

Septins, under Cla4p Regulation, and the Chitin Ring Are Required for Neck Integrity in Budding Yeast

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CLA4, encoding a protein kinase of the PAK type, and *CDC11*, encoding a septin, were isolated in a screen for synthetic lethality with *CHS3*, which encodes the chitin synthase III catalytic moiety. Although Ste20p shares some essential function with Cla4p, it did not show synthetic lethality with Chs3p. *cla4* and *cdc11* mutants exhibited similar morphological and septin localization defects, including aberrant and ectopic septa. Myo1p, which requires septins for localization, formed abnormally wide rings in *cla4* mutants. In cultures started with unbudded cells, an inhibitor of Chs3p activity, nikkomycin Z, aggravated the abnormalities of *cla4* and *cdc11* mutants and gave rise to enlarged necks at the mother-bud junction, leading to cell death. It is concluded that Cla4p is required for the correct localization and/or assembly of the septin ring and that both the septin ring and the Chs3p-requiring chitin ring at the mother-bud neck cooperate in maintaining the neck constricted throughout the cell cycle, a vital function in budding yeast.

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, a chitin ring is formed in the cell wall at the base of an emerging bud, in a reaction requiring chitin synthase III, of which Chs3p is the putative catalytic unit (for reviews see Cabib *et al.*, 1996, 2001). After further bud growth and mitosis, a primary septum is constructed between mother and daughter cell, requiring the joint action of three elements: the septin filament ring, which seems to serve as a scaffold for other components of the septation apparatus (for reviews see Longtine *et al.*, 1996; Gladfelter *et al.*, 2001); the actomyosin contractile ring, which invaginates the plasma membrane inward at cytokinesis (Tolliday *et al.*, 2001); and the chitin synthase II system. This system, with Chs2p as the catalytic subunit, acts in the formation and secretion of chitin into the membrane invagination, giving rise to the primary septum and separating mother and daughter cell (Cabib *et al.*, 1996; Schmidt *et al.*, 2002). Finally, secondary septa are formed from both dividing cells and a trilaminar structure is built,

with the chitin layer in the middle. At this point, the chitin ring encircles the septum and is contiguous to it. After cell separation, the ring ends up as the rim of the bud scar, which remains on the mother cell and contains most of the septum. We have reported earlier (Slater *et al.*, 1985) and confirmed recently (Roh *et al.*, 2002b) that in septin mutants structures morphologically similar to septa are made at ectopic locations in the cell. This result emphasizes both the scaffold function of the septin ring and the autonomous nature of the septation apparatus. It also highlights the need for cellular mechanisms for the organization and retention of the septins at the mother-bud neck.

In contrast with all the changes taking place at the mother-bud junction, the diameter of the neck, as measured from the inner surface of the cell wall, remains unmodified during septation and indeed throughout the cell cycle. The mechanisms that prevent the neck from being enlarged in the face of adjacent growth of the bud are unknown. We found information on these mechanisms in an unexpected way. In an attempt to identify genes necessary for Chs2p function, we had initiated a genetic screen for mutants that, like *chs2*, would show synthetic lethality with a *chs3* mutation. Two genes isolated in that screen, *CLA4* and *CDC11*, coding for a protein kinase of the PAK type (Cvrckova *et al.*, 1995) and for a septin, respectively, did not seem to have a direct role in Chs2p function. On the other hand, a study of mutants in those genes and of their interaction with the *chs3* mutation has shown that septins and the chitin ring formed at bud

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Table 1. Strains used in this study

Strain	Genotype	Source
ECY36-3D	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1</i>	Shaw <i>et al.</i> (1991)
ECY46-4-1B	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2Δ1 chs3::LEU2</i>	Crotti <i>et al.</i> (2001)
ECY101	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG</i> [pEC28]	This study
ECY101-39	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG cla4-39</i> [pEC28]	This study
ECY105	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ste20::URA3</i> [pEC28]	This study
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	Sikorski and Hieter (1989)
YMS134	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cla4::LEU2</i>	This study
YMS189	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG cla4-39 swe1::kanMX6</i> [pEC28]	This study
YMS190	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2 cla4::URA3 swe1::kanMX6</i>	This study
YMS197	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG cla4::URA3</i> [pEC28]	This study
YMS306	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cla4::TRP1</i>	This study
YMS332	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cla4::LEU2 swe1::kan MX6</i>	This study
1238	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 swe1::kanMX6</i>	Research Genetics
DHY103-9B	<i>MATa ura3-52 lys2 his3-Δ200 leu2 tyr1 cdc3-1</i> [pMS55]	Roh <i>et al.</i> (2002b)
AVY1	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ade3::hisG</i>	This study
AVY2	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ade3::hisG leu2::TRP1</i> [pAV1]	This study
AVY2-25	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ade3::hisG leu2::TRP1 cdc11-25</i> [pAV1]	This study
AVY3	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ade3::hisG leu2::TRP1 cdc11-25 cla4::LEU2</i> [pAV1]	This study
AVY5	<i>MATa ura3-52 leu2-2 trp1-1 chs1-23 chs3-1 ade2::hisG ade3::hisG leu2::TRP1 cdc11-25</i>	This study

emergence cooperate in the maintenance of mother-bud neck size. When the function of both systems is compromised simultaneously, the neck enlarges and the cell eventually dies, which shows that neck integrity is essential for viability.

MATERIALS AND METHODS

Strains and Growth Conditions

The yeast strains used in this study are listed in Table 1. Growth media and conditions were as previously described (Schmidt *et al.*, 2002), except that synthetic complete media (SC) were prepared from dropout powders (Qbiogene, Carlsbad, CA).

Strain Construction

General methods of DNA manipulation were as described in Ausubel *et al.* (1994). Yeast transformation was carried out with the lithium acetate method (Ito *et al.*, 1983). Deletion of *ADE2* in ECY36-3D was carried out with a deletion cassette from ATCC vector 99604 (pΔADE2; Aparicio *et al.*, 1991), according to instructions from the supplier. The *URA3* initially inserted in the *ADE2* gene between two *hisG* was eliminated by growth on uracil-containing medium and plating on fluoroarotic acid medium. The resulting strain, ECY101, was transformed with pEC28 to generate ECY101[pEC28]. This strain was used for mutagenesis and red-white selection.

STE20 was disrupted in strain ECY101[pEC28] by transformation with a *ste20::URA3* cassette cut from plasmid pEL45 (Leberer *et al.*, 1992) with *SalI* and *XbaI*, to yield ECY105 (Table 1).

The *cla4::LEU2* gene disruption was achieved by transforming yeast with *SphI*/*SmaI*-cut plasmid pFD26 (Cvrcková *et al.*, 1995). For disruption of *CLA4* with *URA3* or *TRP1*, plasmid pMS17, was cut with *MluI*/*XcmI*. The DNA fragment was then blunted and ligated to the reporter genes, prepared as follows. A 1.20-kb *URA3* fragment or a 1.0-kb *TRP1* fragment was generated by PCR, blunted, and phosphorylated. The blunt-ended ligations yielded plasmid pMS32 (*URA3*) and pMS46 (*TRP1*), respectively. The disruption fragments were amplified by PCR from these plasmids, using prim-

ers *CLA4UP*: 5'-AGTAGAGGAGATCTACAAACTTGA-3' and *CLA4DOWN*: 5'-GATATGCTTCTAGAAATAGTTGTGTG-3'.

Disruption of *SWE1* with the *kanMX6* module was performed by amplifying the *swe1::kanMX6* allele from strain 1238 (Invitrogen, Carlsbad, CA) via PCR with primers *SWE1UP*: 5'-TTGAACATTGGCGT-GCCC-3' and *SWE1DOWN*: 5'-TTATCTGCTACATCTGTAA-3'.

Disruption of *MYO1* was obtained as described by Schmidt *et al.* (2002).

