i>	This study
DLY5420	<b>a</b> <i>GAL1p-SWE1::LEU2 elm1::URA3</i>	This study
DLY5421	<b>a</b> <i>GAL1p-SWE1::LEU2 cla4::TRP1</i>	This study
DLY5422	<b>a</b> <i>GAL1p-SWE1-myc::URA3 nap1::kan<sup>R</sup></i>	This study
DLY5816 <sup>†</sup>	α <i>bni4::TRP1</i>	J. Pringle**
DLY5935	<b>a</b> <i>GAL1p-SWE1::LEU2 gin4::kan<sup>R</sup> cla4::TRP1</i>	This study
DLY5936	<b>a</b> <i>GAL1p-SWE1::LEU2 gin4::kan<sup>R</sup> nap1::kan<sup>R</sup></i>	This study
DLY5937	α <i>GAL1p-SWE1::LEU2 nap1::kan<sup>R</sup> cla4::TRP1</i>	This study
DLY5939	<b>a</b> <i>GAL1p-SWE1::LEU2 rgal1::TRP1 rga2::URA3</i>	This study
DLY6117 <sup>†</sup>	<b>a</b> <i>bud3::HIS3</i>	J. Pringle**
DLY6118 <sup>†</sup>	<b>a</b> <i>bud4::HIS3</i>	J. Pringle**
DLY6133	α <i>GAL1p-SWE1-12myc::URA3 cln1::TRP1 cln2::LEU2</i>	This study
DLY6135	α <i>GAL1p-SWE1-12myc::URA3 cln2::LEU2 cln3</i>	This study
DLY6136 <sup>†</sup>	<b>a</b> <i>GAL1p-SWE1::LEU2 bnr1::HIS3</i>	This study
DLY6137 <sup>†</sup>	α <i>GAL1p-SWE1::LEU2 bud2::HIS3</i>	This study
DLY6138 <sup>†</sup>	α <i>GAL1p-SWE1::LEU2 bud5::HIS3</i>	This study
DLY6139 <sup>†</sup>	α <i>GAL1p-SWE1::LEU2 bni4::TRP1</i>	This study
DLY6141 <sup>†</sup>	<b>a</b> <i>GAL1p-SWE1::LEU2 bud3::HIS3</i>	This study
DLY6142 <sup>†</sup>	<b>a</b> <i>GAL1p-SWE1::LEU2 bud4::HIS3</i>	This study
DLY7326	<b>a</b> /α <i>GAL1p-CLB1<sup>Δ152</sup>::LEU2/leu2</i>	This study
DLY7327	<b>a</b> /α <i>cdc42</i> <sup>V36T,K94E</sup> / <i>cdc42</i> <sup>V36T,K94E</sup> <i>GAL1p-CLB1<sup>Δ152</sup>::LEU2/leu2</i>	This study
DLY7452 <sup>†</sup>	<b>a</b> <i>cdc12-6 GAL1p-SWE1::LEU2</i>	This study
JMY1141 <sup>‡</sup>	<b>a</b> <i>cdc12-6</i>	This study
JMY1488	<b>a</b> <i>cdc12-6::LEU2</i>	This study
MOSY148	<b>a</b> <i>cla4::TRP1</i>	Marquitz et al., 2002
RSY136	<b>a</b> <i>bar1 GAL1p-SWE1-myc::URA3</i>	Sia et al., 1998

\*All strains in the BF264-15Du strain background (*ade1 his2 leu2-3,112 trp1-1<sup>a</sup> ura3Δns*) Richardson et al., 1989, except where indicated.

<sup>†</sup>YEF473 strain background (*his3 leu2 trp1 ura3 lys2*) Bi and Pringle, 1996.

<sup>‡</sup>Spore colony from dissection of *cdc12-6/cdc12-6* diploid in the prototrophic C276 strain background, which was a gift from John Pringle (University of North Carolina, Chapel Hill).

<sup>§</sup>C. Wittenberg, Scripps Research Institute, La Jolla, CA; <sup>¶</sup>E. Bi, University of Pennsylvania, Philadelphia, PA; <sup>\*\*</sup>J. Pringle, University of North Carolina, Chapel Hill; <sup>††</sup>F. Cross, Rockefeller University, New York, NY.



**Fig. 1.** Septins contribute to shaping the wide necks in *cdc42<sup>V36T,K94E</sup>* mutants and the base of the bud in wild-type cells. (A) Strains DLY3067 (*GAL1p-CDC42*) and DLY4412 (*cdc12-6 GAL1p-CDC42*) were transformed with pMOSB55 (*CDC42*) or pMOSB57 (*cdc42<sup>V36T,K94E</sup>*). Cells were photographed after growth to exponential phase at 24°C, the permissive temperature for *cdc12-6* mutants, on dextrose-containing medium. (B) The same strains were grown to stationary phase, stained with FITC-ConA to visualize cell wall mannan, washed, and inoculated into fresh medium at 37°C, the restrictive temperature for *cdc12-6* mutants. Morphology of new buds (unstained by FITC-ConA) was documented 3 hours later. Bar, 10  $\mu$ m. (C) Strains DLY4035 (*GAL1p-SWE1*) and DLY7452 (*cdc12-6 GAL1p-SWE1*) were grown to stationary phase in raffinose-containing medium at 24°C, and inoculated into galactose-containing medium (to induce Swe1p expression) at 37°C (to inactivate septins). Cell morphology was documented 5 hours later. Bar, 5  $\mu$ m.

that misorganized septins can direct aberrant secretion and cell wall deposition.