Deletion of *ADE3* in ECY101 was carried out with an *ade3::hisG-URA3-hisG* deletion cassette amplified by PCR from plasmid pAV4. From the resulting strain, *URA3* was eliminated by growth on uracil-containing medium followed by plating on fluoroarotic acid medium. The resulting strain, AVY1, was transformed with pAV1 to generate AVY1[pAV1].

Deletion of *LEU2* in AVY1[pAV1] was done with a disruption cassette cut out from pAV10 by *HpaI*-*SphI* double digestion, thus generating strain AVY2. This strain was used for mutagenesis and red-white selection.

To check the effect of a *CLA4* deletion in the *cdc11-25* mutant (AVY2-25), the *CLA4* gene was disrupted with a *cla4::LEU2* disruption cassette as described above.

All disruptions were confirmed by PCR.

Construction of a *cdc11-25 chs3-1* double mutant, AVY5, was done by segregating the plasmid pAV1 from the strain AVY2-25. This was achieved by streaking AVY2-25 on YEPD-agar containing 1 M sorbitol at 26°C. Those cells that lost the plasmid formed white sectors or white colonies. Loss of plasmid was confirmed by Calcofluor White staining, growth on Calcofluor White, ability to grow on fluoroarotic acid, and uracil auxotrophy at 26°C.

Plasmid Construction

To overexpress a nonfunctional *chs3R995A* allele, the mutant allele contained in plasmid pHV7-37 (C. Roncero) was excised with *Clal* and *SalI* and cloned into YEp352 cut with the same enzymes, yielding vector pMS75 (Table 2). To construct pAS8, the *CHS2* ORF contained in the multiple cloning site of YEp352 (pEC2, Ford *et al.*, 1996) was cut out with *EcoRV* and *SacI* and ligated to *SmaI*/*SacI* digested vector pRS314.

To obtain pMS76, the *LEU2*-containing *PvuI* fragment from pLP17 (Lippincott and Li, 1998b) was replaced with the *HIS3*-containing

Table 2. Plasmids used in this study

Plasmid	Description	Source
Library in pRS200		ATCC 77164
pΔADE2	p[<i>ade2::hisG::URA3::hisG</i>]	Aparicio <i>et al.</i> (1991) (ATCC 99604)
pEC2	YEp352 <i>CHS2</i>	Ford <i>et al.</i> (1996)
pEC28	pRS412 <i>CHS3</i>	Schmidt <i>et al.</i> (2002)
pEL45	pBluescript KS+ <i>ste20::URA3</i>	Leberer <i>et al.</i> (1992)
pFD26	<i>cla4::LEU2</i>	Cvrcková <i>et al.</i> (1995)
pFLC1	p <i>GALISTE20</i>	S. Davis
pHV7-37	YCp50 <i>chs3R995A</i>	C. Roncero
pHV8	YEp352 <i>CHS3</i>	Valdivieso <i>et al.</i> (1991)
pLP17	pRS315 <i>CDC12-GFP</i>	Lippincott and Li (1998b)
pNKY50	YEp24 <i>hisG::URA3::hisG</i>	Alani <i>et al.</i> (1987)
pSM491	Contains triple HA tag	S. Michaelis
pMS17	pRS200 <i>CLA4</i>	This study
pMS32	pRS200 <i>cla4::URA3</i>	This study
pMS46	pRS200 <i>cla4::TRP1</i>	This study
pMS39	From pRS200 library	This study
pMS55	pRS316 <i>MYO1-GFP</i>	Schmidt <i>et al.</i> (2002)
pMS63	pRS426 <i>MYO1-GFP</i>	This study
pMS75	YEp352 <i>chs3R995A</i>	This study
pMS76	pRS313 <i>CDC12-GFP</i>	This study
pMS79	YEp352 <i>chs3R995A::HA</i>	This study
pMS80	YEp352 <i>CHS3::HA</i>	This study
pRS316 <i>CDC3GFP</i>	<i>CDC3-GFP</i>	M. Longtine
p366	YCp50 with <i>URA3</i> replaced by <i>LEU2</i>	ATCC 77163
Library in p366		ATCC 77162
pFD10	YCp50 <i>ADE3</i>	A. Bender
pAS8	pRS314 <i>CHS2</i>	This laboratory
pLP8	pRS315 <i>MYO1-GFP</i>	Lippincott and Li, (1998a)
p19	From p366 library	This study
pAV1	YCp50 <i>CHS3ADE3</i>	This study
pAV4	YCp50(<i>URA3</i> cut out) + <i>ade3::hisG-URA3-hisG</i>	This study
pAV10	YEp351 <i>leu2::TRP1</i>	This study
pAV11	p366 <i>mog1HOC1CDC11mig1</i>	This study
pAV12	YEp351 <i>CDC11</i>	This study
pAV13	p366 <i>CDC11</i>	This study
pAV17	YEp351 <i>cdc11-25</i>	This study
pAV21	pRS425 <i>MYO1-GFP</i>	This study

PvuII fragment from pRS313. For construction of the screening plasmid, pAV1, a 4.6-kb *PvuII* fragment containing the *CHS3* gene was cut out from pHV8 and ligated to pFD10 at the *SmaI* site.

For the construction of an *ADE3* disruption cassette, a 2.5-kb fragment containing the *URA3* gene was removed from pFD10 by *BamHI* digestion. The remaining plasmid was religated and a 1665-base pair *AgeI-PvuII* fragment was replaced by a 3.8-kb *BamHI-BglII* fragment of *hisG-URA3-hisG* from plasmid pNKY50 after Klenow filling-in treatment. This yielded plasmid pAV4.

To construct a *leu2::TRP1* disruption cassette, a 5000-base pair *AgeI-EcoRV* fragment from YEp351 was replaced with a blunt ended 1.0-kb *TRP1* fragment amplified by PCR from pRS314, yielding plasmid pAV10.

To clone *CDC11*, the gene was amplified by PCR from p19 with oligonucleotides *CDC11HindIII*UP: 5'-CTGTAAATTAACAAG-CTTTTATAAATAT-3' with an engineered *HindIII* site and *CDC11SphI*DN: 5'-CTCATTGGCATGCAATTTGG-3' with an engineered *SphI* site. The PCR product was digested with *HindI-SphI* and ligated to p366 digested with the same enzymes, resulting in pAV13.

To clone *CDC11* in YEp351, the *CDC11* PCR product from p19 with the above mentioned oligonucleotides was blunt-ended, phosphorylated, and ligated to YEp351 at the *SmaI* site, yielding plasmid pAV12.

pAV21 was constructed as follows: an ~8-kb *PvuII* fragment from pLP8 was ligated to the large *PvuII* fragment from pRS426 to yield pMS63. From pMS63, the ~8-kb *PvuII* fragment was cut and ligated to the 5.2-kb *PvuII* fragment of pRS425 to yield pAV21.

Mutagenesis

Mutagenesis of strain EY101 with ethyl methane sulfonate was carried out as described in Ausubel *et al.* (1994). Mutagenized cells were plated on SD agar containing nutritional requirements and 10 µg/ml adenine. White colonies were transferred to plates with the same medium and cell morphology was checked after growth by phase contrast microscopy. Those colonies showing similarity to *chs2* mutants, i.e., clumps of cells with some cells of aberrant shape, were picked for further study.

The same method was used with strain AVY2, except that red colonies were selected.