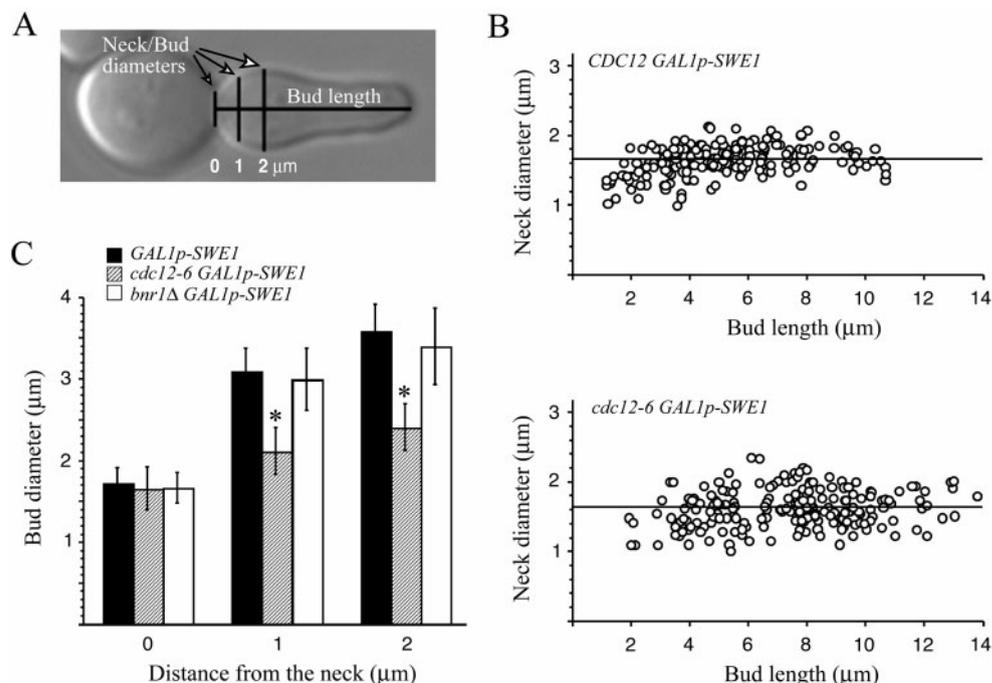
If misorganized septins direct improper growth in mutant cells, do well-organized septins also target growth in wild-type cells? The most striking perturbation of bud shape in septin-mutant strains is the bud elongation cycle delay that occurs as a result of the Swe1p-mediated cell cycle delay. However, septin-mutant buds appear distinct from wild-type buds even when they are very small (as first pointed out to us by John Pringle, UNC-Chapel Hill), raising the possibility that additional aspects of bud growth are perturbed. To determine whether this was the case, we compared the buds made by *cdc12-6* cells lacking any assembled septins to those made by cells that were arrested in G2 by overexpression of Swe1p (Fig. 1C). In both cases, cells made elongated buds that grew predominantly at the bud tip. However, the Swe1p-overexpressors with intact septins had clear bulges on the bud side of the neck, whereas the cells without localized septins maintained a more narrow bud diameter extending from the base of the bud (Fig. 1C). To perform a more quantitative comparison, the bud length and neck and bud diameters of 200 *cdc12-6 GAL1p-SWE1* and control *CDC12 GAL1p-SWE1* cells were measured using DIC microscopy (Fig. 2A,B). Several conclusions emerge from this analysis. First, neck diameter does not change as a function of bud length (2–14  $\mu$ m) in either strain (Fig. 2B), indicating that once the neck is formed it is not remodeled upon further bud growth (at least under conditions of Swe1p-mediated cell cycle arrest). Second, neck diameter was very similar in wild-type and septin mutant cells (Fig. 2B,C), indicating that septins are not required to ‘constrict’ the neck or focus the initial outgrowth of the bud. Third, bud diameter 1  $\mu$ m or 2  $\mu$ m distal to the neck was clearly greater in wild-type than in septin mutant cells (Fig. 2C). This difference was highly significant statistically ( $P < 0.001$ ), indicating that assembled septins are required for the normal bulging of the base of the bud. Thus, septins direct the deposition of new cell wall to the bud side of the neck, expanding the base of the bud to generate normal hourglass shape of the neck in wild-type cells.

Septins recruit the formin protein Bnr1p to the mother side of the neck, where Bnr1p nucleates actin cables that extend into the mother (Kamei et al., 1998; Pruyne et al., 2004). Thus, the role of septins in shaping the base of the bud could reflect their role in organizing actin cables that deliver new vesicles from the mother cell to the neck. To test whether Bnr1p was required for the bulging of the base of the bud, we repeated the bud morphometry using *bnr1 $\Delta$  GAL1p-SWE1* mutants. Unlike septin mutants, in *bnr1 $\Delta$*  mutants the bud diameter was normal 1  $\mu$ m and 2  $\mu$ m from the neck (Fig. 2C). As *bnr1 $\Delta$*  mutants lack neck-nucleated cables (Pruyne et al., 2004), we conclude that the role of septins in shaping the base of the bud is not mediated by their effects on actin cables, but more probably reflects a role of septins in targeting secretion (see Discussion).

#### Influence of cell morphology and cell cycle on septin organization

The results described above indicate that septins directly influence cell shape. Several observations suggest that the converse might also be true, and that Swe1p-dependent bud

**Fig. 2.** Septins, but not Bnr1p, contribute to shaping the base of the bud. Strains DLY4035 (*GAL1p-SWE1*), DLY7452 (*cdc12-6 GAL1p-SWE1*) and DLY6136 (*bnr1Δ GAL1p-SWE1*) were grown to stationary phase in raffinose-containing medium at 24°C, stained with FITC-ConA, washed, and inoculated into galactose-containing medium (to induce Swe1p expression) at 37°C (to inactivate septins). Bud morphology was documented 5 hours later by DIC microscopy, and scored for new buds (unlabeled with FITC-ConA). (A) Image showing the measurements of bud length, neck diameter and bud diameter at 1 μm and 2 μm from the neck. (B) Plots of neck diameter versus bud length for wild-type (DLY4035; *CDC12*) and septin mutant (DLY7452, *cdc12-6*) buds. Each circle represents one cell ( $n=200$ ). (C) Quantitation of neck diameter and bud diameter at 1 μm and 2 μm from the neck. Values are mean ± standard deviation ( $n=130$  for the wild-type,  $n=184$  for the *cdc12-6* and  $n=125$  for the *bnr1Δ* strains) Asterisk indicates that the bud diameter difference between *cdc12-6* and wild-type cells was statistically significant ( $P<0.001$ ).