Cloning and Sequencing of *CLA4* and *cla4-39*

Mutant strain EY101-39 was transformed with a genomic library contained in vector pRS200 (ATCC 77164). Transformants were screened for their ability to segregate the *ADE2*-containing plasmid pEC28, leading to the appearance of red sectors, and for the correc-

tion of the mutant morphology. Plasmid pMS39 was isolated from two of the transformants. pMS39 induced sectoring of transformant colonies and corrected the mutant morphology after retransformation into the original mutant. The DNA contained in the cloning site of pMS39 was sequenced using T3 and T7 primers, the ABI prism sequencing kit, and the ABI-prism 310 sequencer according to manufacturer's (Perkin Elmer-Cetus, Oak Ridge, TN) instructions. The insertion in the cloning site was found to originate from chromosome XIV, position 71828–63727. Removing a *SgrAI/EcoRI* fragment from this vector left *CLA4* as the only intact reading frame in the insert. This truncated plasmid pMS17 still complemented both morphology and sectoring phenotype of ECY101-39; therefore, the mutant allele was designated *cla4-39*. To determine the nature of the *cla4-39* mutation, the allele was sequenced from ECY101-39 chromosomal DNA as described above.

Cloning and Sequencing of *cdc11-25*

The mutant strain AVY2-25 was transformed with a genomic library in the vector p366 (ATCC 77162). The transformants were screened for their ability to segregate the *ADE3 CHS3* containing plasmid pAV1, leading to the formation of white sectors and for the correction of the mutant morphology. From three of the transformants, plasmid p19 was isolated, which was able to induce sectoring of the transformant colonies and complement the mutant morphology when transformed into the original mutant AVY2-25. The DNA insert in p366 cloning site was sequenced (Seqwright DNA Sequencing) with oligos: 5'-GCCACTATCGACTACGCGATC-3' and 5'-GTGGCGCCGGTGATCCGCT-3'. The insert in p19 was found to be from chromosome X at position 572600–583595. A 7-kb *HindIII* fragment of p19 (pAV11) was able to induce sectoring and complement mutant morphology of AVY2-25. The ORFs in this fragment were further subcloned. It was found that *CDC11* was the only ORF able to induce the sectoring phenotype and complementation of the mutant morphology.

To determine the position of the *cdc11-25* mutation, the mutant DNA allele was recovered from AVY2-25 by gap repair (Rothstein, 1991). Gap-repaired plasmid pAV17 was isolated from the colonies, which showed a restriction pattern identical to pAV12 but was

unable to complement the mutant phenotype of *cdc11-25* mutant and did not induce sectoring. The insert was sequenced with several oligonucleotides from the flanking regions of *CDC11*. The sequences revealed a G-A change at position 95, changing the GGA triplet codon for glycine to glutamic acid (G32E).

Quantifying *Chs3^{R995A}* Expression

To quantify the expression of the mutant *Chs3p*, we introduced a triple-HA tag into the N-terminus of the protein. The tag was amplified from pSM491 with primers CHS3HAUP: 5'-GGCCG-CACCGGTTACCCATACGATGTTCT-3' and CHS3HADOWN: 5'-GCGGCCACCGGTAGCAGCGTAATCTGGAAC-3' (engineered *AgeI* sites in bold). Plasmids pHV8 and pMS75, encoding the wild-type and mutant *Chs3p*, respectively, were linearized with *AgeI* and ligated to the PCR fragments, yielding plasmids pMS79 (*chs3R995A::HA*) and pMS80 (*CHS3::HA*). Both plasmids were then introduced into strain ECY46-4-1B (*chs3::LEU2*). Calcofluor White staining of transformants showed that pMS80 gave rise to wild-type chitin synthase III activity, whereas pMS79 did not. Sample preparation and Western blotting were as described previously for Spa2HA (Schmidt *et al.*, 2002).

Electron Microscopy

Electron microscopy was carried out as previously described (Schmidt *et al.*, 2002).

Fluorescence Microscopy

Fluorescence of GFP-tagged proteins was observed as already reported (Roh *et al.*, 2002b). Septin rings showing breaks in continuity or an asymmetric distribution of septin-GFP were scored as abnormal.

Isolation of Single, Unbudded Cells

Single, unbudded cells were isolated by centrifugation on sucrose gradients as already described (Drgonová *et al.*, 1999), except that

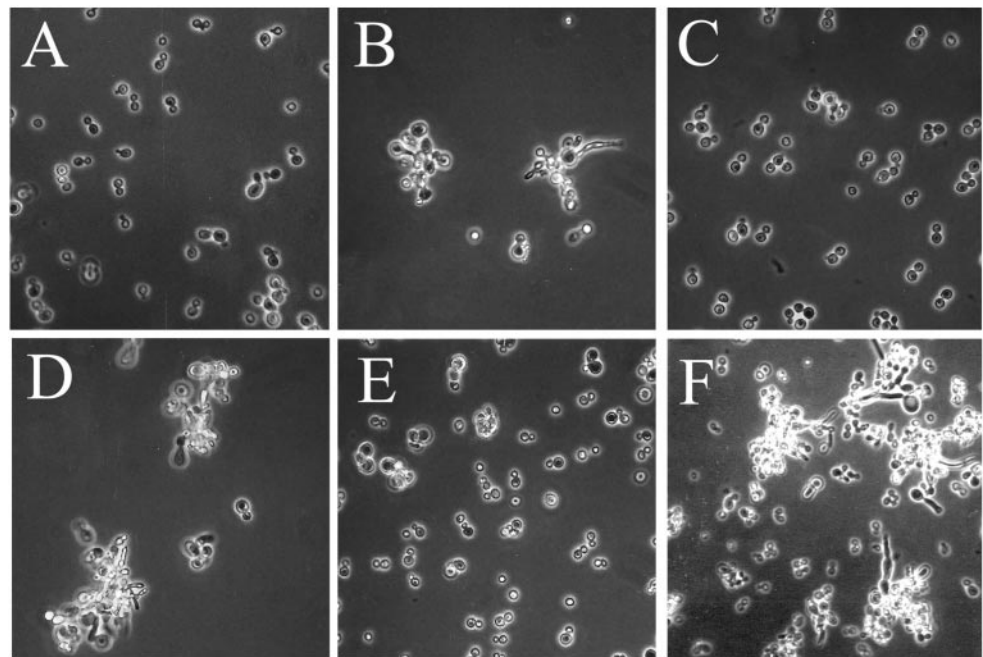


Figure 1. Morphology of different strains related to the isolation of a *cla4* mutant. (A) Wild-type (ECY101). (B) ECY101-39 (*cla4-39* mutant). (C) ECY101-39 transformed with pMS17 (pRS200CLA4). (D) YMS197 (ECY101 in which *CLA4* was disrupted). (E) ECY105 (ECY101 in which *STE20* was disrupted). (F) YMS197[pFLC1] (*cla4::URA3* with *STE20* overexpressed by growth in 2% galactose medium).

methylmannoside was not added to the cell suspension before sonication.

RESULTS

Isolation of *CLA4* in a Screen for Synthetic Lethality with *CHS3*

A genetic screen for mutants that would be inviable in a *chs3* background was conducted with a modification of the red-white selection (Bender and Pringle, 1991; see MATERIALS AND METHODS). In this modification, an *ade2Δ chs3-1* strain that can lose a plasmid containing *ADE2* and *CHS3* (ECY101[pEC28]) gives rise to white colonies with red sectors, whereas strains containing a mutation that is synthetically lethal with *chs3-1* cannot lose the plasmid and therefore remain white. To increase the probability of selecting mutants compromised in septation, only white colonies whose cells showed some clumping were chosen for further study. One such strain was mutant ECY101-39 (Figure 1B).

After transformation of ECY101-39 with a genomic DNA library, one plasmid was found to restore the sectoring. The insert was subcloned and it was found that a fragment containing as the only ORF *CLA4*, which encodes a protein kinase of the PAK type (Cvrcková *et al.*, 1995), was able to complement both the morphology and the sectoring defect of ECY101-39 (Figure 1C); therefore, the mutation was designated as *cla4-39*. By appropriate crosses it was determined that ECY101-39 carries a recessive mutation in the *CLA4* locus. Sequencing of the allele detected a G-A transition at position 200, turning the TGG triplet coding for Trp⁶⁷ into a TAG stop codon and leaving a 66-amino acid Cla4p fragment that is not functional (Benton *et al.*, 1997). In fact, a null mutant of *CLA4* behaved as *cla4-39* with respect both to lack of sectoring and morphology (Figure 1D).

Although by the sectoring assay *cla4Δ* and *chs3-1* behaved as synthetically lethal, we were able to obtain a *cla4Δ chs3Δ* double mutant by deleting *CLA4* in a *chs3::LEU2* strain. The double mutant, however, grew very slowly, giving rise to large aggregates and was temperature sensitive (our unpublished results). Even at 30°C, the cells showed a wide neck and many cells lysed, which may explain the slow growth. Furthermore, null mutations of *Bni4p*, which is needed for binding of *Chs3p* to the septin ring (DeMarini *et al.*, 1997), and of *Chs4p*, which is required both for that binding (DeMarini *et al.*, 1997) and for enzymatic activity of *Chs3p* (Choi *et al.*, 1994a; Trilla *et al.*, 1997), clearly aggravated the morphological defect of a *cla4Δ* strain, especially when the double mutants were shifted from minimal medium to YEPD (our unpublished results).

STE20 Does Not Complement the Observed *cla4* Defect

CLA4 and *STE20*, which encodes another kinase of the PAK type (Leberer *et al.*, 1992) have been found to be synthetically lethal (Cvrcková *et al.*, 1995), therefore it has been postulated that they have some function(s) in common. To explore whether any such function was involved in the defects observed in the *cla4* mutants, we deleted *STE20* in the strain used for mutagenesis (ECY101[pEC28]). The resulting strain showed normal sectoring and wild-type morphology (Figure 1E). Moreover, when *STE20* was overexpressed in a

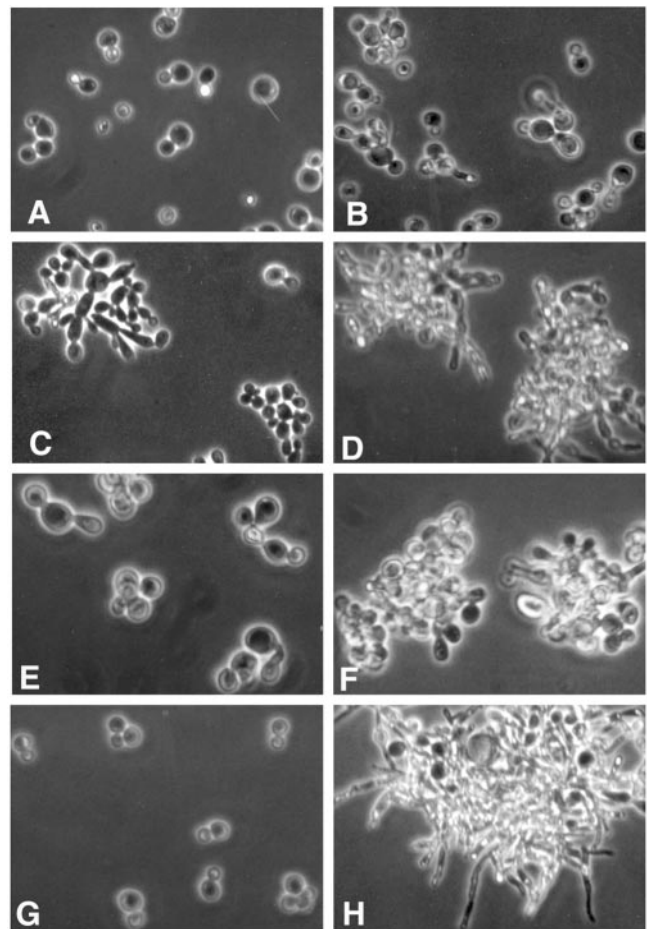


Figure 2. Morphology of the *cdc11-25* mutant isolated in the screen for synthetic lethality with *chs3-1* and of related strains. (A and B) AVY2-25 (*cdc11-25*) at 26°C in minimal and YEPD medium, respectively. (C and D) AVY2-25 at 30°C in minimal and YEPD medium, respectively. (E and F) AVY5 (*cdc11-25 chs3-1*) at 26°C in minimal and YEPD medium, respectively. (G) AVY2-25 transformed with pAV13 (YE351CDC11) at 30°C in minimal medium. (H) AVY3 (*cdc11-25 cla4::LEU2*) at 26°C in minimal medium.

cla4Δ strain by placing it under the control of the *GAL1* promoter, the morphological defects of the strain were unchanged (Figure 1F). We conclude that the defects observed in the *cla4* mutants reveal function(s) of Cla4p that are not shared with Ste20p.

CDC11 Shows Synthetic Lethality with *CHS3*

A screen similar to that outlined above but done exactly as described by Bender and Pringle (1991) was carried out. In this case, the original strain, AVY2, contained both *ade2* and *ade3* deletions, as well as a *chs3-1* mutation and a plasmid carrying *CHS3* and *ADE3*. With this approach, the colonies that cannot lose the plasmid are red and those that can lose it show white sectors. After mutagenesis, a red colony was isolated that showed cell clumps and elongated cells when the culture was grown at 30°C (Figure 2, C and D). The

morphology was closer to that of wild-type at 26°C (Figure 2, A and B).

After transformation with a genomic library, one plasmid was found to complement the defect. The insert was subcloned and a plasmid containing *CDC11*, which codes for a septin, as the only ORF was able to complement both the morphological and the sectoring defect of strain AVY2-25 (Figure 2G). Crosses with *cla4Δ* and *chs3Δ* strains showed that the mutant does not harbor a mutation in *CLA4* and that the mutation is recessive. Sequencing located the *cdc11-25* mutation at position 32, changing a GGA triplet codon for glycine to GAA for glutamic acid.

As in the case of the *cla4* mutant, it was possible to obtain a *cdc11-25 chs3-1* double mutant (see MATERIALS AND METHODS). The double mutant, although viable, showed a more aberrant morphology than *cdc11-25* both at 26 and 30°C in minimal medium (Figure 2, E and F) and did not grow in YEPD at 30°C. We also disrupted *CLA4* in the *cdc11-25* mutant, which resulted in a much more abnormal morphology (Figure 2H).

Both *cla4* and *cdc11* Mutants Show Septin Defects That are Exacerbated by a *chs3* Mutation

Because both a *cla4* strain and a septin mutant, *cdc11-25*, showed synthetic lethality in a red-white screen and because some septin defects were previously observed in *cla4* strains (Cvrcková *et al.*, 1995; Longtine *et al.*, 2000), we decided to examine in detail the septin organization and localization in both *cla4Δ* and *cdc11-25* strains. Septins fused to GFP were used for visualization of the septin rings. A *cla4::TRP1* mutant showed many more aberrant rings than wild-type as well as some mislocalized patches (Figure 3B and Table 3). A manifestation of the *cla4* phenotype is the formation of elongated buds (see above and Cvrcková *et al.*, 1995; Longtine *et al.*, 2000). To find out whether this modality of growth had given rise to the septin defects, we introduced in a *cla4Δ* strain a *swe1* deletion that eliminates the elongation (Longtine *et al.*, 2000). The double mutant, although clumpy, did not show elongated buds (Figure 3E), as expected, but the septin defect was similar to that of a single *cla4Δ* mutant (Figure 3C and Table 3). Although a *swe1* deletion suppresses lethality of a double *cla4 ncs1* mutant (Mitchell and Sprague, 2001) a *cla4 swe1* double mutant, either in the ECY101-39 background (YMS189) or in the YPH499 background (YMS190), still showed synthetic lethality with *chs3*, as shown by lack of sectoring (see also below).