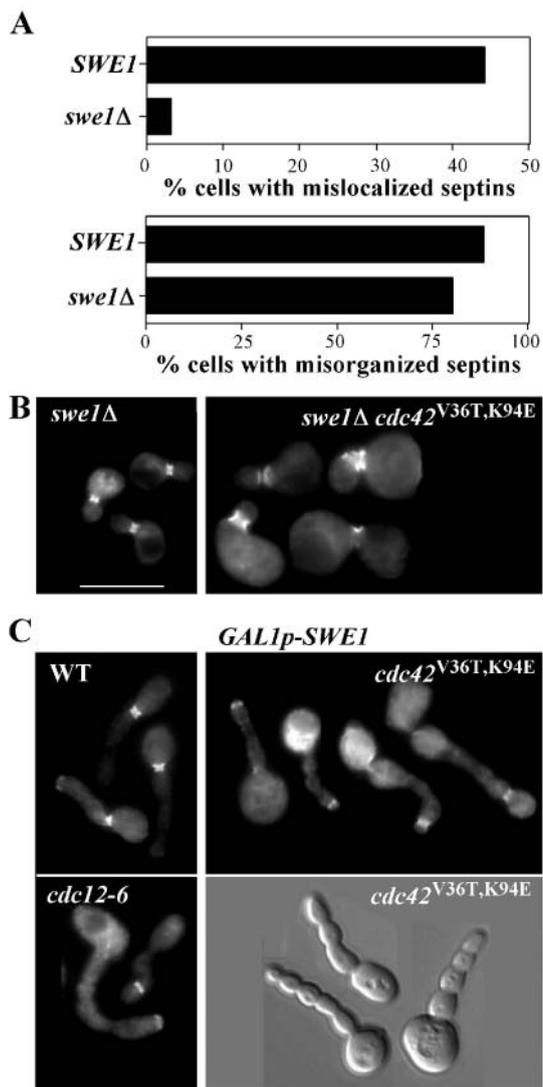
elongation might contribute to septin mislocalization. In particular, mislocalization of septins towards the bud tip is frequently associated with elongated buds (Bouquin et al., 2000; Gladfelter et al., 2004; Goehring et al., 2003; Versele and Thorner, 2004; Weiss et al., 2000), and in the case of *ste20Δ cla4-as3* mutants, deletion of *SWE1* largely reversed this septin mislocalization (Weiss et al., 2000). Similarly, we found that the *cdc42<sup>V36T,K94E</sup> swe1Δ* double mutant exhibited many fewer cells with mislocalized septins part-way into the bud or at the bud tip, compared to the *cdc42<sup>V36T,K94E</sup>* single mutant (Fig. 3A). Although septins were localized to the neck in the *cdc42<sup>V36T,K94E</sup> swe1Δ* strain, septin staining was often still misorganized, so that staining was faint, smeared or extended (Fig. 3A,B). Thus, Swe1p does not account for all of the septin defects in *cdc42<sup>V36T,K94E</sup>* cells, but it does increase the proportion of cells with septins mislocalized towards the bud tip.

Cell cycle arrest due to Swe1p overexpression does not, in and of itself, cause significant septin mislocalization (Gladfelter et al., 2002) (Fig. 3C). However, overexpression of Swe1p in *cdc42<sup>V36T,K94E</sup>* cells increased the frequency of mislocalized septin rings to 92%, and concomitantly reduced the proportion of cells that retained detectable septin staining at the neck (Fig. 3C, Table 2). Although *cdc42<sup>V36T,K94E</sup>* cells with more than one septin ring were quite rare, such cells became common (28%) upon Swe1p overexpression (Fig. 3C, Table 2). Interestingly, the elongated buds of these cells contained variable numbers of bulges along their length (Fig. 3C), in contrast to G2-arrested wild-type cells, which formed smooth buds (Fig. 1C). In such cells, septin staining frequently coincided with a narrowing (or 'neck') between the most distal two bulges. Given the data presented in Fig. 2 indicating that septin rings promote

bulging on the bud side of the neck, it seems probable that the bulges in these buds were caused by septin rings that localized (transiently) to the 'necks' between the bulges (see Discussion).

#### Basis for Swe1p-induced septin mislocalization

The results described above indicate that Swe1p can strongly influence septin localization. Swe1p phosphorylates and inhibits Cdc28p, causing cell cycle arrest and continued polarization of growth (Booher et al., 1993). Overexpression of the stoichiometric Clb/Cdc28p inhibitor Sic1p induced similar septin mislocalization to that caused by overexpression of Swe1p (data not shown), consistent with the hypothesis that inhibition of Clb/Cdc28p is responsible for the effect of Swe1p on septin localization. Clb/Cdc28p inhibition arrests the cell cycle, allowing more time for septin defects to develop. If simply providing more time were sufficient to explain the effect of Swe1p, then arresting the cell cycle at any stage (after bud formation) might have a similar effect. To test whether that was the case, we arrested *cdc42<sup>V36T,K94E</sup>* cells with high Clb/Cdc28p activity by overexpressing Clb1p<sup>Δ152</sup>, a form of Clb1p lacking the destruction box (Ghiara et al., 1991). Even after 4 hours of arrest, these cells maintained robust (though misorganized) septin staining at the neck (Fig. 4A); mislocalized septins were found in only 12% of arrested cells, as compared with 92% of cells arrested by Swe1p. Similar results were obtained upon arrest with the microtubule-depolymerizing drug nocodazole (Fig. 4B). Thus, arrest with low Clb/Cdc28p activity (Swe1p or Sic1p overexpression) promotes septin mislocalization but arrest with high Clb/Cdc28p activity (Clb1p<sup>Δ152</sup> overexpression or nocodazole) does not, suggesting that septin mislocalization requires



**Fig. 3.** Swe1p-dependent G2 delay promotes assembly of ectopic septin rings. (A,B) Strains DLY4037 (*cdc42*<sup>V36T,K94E</sup>), DLY1028 (*swe1Δ*) and DLY4136 (*cdc42*<sup>V36T,K94E</sup> *swe1Δ*) were grown to exponential phase in YEPD at 30°C and processed to visualize septin distribution. The proportion of budded *cdc42* or *cdc42 swe1* cells displaying ectopic septins in the bud (top graph: mislocalized septins) or defective (faint, patchy, or absent) septins at the neck (bottom graph: misorganized septins) were scored. Representative cells (B) show that septins at the neck are still defective in *cdc42*<sup>V36T,K94E</sup> cells that lack Swe1p. (C) Strains RSY136 (*GAL1p-SWE1*), DLY4141 (*cdc42*<sup>V36T,K94E</sup> *GAL1p-SWE1*), and DLY4838 (*cdc12-6 GAL1p-SWE1*) were grown to exponential phase in sucrose-containing medium at 24°C, induced to overexpress Swe1p by addition of galactose (2% final concentration), and processed to visualize septin distribution or cell morphology after a further 4 hours at 24°C. Bar, 10 μm.

inhibition of Clb/Cdc28p. The most obvious difference between cells arrested with low versus high Clb/Cdc28p is that Cdc28p inhibition prevents the apical-isotropic switch, thereby retaining Cdc42p at the bud tip and promoting continued polarized growth.