We wanted to determine the effect of a Chs3p defect on the septin organization of *cla4Δ* mutants. Because the double mutant *cla4Δ chs3Δ* grows very poorly, an alternative procedure to abolish Chs3p activity in a *cla4* strain was used, by adding to the cultures nikkomycin Z, a specific inhibitor of chitin synthase III (Choi *et al.*, 1994b; Gaughran *et al.*, 1994). To observe septin rings during budding, when they are formed, we isolated single, unbudded cells by centrifugation on sucrose gradients and incubated them in the absence or presence of the inhibitor. Addition of nikkomycin Z to wild-type already caused the appearance of abnormal rings (Table 4) in a proportion similar to that found in a random culture of a *chs3Δ* mutant (Table 3 and Figure 3D). In a *cla4* null mutant, nikkomycin Z greatly increased the proportion of aberrant rings and mislocalized patches (Table 4). Finally, a *cla4Δ swe1Δ* double mutant showed more aberrant rings

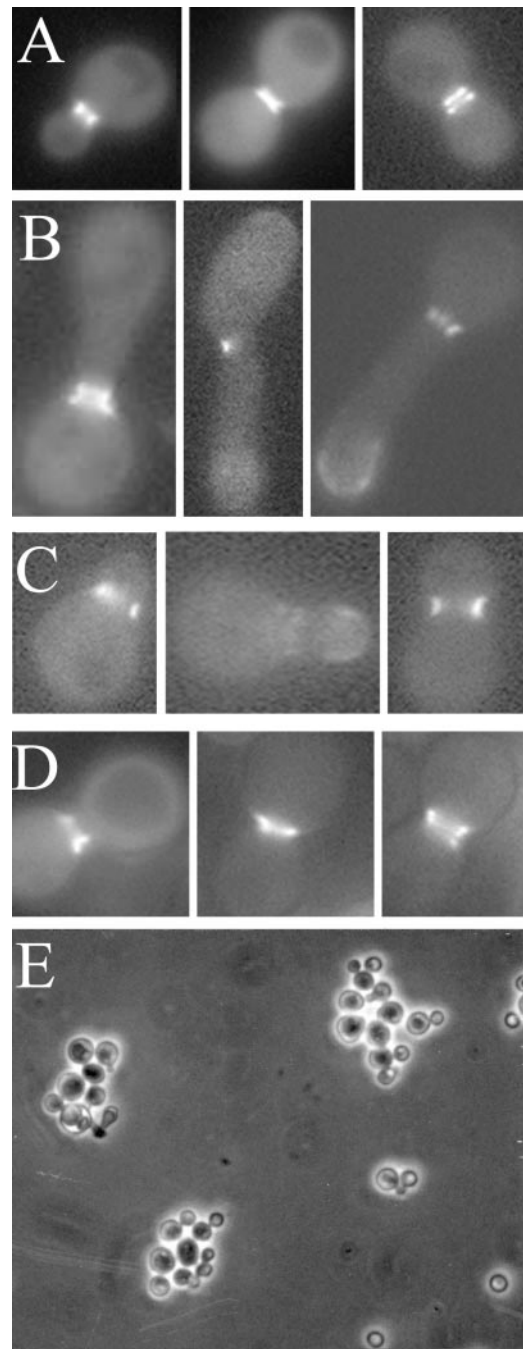


Figure 3. Abnormalities in septin ring organization in *cla4Δ*, *cla4Δ swe1Δ* and *chs3Δ* mutants. All four strains shown were transformed with plasmid pMS76 (pRS313CDC12-GFP). (A) Wild-type (YPH499). (B) *cla4Δ* (YMS306). (C) *cla4Δ swe1Δ* (YMS332). (D) *chs3Δ* (ECY46-4-1B). (E) Morphology of *cla4Δ swe1Δ* double mutant. Introduction of the *swe1* mutation eliminated bud elongation (compare with Figure 1, B or D), although not cell aggregation of a *cla4* strain.

than the single *cla4Δ* mutant, and nikkomycin addition resulted in many more mislocalized patches (Table 4), thus confirming the above-mentioned sectoring results. The sep-

Table 3. Septin localization in different strains

Strain ^a	Genotype	Normal rings (%) ^b	Abnormal rings (%) ^b	Mislocalized patches (%) ^b	Total rings and patches analyzed
YPH499	Wild type	84	16	0	43
YMS134	<i>cla4Δ</i>	39	50	11	62
ECY46-4-1B	<i>chs3Δ</i>	55	43	2	56
YMS332	<i>cla4Δ swe1Δ</i>	32	44	24	72

^a Strains were transformed with pRS316CDC3-GFP.

^b Percentages are relative to the total number of rings and patches observed. For criteria in scoring of abnormal rings, see MATERIALS AND METHODS.

tin abnormalities produced by the addition of nikkomycin Z, although more numerous, were similar in aspect to those shown in Figure 3.

Because the localization of Myo1p, a component of the actomyosin contractile ring, depends on septins (Lippincott and Li, 1998a), we examined the distribution of a Myo1p-GFP construct in a *cla4Δ* mutant (strain YMS134[pMS55]). The Myo1-GFP rings in the mutant were often somewhat blurred and almost twice the diameter of the wild-type rings (Figure 4, A and B) a finding consistent with a septin ring defect.

Finally, because of the separation defect in *cla4* strains, which results in clumping (Figure 1B), mutant cells were observed by electron microscopy (Figure 5). Some septa were essentially normal, with a typical trilaminar structure (Shaw *et al.*, 1991; Schmidt *et al.*, 2002). Many others, however, had an aberrant structure, with frequent lacunae and multiple intersecting chitin layers (Figure 5, C and D). Multiple septa were also observed in some cases (Figure 5B). A few septa were completely delocalized (Figure 5A), like those found in septin mutant at a nonpermissive temperature (Slater *et al.*, 1985; Roh *et al.*, 2002b), again pointing to a septin defect.

Not unexpectedly, the *cdc11-25* strain showed abnormal and delocalized septin rings when grown at 30°C, a defect that was aggravated by growth in rich medium (Table 5).

The defect was further exacerbated by the presence of a *chs3-1* mutation (Figure 6 and Table 5). In accordance with the high proportion of mislocalized septins in the mutant, ectopic septa were seen at high frequency by electron microscopy (Figure 7, A and C). The septa with normal localization had structures resembling those shown in Figure 5 of the *cla4Δ* septa (Figure 7A), including the presence of multiple septa (Figure 7B).

A Simultaneous Defect in Chitin Synthase III and Septins Causes Enlargement of the Mother-Bud Neck

Why is the defect of the *cla4* or the *cdc11-25* mutant much aggravated by a *CHS3* deletion? One way to look into this problem is to ask another question, i.e., whether the mere presence of the Chs3 protein is sufficient to prevent the defect seen in the double mutant or whether the enzymatic activity of the protein is also required. This question arises because Chs3p is known to be connected to a septin, Cdc10p, through Chs4p and Bni4p (DeMarini *et al.*, 1997). This linkage is necessary for the appropriate localization of Chs3p, but it might reciprocally help to retain the septins in their location. We approached this question in two ways. One was to express, in a *cla4Δ chs3Δ* strain or in a *cdc11-25 chs3-1* strain, a *CHS3* gene containing a single mutation in the putative active site, *chs3^{R993A}*. The mutation causes loss of

Table 4. Changes in septin localization in different strains upon inhibition of Chs3p with nikkomycin Z^a

Strain ^b	Genotype	Nikkomycin Z ^c	Normal rings (%)	Abnormal rings (%)	Mislocalized patches (%)	Total rings and patches analyzed
YPH499	wt	–	93	7	0	56
		+	55	45	0	53
YMS134	<i>cla4Δ</i>	–	60	32	8	53
		+	4	69	27	134
YMS322	<i>cla4Δ swe1Δ</i>	–	32	58	8	52
		+	0	46	54	60

^a Single, unbudded cells were isolated in sucrose gradients as indicated in MATERIALS AND METHODS. Cells were incubated in SC medium lacking uracil, until most cells had well-developed buds (2.5–6 h, depending on the strain). The septin distribution was determined by fluorescence microscopy as outlined in MATERIALS AND METHODS. Percentages are referred to the total number of rings and patches observed.

^b All strains were transformed with pRS316-CDC3-GFP.

^c When added, the final concentration of nikkomycin Z was 1 mM.

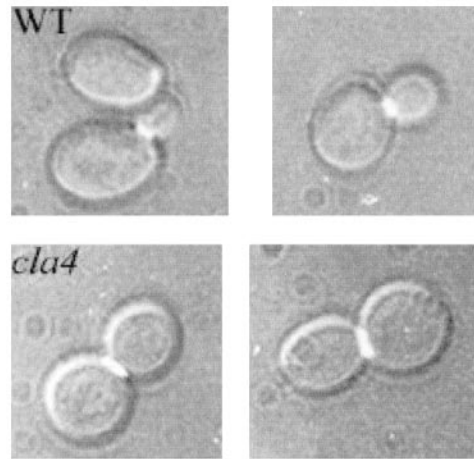
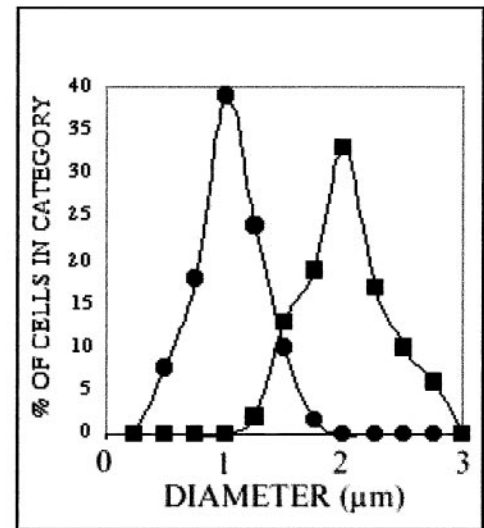


Figure 4. *cla4Δ* mutants have wider Myo1p-GFP rings than wild-type. Wild-type is YPH499[pMS55]; the *cla4Δ* strain is YMS134[pMS55]. At left, fluorescence images superimposed to Nomarski images. Panel at right, statistical measurements of ring diameter: circles, wild-type; squares, *cla4* mutant.



Chs3p activity in vitro and of function in vivo (Cos *et al.*, 1998). If only the presence of the protein were sufficient, expression of this protein in a plasmid should suppress the defect of the double mutants. However, the neck widening,

clumping, and lysis of the original *cla4Δ chs3Δ* or the *cdc11-25 chs3-1* strain were not affected by the presence of *chs3^{R993A}*, either on a centromeric (pHV7-37) or a high-copy (pMS75) plasmid (unpublished results). Western blots of extracts from cells containing a *chs3* null mutation and a high-copy plasmid expressing HA-fusions with wild-type or mutated *CHS3* (see MATERIALS AND METHODS) showed similar levels of both Chs3 proteins (unpublished results). Furthermore, the wild-type *HA-CHS3* conferred Calcofluor White sensitivity to the *chs3-1* strain, but the mutated gene did not (unpublished results).

The other experiment consisted in inhibiting the activity of chitin synthase III in vivo with the competitive inhibitor nikkomycin Z, as described above. In wild-type, nikkomycin Z does not affect growth, although it largely abolishes Calcofluor staining, because the chitin that requires Chs3p for synthesis is not made (see also Figure 8B). In these experiments, we again started from single, unbudded cells. When *cla4Δ* cells were incubated without inhibitor, they budded in a fairly normal way, with an occasional elongated bud, as seen in random culture (Figure 8A). The formation of the chitin ring at the mother cell-bud neck and hence the localization of Chs3p appeared also to be normal in these cells, as detected with Calcofluor White (Figure 8B). In the presence of nikkomycin Z, however, the buds became extremely long and the neck between mother cell and bud widened, so that many cell pairs looked like long tubes, with little constriction between the two cells (Figure 8, A and B). Staining with Calcofluor White showed that nikkomycin Z prevented deposition of chitin at the neck both in wild-type and in the *cla4Δ* strain, although some diffuse fluorescence of unknown origin sometimes remained in the mutant (Figure 8B). The rapid inhibition of chitin ring formation upon the addition of nikkomycin Z before budding strongly supports the notion that the compound acts as an inhibitor of chitin synthase III in vivo, rather than interfering with expression of Chs3p. Furthermore, in previous work we observed that turning off the *CHS3* gene did not result in a decrease of enzyme activity for many hours (Choi *et al.*, 1994b). Accordingly, we found that turning off *CHS3* (under a *MET3* pro-

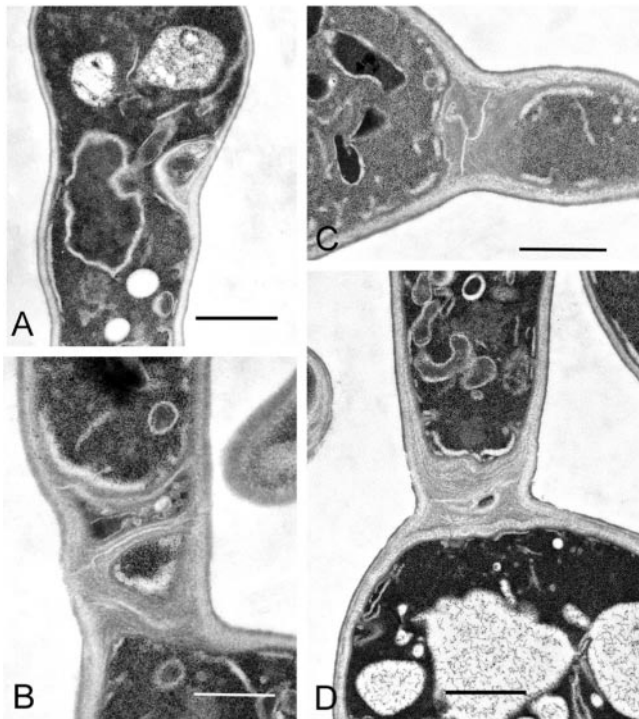


Figure 5. Electron microscopy of abnormal and delocalized septa in a *cla4Δ* mutant (strain YMS134). In A, a delocalized septum is shown (cf. Slater *et al.*, 1985; Roh *et al.*, 2002b). In B, the multiple septa may have resulted from repeated “landings” of septins unable to bind well to their normal locations. Note in C and D the meandering chitin lines and, in D, a lacuna. Bars, 1 μm.

Table 5. Septin localization in *cdc11-25* strains

Strain	Temp (°C)	Medium	Normal rings (%) ^a	Abnormal rings (%) ^a	Mislocalized patches (%) ^a	Total rings and patches observed
AVY2-25[pLP17] ^b	26	Minimal	89	8	3	63
	30	Minimal	62	32	6	82
	26	YEPD	81	13	6	126
	30	YEPD	14	43	43	103
AVY5 ^c -[pRS316-CDC3-GFP]	26	Minimal	53	41	6	80
	30	Minimal	32	44	24	72
	26	YEPD	0	32	68	28

^a Percentages are relative to the total number of rings and patches observed. For criteria in scoring of abnormal rings, see MATERIALS AND METHODS.

^b Strain containing *cdc11-25* and *CDC12-GFP* in plasmid.

^c Strain containing *cdc11-25* and *chs3-1*. This strain does not grow in YEPD at 30°C.

moter) in a *cla4Δ* strain required overnight incubation to detect changes in septins or morphology (our unpublished results).

Neck diameters were measured in the Calcofluor-stained cells as photographed with fluorescence filters, because the cell contour was easier to see under those conditions. After 4 h of incubation at 30°C, the average diameter of the necks of *cla4Δ* in the presence of nikkomycin Z was almost double of that in the control (Figure 8B and Table 6). No such increase was seen in a wild-type strain (Figure 8B and Table 6). The possibility that in the *cla4Δ* strain the necks formed in the presence of nikkomycin Z were already wide at bud emergence was explored by measuring neck diameters of very small buds. The results showed only a small increase in nikkomycin Z-treated cells, ascribable to enlargement after budding (Table 6). This clearly indicates that the neck widens during bud growth. The *cla4Δ* cells incubated in the presence of the inhibitor appeared to bud only once, although branches sometimes appeared on the long tubes. Again, the results were not changed appreciably by the presence of a *swe1* deletion in addition to the *cla4* deletion

(Table 6), except that the buds formed in the presence of nikkomycin Z were less elongated in this case.