Septins at the bud tip could reach that location in two very

different ways. First, septin rings initially assembled at the neck might move or drift as a unit towards the bud tip. This scenario is attractive in that septin rings located part-way into the bud are easily interpreted as septins ‘in transit’. Alternatively, septins initially assembled at the neck might gradually disassemble into their constituent filaments or subunits, and these free subunits might then be reassembled into new rings at the bud tip. This scenario, in turn, is attractive in that cells with septins located both at the neck and at the bud tip are easily interpreted as intermediate stages in which the neck ring has not yet fully disassembled.

To determine whether septin subunits released from the neck would assemble at the bud tip, we made use of temperature-sensitive *cdc12-6* mutants. Cells were allowed to form buds and assemble septins at the neck at the permissive temperature, and then shifted to the restrictive temperature to disassemble the septin rings. After 30 minutes at 37°C no detectable septin structures remained (Fig. 5A). The cells were then shifted back down to the permissive temperature and monitored to determine where septins reassembled in budded cells. Septin staining became detectable 10 minutes after shift down, and septins reassembled at the bud tip in many, though not all (see below), of the budded cells. In no case did septins reassemble at the neck. This result suggests that in the absence of assembled septins, no ‘septin anchor’ remains at the neck. Rather, factors located at the bud tip are capable of directing new septin assembly in G2.

At later times following shift to 24°C, the proportion of cells with septin staining at the bud tip decreased, concomitant with an increase in the number of cells containing septin rings partway into the bud (Fig. 5A,B). These buds were somewhat elongated, presumably as a result of the Swe1p-dependent G2 delay caused by the septin perturbation. Thus, it appears that the septins initially reassemble in a ring at the bud tip, which then remains stationary as the bud continues to grow, in effect creating a new neck.

In addition to the cells described above, by 30 minutes following shift-down many unbudded cells had formed septin rings, and by 60 minutes these cells had formed buds and showed bud-neck septin staining as expected (Fig. 5C). However, about 25% of cells had not reassembled septins even after 60 minutes of shift-down. In some instances, it appeared that large-budded cells delayed septin assembly until the next cell cycle. We speculated that these might represent cells that had already activated Clb/Cdc28p complexes prior to the temperature shift. To test whether Clb/Cdc28p activation would prevent septin reassembly, we first arrested *cdc12-6* cells in mitosis with high Clb/Cdc28p activity using nocodazole, and then repeated the temperature shift-up, shift-down protocol while maintaining the mitotic arrest. In this case, none of the cells reassembled septins even 90 minutes after shift down (Fig. 5D). Thus, the capacity to reassemble septins upon shift-down is eliminated once Clb/Cdc28p is activated, presumably because the cells are no longer polarized. In aggregate, these findings indicate that factors at the bud tip are able to direct the formation of a new septin ring at the tip, but that this ability is lost following Clb/Cdc28p activation. In wild-type cells, the properly assembled septin hourglass must somehow prevent the utilization of the bud-tip-localized septin-assembly pathway (see Discussion).

**Table 2. Septin localization in cells arrested in G2 by Swe1p overexpression**

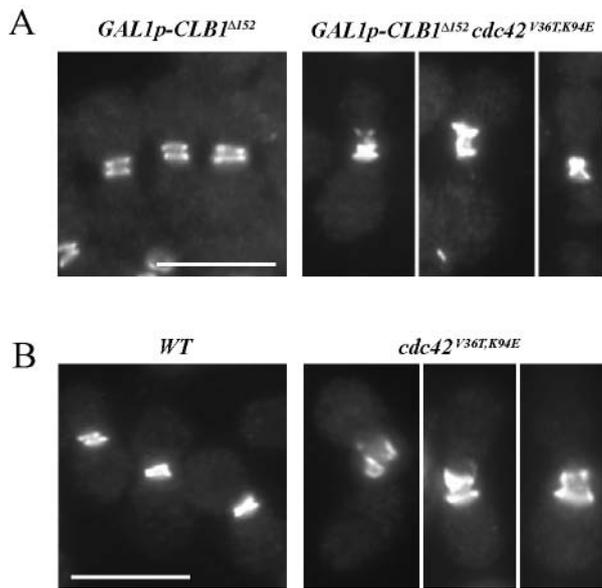
Strain	% of cells with septins at:						
	Neck	Tip	Bud	Neck+Bud	Neck+Tip	Bud+Tip	None
<i>GAL1p-SWE1</i>	93	0	0	<1	6	0	<1
<i>cdc42<sup>V36T,K94E</sup></i>	69	13	10	4	0	0	4
<i>GAL1p-SWE1 cdc42<sup>V36T,K94E</sup></i>	4	32	32	7	12	9	4
<i>cdc12-6</i>	35	12	19	3	3	4	24
<i>GAL1p-SWE1 cdc12-6</i>	11	43	15	7	11	0	13
<i>cla4Δ</i>	90	0	2	4	0	0	4
<i>GAL1p-SWE1 cla4Δ</i>	87	0	<1	3	9	0	<1
<i>elm1Δ</i>	64	20	2	0	3	1	10
<i>GAL1p-SWE1 elm1Δ</i>	23	7	0	15	50	4	1
<i>gin4Δ</i>	95	0	2	0	0	0	3
<i>GAL1p-SWE1 gin4Δ</i>	85	3	1	6	4	0	<1
<i>nap1Δ</i>	94	0	1	3	0	0	2
<i>GAL1p-SWE1 nap1Δ</i>	89	<1	0	9	2	0	0
<i>rga1Δ rga2Δ</i>	93	3	2	1	0	0	1
<i>GAL1p-SWE1 rga1Δ rga2Δ</i>	73	1	4	0	0	6	16
<i>gin4Δ cla4Δ</i>	67	8	7	2	4	1	11
<i>GAL1p-SWE1 gin4Δ cla4Δ</i>	26	15	10	21	14	8	6
<i>gin4Δ nap1Δ</i>	92	0	5	0	0	0	3
<i>GAL1p-SWE1 gin4Δ nap1Δ</i>	73	2	4	8	7	2	4
<i>nap1Δ cla4Δ</i>	92	0	0	6	0	0	2
<i>GAL1p-SWE1 nap1Δ cla4Δ</i>	79	0	1	7	6	1	6
<i>bni1Δ</i>	91	1	0	2	5	0	1
<i>GAL1p-SWE1 bni1Δ</i>	62	5	8	18	4	0	3
<i>bnr1Δ</i>	92	0	2	3	1	0	1
<i>GAL1p-SWE1 bnr1Δ</i>	84	3	1	3	8	<1	<1
<i>bud2Δ</i>	95	0	0	2	2	0	1
<i>GAL1p-SWE1 bud2Δ</i>	90	1	0	4	4	0	1
<i>bud5Δ</i>	96	0	0	3	0	0	1
<i>GAL1p-SWE1 bud5Δ</i>	74	2	1	12	5	3	3
<i>bni4Δ</i>	90	0	0	4	5	1	0
<i>GAL1p-SWE1 bni4Δ</i>	72	1	2	17	3	5	0
<i>bud3Δ</i>	96	0	0	0	3	0	1
<i>GAL1p-SWE1 bud3Δ</i>	73	1	1	16	6	2	1
<i>bud4Δ</i>	94	0	0	1	4	0	1
<i>GAL1p-SWE1 bud4Δ</i>	80	0	0	18	2	0	0
<i>cln2Δ cln3Δ</i>	86	0	0	1	10	0	3
<i>GAL1p-SWE1 cln2Δ cln3Δ</i>	70	4	2	5	14	0	5
<i>cln1Δ cln2Δ</i>	64	10	1	2	17	2	4
<i>GAL1p-SWE1 cln1Δ cln2Δ</i>	30	22	15	3	15	5	10

More than 200 cells scored for each strain.

### Effect of Swe1p-mediated G2 arrest on other septin organization mutants

Based on these findings, the dramatic effects of Swe1p on septin mislocalization in *cdc42<sup>V36T,K94E</sup>* cells can be understood as a consequence of two collaborating effects. First, Swe1p delays cell cycle progression, providing time for septins to be released from the poorly organized hourglass at the neck. Second, Swe1p maintains the ability of bud-tip-localized septin-assembly factors to recruit the liberated septins to form an ectopic ring. If this analysis is correct, then Swe1p might promote septin mislocalization in *any* mutant possessing a defective septin hourglass structure. To test this hypothesis, we examined the effects of Swe1p overexpression in a panel of strains with known defects in septin organization (Table 2). Even at the permissive temperature, septin mislocalization in *cdc12-6* cells was dramatically exacerbated upon overexpression of Swe1p (Fig. 3C, Table 2). Similar effects were observed in *elm1Δ* mutants (Blacketer et al., 1993; Bouquin et al., 2000), and lesser but still significant degrees of septin mislocalization were induced in *gin4Δ* mutants

(Longtine et al., 1998) upon Swe1p overexpression (Table 2). Although *cla4Δ* and *nap1Δ* single mutants (Longtine et al., 2000) exhibited a very low level of septin mislocalization, Swe1p overexpression did induce significant septin mislocalization in *cla4Δ nap1Δ* double mutants (Table 2). More severe effects were observed in *gin4Δ nap1Δ* and *gin4Δ cla4Δ* mutants, and similar phenotypes were induced in *rga1Δ rga2Δ* mutants lacking two of the Cdc42p-directed GTPase activating proteins implicated in septin organization (Caviston et al., 2003; Gladfelter et al., 2002) (Table 2). Thus, Swe1p overexpression led to a significant drop in the proportion of mutant cells exhibiting a normal-looking hourglass and a concomitant rise in the proportion of cells displaying ectopic septin rings or patches within the bud in all cases. The susceptibility of the different mutants to the septin-mislocalizing influence of Swe1p was variable, perhaps indicating differences in the degree to which the septin hourglass is destabilized in each mutant (see Discussion). Nevertheless, this survey shows that Swe1p action is not specific to the *cdc42<sup>V36T,K94E</sup>* mutant.



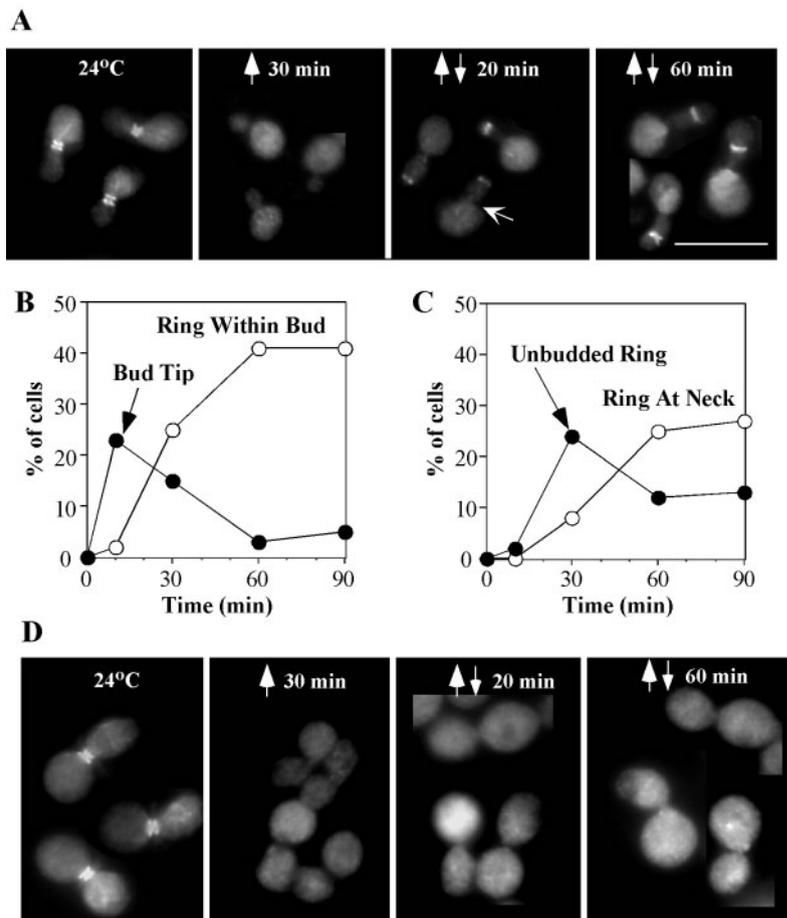
**Fig. 4.** Arrest with high Clb/Cdc28p activity maintains neck-localized septins. (A) Strains DLY7326 (*GAL1p-CLB1 $\Delta$ 152*) and DLY7327 (*cdc42<sup>V36T,K94E</sup> GAL1p-CLB1 $\Delta$ 152*) were grown to exponential phase in sucrose-containing medium at 29°C, induced to overexpress Clb1p $\Delta$ 152 by addition of galactose (2% final concentration), and processed to visualize septin distribution after a further 4 hours. (B) Strains DLY5 (WT) and DLY5080 (*cdc42<sup>V36T,K94E</sup>*) were grown to exponential phase in YEPD at 29°C and nocodazole was added to 15  $\mu$ g/ml to arrest cells in mitosis. After 3 hours of arrest cells were fixed and processed to visualize septin distribution. Bar, 10  $\mu$ m.