Chitin synthase III is responsible for the formation of the chitin ring that is laid down at the onset of budding. If the effect of nikkomycin Z is due only to inhibition of ring formation, the results should not be changed by withdrawal of the inhibitor after the time for ring deposition has elapsed. This was indeed the case. In one experiment, cells were incubated with nikkomycin Z until small buds appeared in most of them, then centrifuged, washed, and suspended in fresh medium. On further incubation the cells showed the same morphological changes as those that were constantly incubated with nikkomycin Z (unpublished results).

The results with the *cdc11-25* strain were similar to those with the *cla4Δ* mutant (Figure 8B), although the widening of the neck was less pronounced. The diameter increased ~50% in the presence of nikkomycin Z (Figure 8B and Table 6). Similar results were obtained with another septin mutant, *cdc3-1* (Figure 8B and Table 6).

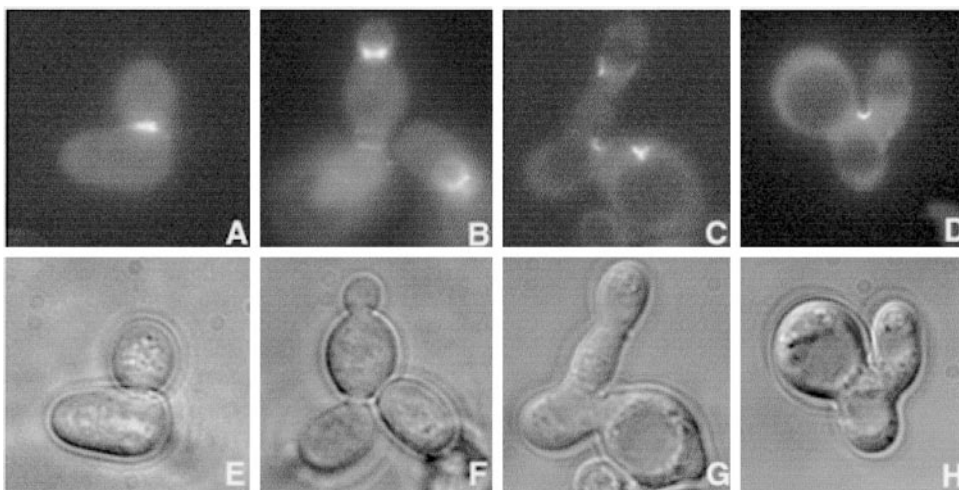


Figure 6. Septin defects in *cdc11-25* strains. Strains were grown in minimal medium at 30°C. A–D, fluorescence images; E–H, Nomarski images. (A, B, E, and F) Strain AVY2-25[pLP17] (*cdc11-25*); (C, D, G, and H) strain AVY5[pRS316-CDC3-GFP] (*cdc11-25 chs3-1*).

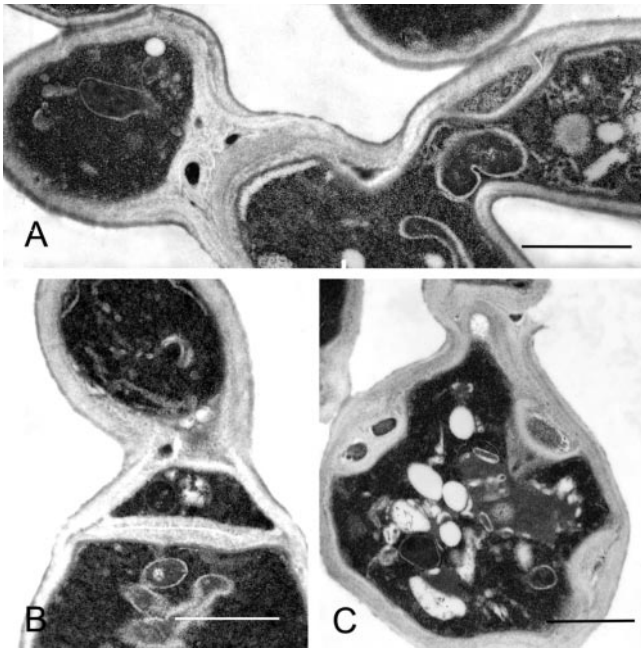


Figure 7. Electron microscopy of abnormal and delocalized septa in *cdc11-25*. In A and C, delocalized septa are shown. See also in A and B, septa with lacunae. In B, the double septum resembles the triple one in Figure 5B. Bars, 1 μ m.

DISCUSSION

cla4 and *cdc11* Mutants Show Similar Septin Abnormalities

Two genes, *CLA4* and *CDC11*, were isolated in our screen for synthetic lethality with *CHS3*. The appearance of *CDC11*, coding for a septin, in the screen was not unexpected, because a similar finding was briefly mentioned by Osmond *et al.* (1999). On the other hand, the isolation of *CLA4* was surprising. Cla4p is a protein kinase of the PAK type (Cvrcková *et al.* 1995). *cla4* mutants have elongated buds and some cytokinesis defect (Cvrcková *et al.*, 1995; Holly and Blumer, 1999; Longtine *et al.*, 2000; Weiss *et al.*, 2000), but the mechanism of action of Cla4p, an effector of Cdc42p (Benton *et al.*, 1997) has not been determined. Cla4p appears to be functionally redundant with another kinase, Ste20p, based on the finding that *CLA4* and *STE20* are synthetically lethal (Cvrcková *et al.*, 1995). The function described here, however, is not shared with Ste20p, because deletion of *STE20* did not result in the same defect and overexpression of Ste20p did not correct the morphology of the *cla4* mutant. In previous work Cla4p was usually studied in a *ste20* background; therefore, the specific functions of Cla4p and those shared with Ste20p were observed together (see, e.g., Holly and Blumer, 1999; Weiss *et al.*, 2000).

The finding that both *CLA4* and *CDC11*, which codes for a septin, show synthetic lethality with *CHS3* suggested that Cla4p may be involved in septin function. Cvrcková *et al.* (1995) and Longtine *et al.* (2000) observed abnormalities in septin organization in a *cla4* mutant. We confirmed and extended their results (Figure 3, Tables 3 and 4). Both *cla4* and *cdc11-25* mutants showed frequent and very similar

abnormalities in septa as well as delocalized septal structures (Figures 5 and 7). The aberrant actomyosin contractile rings found in the *cla4* Δ strain may result from the septin defect, because Myo1p localization depends on septins (Lippincott and Li, 1998a). In turn, the mislocalization of the contractile ring may lead to the abnormal septa as a result of an irregular binding to the plasma membrane, resulting in erratic invagination and abnormal deposition of primary septum chitin (Roh *et al.*, 2002b; Schmidt *et al.*, 2002). As for the ectopic septa, we previously found them in *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants and concluded that they resulted from attachment of defective septin rings to aberrant sites, where they served as scaffolds for an improperly placed septum (Slater *et al.*, 1985; Roh *et al.*, 2002b). From all the above we conclude that Cla4p is necessary for normal septin function, either for attachment of the septin filaments to the plasma membrane, or for organization of the filaments, or both. An attractive hypothesis is that Cla4p, directly or indirectly, causes a modification of some protein in the plasma membrane at the bud neck region, which enables it to bind the septin filaments and/or to organize the septin ring. This would provide an explanation for the more aberrant phenotype of *cla4* Δ *cdc11-25* double mutants compared with the single mutants, because in the double mutant both the septins and their receptor would be defective.