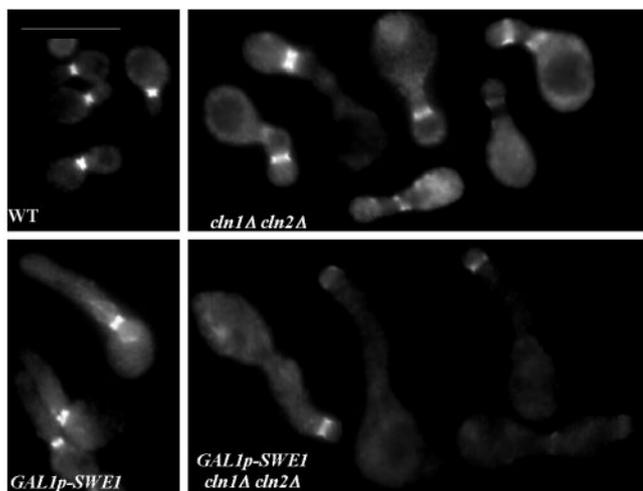
#### Identification of other mutants with defects in septin organization

The findings reported above indicate that relatively mild defects in septin organization at the mother-bud neck can be exacerbated by Swe1p overexpression. In many cases, the arrested cells had a dramatic 'bud chains' phenotype in which the elongated buds displayed irregular bulges along their length (e.g. Fig. 3C). A similar 'bud chains' phenotype has been noted (Fred Cross, personal communication) in *cln1 $\Delta$  cln2 $\Delta$*  mutants arrested by overexpression of Sic1p. *CLN1* and *CLN2* encode two of three G1 cyclins in yeast, and we found that *cln1 $\Delta$  cln2 $\Delta$*  mutants displayed aberrant septin staining patterns that were dramatically exacerbated upon Swe1p overexpression (Fig. 6 and Table 2). *cln2 $\Delta$  cln3 $\Delta$*  mutants also showed septin defects, although to a much lesser degree (Table 2). These results suggest that G1 cyclins are important for establishment or maintenance of the septin ring/hourglass. Other reports of strains with 'bud chains' phenotypes may similarly reflect a combination of septin defects and cell cycle arrest, as discussed below.

#### Fig. 5. Septins disassembled from the neck assemble into new rings at the bud tip during G2 but not M phase.

(A) Strain JMY1141 (*cdc12-6*) was grown to exponential phase in YEPD at 24°C, shifted to 37°C for 30 min, and then shifted back down to 24°C. Samples were taken before the shift (24°C), 30 minutes after shift-up ( $\uparrow$ ), and at various times after shift-down ( $\uparrow\downarrow$ ). Arrow indicates original neck. For each sample following shift-down, we quantitated the percentage of cells with septins assembled at the tip or within the bud (B) or as rings in unbudded cells or at the neck (C). These categories were separated in the interests of clarity, and the remaining cells (25%, not graphed) showed no septin staining. (D) Cells were arrested in mitosis (75% large budded cells) by treatment with 15  $\mu$ g/ml nocodazole for 2 hours at 24°C and then subjected to the same shift-up/shift-down regimen as above. Bar, 10  $\mu$ m.





**Fig. 6.** Septin organization in *cln1Δ cln2Δ* cells. Top panels: strains DLY5103 (WT) and DLY5102 (*cln1 cln2*) were grown to exponential phase in YEPD at 30°C, and the cells were fixed and processed to visualize septins. Bottom panels: strains DLY5106 (*GAL1p-SWE1*) and DLY5104 (*cln1 cln2 GAL1p-SWE1*) were grown to exponential phase in YEPsucrose at 30°C, induced to express Swe1p by addition of galactose (2% for 5 hours), and the cells were fixed and processed to visualize septins. Bar, 10 μm.

## Discussion

The findings described above reveal an intimate series of connections between septin organization, cell cycle and cell shape that help to explain the complex phenotypes of mutants affecting septins. Although our analysis was focused on a particular *cdc42* mutant, the connections described here apply to many other mutants, uncover previously unappreciated septin defects associated with various mutants, and suggest explanations for various published phenotypes. In addition, they support unexpected roles for septins in wild-type cells.

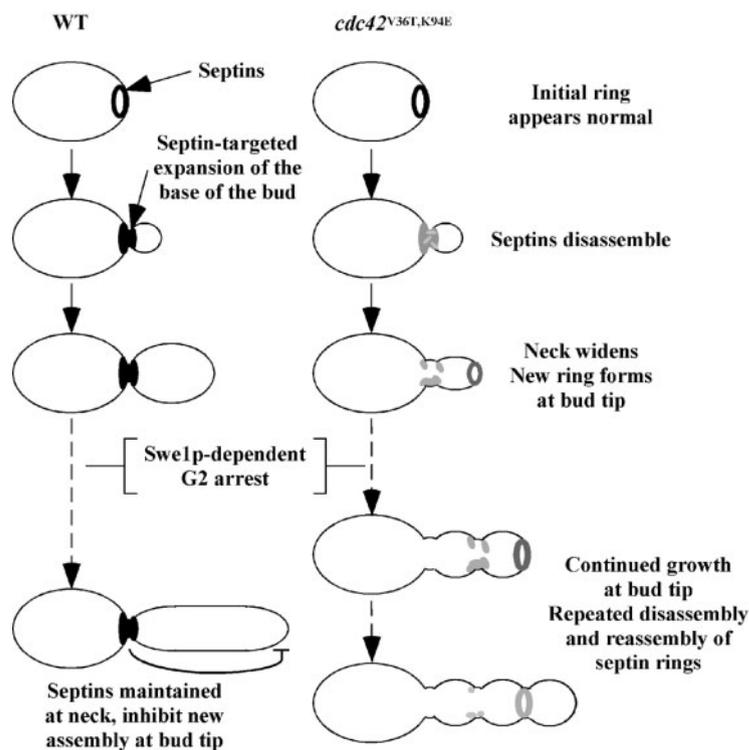
### Septins direct cell growth to shape the base of the bud

Previous studies have noted the frequent correlation between defects in septin organization and aberrant shape of the mother-bud neck (Cvrckova et al., 1995; Gladfelter et al., 2002; Gladfelter et al., 2004; Longtine et al., 1998; Longtine et al., 2000). However, as neck shape was less affected in the total absence of septin structures than in mutants with misorganized septins, it has been unclear whether septin organization and neck shape were causally linked or whether defects in these processes were pleiotropic effects of the underlying mutations. A recent study documented a striking temporal correlation between septin reorganizations and the pattern of cell growth in *cdc42<sup>V36G</sup>* mutants (Caviston et al., 2003), supporting a causal role for septins in dictating cell shape. Our observation that the broad necks observed in *cdc42<sup>V36T,K94E</sup>* mutants were dependent on assembled septins indicates that misorganized septins do contribute to aberrant neck shape, and our findings indicate that septins play an

important role in generating the normal ‘hourglass-shaped’ neck in wild-type yeast.

In the absence of assembled septins, growth was directed to the bud tip by actin cables, and the base of the bud remained cylindrical. In the presence of normal septins, the base of the bud bulged out, forming the hourglass. Septins are known to impact the distribution of actin cables by recruiting/activating the formin Bnr1p (Pruyne et al., 2004), but we found that Bnr1p was not required for the septin-dependent shaping of the base of the bud. Unlike the chitin-rich bud scar on the mother side of the neck, the cell wall at the base of the bud is rich in mannan that is brightly labeled by the lectin Con A (Lew and Reed, 1993), derived from mannanose-rich glycoproteins that are delivered through the secretory pathway, suggesting that septins target secretory vesicles to the base of the bud (Fig. 7, left). Studies in mammalian cells have identified interactions between septins and proteins that target fusion of secretory vesicles with the plasma membrane (Dent et al., 2002; Kartmann and Roth, 2001; Trimble, 1999), and we speculate that similar interactions in yeast cells might underlie septin-dependent targeting of secretion to the base of the bud.

Examination of cells with buds of different sizes indicated that the septin-dependent remodeling of the base of the bud occurred very soon after bud emergence. The fact that bulging was restricted to the bud side of the neck while the middle of the neck remained narrow suggests that this remodeling is tightly controlled in wild-type cells. In contrast, the entire neck region was broadened in a septin-dependent manner in *cdc42<sup>V36T,K94E</sup>* mutants (Fig. 7, right), suggesting that the normal restrictions on septin-mediated secretion were relaxed, allowing an inappropriate amount of secretion in the neck region.



**Fig. 7.** Proposed genesis of the *cdc42<sup>V36T,K94E</sup>* phenotype. See text for details.

This raises the interesting question of how septin-directed secretion is normally limited.

When subjected to G2 arrest, various mutants displayed ectopic septin rings within the bud, and these rings also caused bulging, giving the appearance of ectopic ‘necks’ along the length of the bud (Fig. 7, right). This observation indicates that septin-directed secretion can occur during G2 as well as G1/S (the normal time of bud emergence). However, wild-type cells arrested in G2 did not continue to expand the base of the bud (Fig. 2), suggesting that factors other than cell cycle progression must be able to limit septin-directed secretion. One attractive way to account for that limitation would be that once septins have remodeled the neck from a cylinder into an hourglass, that shape induces a change in the septin cortex that halts septin-mediated secretion.

#### The septin hourglass at the neck inhibits the assembly of new septin rings

We found that when the septin hourglass at the neck was disassembled by shift of *cdc12-6* cells to the restrictive temperature, and then allowed to reassemble by shifting back to the permissive temperature, new septin rings assembled at the bud tip. This result implies that budded cells contain factors capable of directing new septin ring assembly at the bud tip. The capacity to assemble septins at the bud tip was lost following activation of Clb/Cdc28p. Many of the same proteins that are localized to the presumptive bud site at the time of initial septin ring assembly are subsequently localized to the bud tip and then dispersed upon Clb/Cdc28p activation (Lew and Reed, 1995b). Thus, it seems probable that Cdc42p and other proteins that normally promote septin ring assembly in late G1 prior to bud emergence remain competent to promote septin ring assembly at the bud tip until they are dispersed in response to Clb/Cdc28p. However, our data do not rule out models in which Clb/Cdc28p plays a more direct role in blocking septin assembly at the bud tip.

If factors at the bud tip are capable of directing septin ring assembly, then why don't septin rings normally assemble at that site? The finding that elimination of the septin hourglass at the neck allowed subsequent septin assembly at the bud tip implies that neck-localized septins can somehow prevent the bud-tip factors from assembling new rings (Fig. 7, left). What is the basis for this inhibitory effect? In principle, assembly of a septin hourglass at the neck could ‘use up’ a limiting septin assembly factor, such that once the neck ring is assembled there is not enough of this factor left to assemble another ring at the bud tip. Similarly, a cooperative septin assembly process might make new factors more likely to join the existing hourglass than to form a new ring at the tip. Disassembly of the hourglass would liberate the factor(s), allowing new rings to assemble. An alternative hypothesis would be that the septin hourglass at the neck activates a signaling pathway that inhibits septin assembly at the bud tip. This hypothesis also seems plausible, as many signaling proteins are localized to the neck (Gladfelter et al., 2001b), and mutations in some of these do allow assembly of ectopic septin rings in addition to the septin hourglass. Moreover, the hypotheses are not mutually exclusive and both types of processes may contribute to restricting septin assembly in wild-type cells.

#### Genesis of the *cdc42*<sup>V36T,K94E</sup> mutant phenotype

We suggest the following chain of events to account for the constellation of phenotypes exhibited by *cdc42*<sup>V36T,K94E</sup> mutants (see Fig. 7). As suggested previously (Gladfelter et al., 2002), the primary defect is in the initial assembly of an unstable septin ring, leading to loss of septins from the neck with time, explaining the diminished and often patchy septin staining at the neck in these mutants. The poorly organized septin ring misdirects secretion to the neck region (not just to the base of the bud), generating a wide neck. The defective septin ring fails to promote Swe1p inactivation, causing a G2 delay that maintains Cdc42p and other factors polarized at the bud tip. Moreover, the defective septin ring fails to inhibit the assembly of a new septin ring. Thus, some cells generate a new bud-tip-localized ring as the original neck ring disintegrates. As the bud continues to grow, the new septin ring remains behind within the bud.

In *cdc42*<sup>V36T,K94E</sup> mutants lacking Swe1p, on-time activation of Clb/Cdc28p disperses Cdc42p and the other tip-localized factors before they can promote assembly of an ectopic ring. Conversely, in *cdc42*<sup>V36T,K94E</sup> mutants overexpressing Swe1p, the cells are arrested with low Clb/Cdc28p and tip-localized factors are continuously polarized. In these cells, it appears that there are iterative cycles in which septin rings assemble at the bud tip and remain ‘stationary’ as the bud continues to elongate. Like the original ring at the neck, these rings are unstable and eventually disassemble as new rings form at the bud tip, so that septin staining only detects the youngest ring (or two) closest to the bud tip. However, each ring leaves a lasting imprint of its transient existence in the form of a bulge along the length of the bud (Fig. 7).

#### Implications for other mutants

To what degree does our description of the *cdc42*<sup>V36T,K94E</sup> mutant provide insight into septin phenotypes in other mutants? In the view expounded above, the appearance of ectopic septin rings results from several parallel defects, which combine to allow bud tip factors to assemble a new ring. These defects, in turn, stem from an underlying misorganization of the septin hourglass at the neck. One contributory factor in new ring assembly is Swe1p, the degradation of which is dependent on a properly organized septin hourglass (Barral et al., 1999; Longtine et al., 2000). When this pathway was enhanced by overexpressing Swe1p, we found that ectopic rings became more prevalent in many mutants, and were even detected in mutants that do not normally exhibit ectopic septin rings. This finding suggests that all of the mutants may share a common pathway for ectopic ring assembly.

The comparison of strains bearing different mutant lesions revealed marked differences in the frequency of ectopic rings induced upon Swe1p overexpression. In general, there was a correlation between the appearance of new rings and the disappearance of septins at the neck, supporting the possibility that neck-localized factors (perhaps septins themselves) must be released to allow new ring assembly. If this is the case, then a major difference between different strains may lie in the rate at which the hourglass disassembles with time. Recent FRAP studies indicate that septins within the wild-type hourglass are ‘immobile’, but become exchangeable in various mutant strains (Caviston et al., 2003; Dobbelaere et al., 2003). It will be

interesting to determine whether there is a strong correlation between septin exchangeability (as measured by FRAP) and septin disappearance from the neck during Swe1p-induced arrest.

#### Identification of mutants with previously unappreciated defects in septin organization

The discovery that Swe1p-mediated G2 arrest exacerbates what are otherwise rather mild septin defects suggested that imposition of G2 arrest might help to reveal septin abnormalities in mutants that had not previously been implicated as septin defective. Indeed, we identified several such defects, suggesting that Cln1p, Cln2p, Bud3p, Bud4p, Bud5p, Bni1p and Bni4p help to promote proper septin organization. In some cases, other recent studies are also consistent with this view, as considered below.

Bud4p is required for axial bud site selection (Chant and Herskowitz, 1991; Sanders and Herskowitz, 1996), and Iqg1p localization (Osman et al., 2002). However, Bud4p is distantly related to annillin in mammals and Mid2p in *S. pombe*, which have been implicated in septin organization (Berlin et al., 2003; Kinoshita et al., 2002; Tasto et al., 2003), lending credence to the idea that Bud4p also contributes to septin organization in *S. cerevisiae*.

Bni4p tethers chitin synthase III to the septin cortex (DeMarini et al., 1997) and recruits Glc7p, the catalytic subunit of protein phosphatase I, to that site (Kozubowski et al., 2003). As septin phosphorylation probably contributes to proper septin organization (Dobbelaere et al., 2003; Versele and Thorner, 2004), targeting a major phosphatase to the septins seems likely to impact septin organization.

Bni1p is a formin implicated in nucleating actin cables at sites of polarization (Evangelista et al., 1997; Evangelista et al., 2002; Sagot et al., 2002). Bni1p was first identified through genetic interactions with septin mutants, and *bni1* mutants have wide necks (hence the name, BNI=bud neck involved) (H. Fares and J. Pringle, personal communication), so a role for Bni1p in septin organization is entirely consistent with these earlier findings.

Cln1p and Cln2p are related G1 cyclins that trigger bud emergence, spindle pole body duplication and degradation of Sic1p (Lew et al., 1997). Loss of Cln1p and Cln2p is lethal when combined with mutations in septins, as well as mutations in the *CLA4*, *GIN4* and *NAP1* septin organization genes (Benton et al., 1997; Cvrckova et al., 1995; Zimmerman and Kellogg, 2001), consistent with a specific role for these cyclins in promoting septin organization.

Upon cell cycle arrest by Swe1p, mutants with defective septin organization formed elongated buds characterized by irregular bulges and constrictions (see above and Fig. 7). An identical 'bud chains' phenotype with septin rings generally localized to the most distal 'neck' has been described in *zds1Δ zds2Δ* mutants (Bi and Pringle, 1996), as well as *cdc55* mutants (Healy et al., 1991). Both phenotypes are Swe1p-dependent (McMillan et al., 1999b; Wang and Burke, 1997), as is a similar phenotype observed upon exposure of wild-type cells to isoamyl alcohol (Martinez-Anaya et al., 2003). We now suggest that these phenotypes all arise as a result of combining prolonged Swe1p-mediated G2 delay with a mild defect in septin organization, and that the 'bud chains' cell morphology may be useful as a rapid diagnostic for septin defects.

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