Septins and the Chitin Ring Cooperate in Maintaining the Mother-Bud Neck Size

Our results implied an involvement of Cla4p in septin function but did not explain the puzzling interaction between Cla4p or Cdc11p on one side and Chs3p on the other. Because septins retain Chs3p at the correct localization through a Bni4p-Chs4p bridge, it seemed possible that Chs3p, a plasma membrane protein, might exert a reciprocal action and tether the septin ring to the membrane. If so, mere presence of Chs3p, even in an enzymatically inactive form, could be sufficient to stabilize the septin ring. However, either a centromeric or a high-copy plasmid carrying a mutated form of *CHS3* was unable to suppress the defect of a *cla4* Δ *chs3* Δ mutant, although the protein was expressed at normal levels. The Chs3 protein used had a single point mutation, resulting in the change of one of three adjacent arginine residues into alanine in the putative catalytic site of the protein, which eliminates the enzymatic activity (Cos *et al.*, 1998). Therefore, little change would be expected in charge, general conformation, and ability to bind other proteins. On the other hand, inhibition of chitin synthase III activity by nikkomycin Z, a competitive inhibitor that is supposed to act exclusively by displacing the substrate (the structural analog UDP-GlcNAc), not only aggravated the septin abnormalities, but also the morphological defect of a *cla4* mutant. The unbudded cells used in this experiment budded only once and gave rise to very elongated buds, with a much widened neck at the junction with the mother cell (Figure 8B and Table 6). A similar widening of the neck as well as cell lysis were observed in a *cla4* Δ *chs3* Δ double mutant (see RESULTS), although this strain was able to grow slowly, possibly because of some suppressor. Neck widening, albeit less pronounced, was detected in septin mutants, *cdc11-25* and *cdc3-1*, upon treatment with nikkomycin Z (Figure 8B and Table 6). Thus, under conditions in which attachment to the membrane and/or organization of

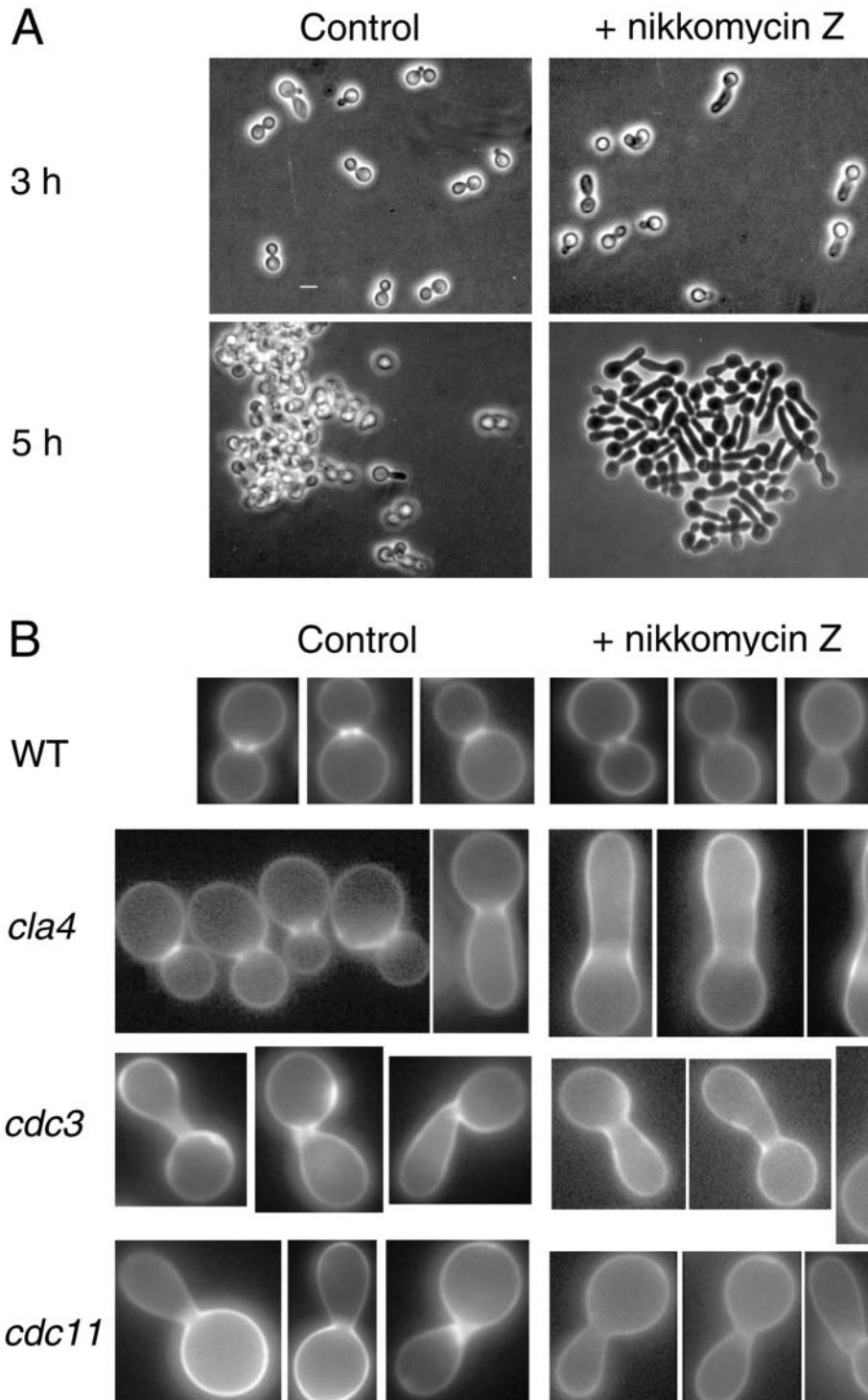


Figure 8. Effect of nikkomycin Z on morphology of wild-type (YPH499) and a *cla4Δ* mutant (YMS306). The cultures were started with single unbudded cells (see MATERIALS AND METHODS) in synthetic complete medium with or without 1 mM nikkomycin Z and incubated at 30°C. (A) Phase contrast images of the cultures of YMS306 at different times. Note in the control a few elongated cells at 5 h. Bar, 10 μm. (B) Fluorescence images of cells of wild-type and different mutants after 4-h incubation without or with nikkomycin Z, followed by staining with Calcofluor White. Note in wild-type cells the absence of a fluorescent chitin ring, when incubated in the presence of nikkomycin Z. Some residual and largely delocalized fluorescence in the *cla4Δ* mutant under the same conditions remains unexplained.

the septins is compromised by a *cla4* or a septin mutation, a defect in Chs3p enzymatic activity results in abnormal growth and expansion of the neck region, which normally is unchanged throughout the cell cycle. This expansion ulti-

mately leads to cell death, which explains the synthetic lethality between *cla4* or *cdc11* and *chs3*.

How does the chitin ring, which requires Chs3p for its synthesis, contribute to prevent growth in the neck region?

Table 6. Effect of nikkomycin Z on mother-bud neck diameter in different strains^a

Strain	Genotype	Incubation time (h)	Temp (°C)	Neck diameter (μm)	
				Control	+Nikkomycin Z
YPH499	wild type	4	30	1.1 ± 0.1 (28)	1.16 ± 0.25 (22)
YMS306 ^b	<i>cla4Δ</i>	2	30	1.35 ± 0.14 (49)	1.65 ± 0.2 (53)
YMS306	<i>cla4Δ</i>	4.5	30	1.4 ± 0.24 (64)	2.6 ± 0.3 (68)
YMS332	<i>cla4Δswe1Δ</i>	6.5	30	0.97 ± 0.25 (41)	2.6 ± 0.45 (32)
AVY2-25	<i>cdc11-25</i>	6.5	26	1.1 ± 0.1 (42)	1.5 ± 0.18 (36)
AVY2-25	<i>cdc11-25</i>	4	30	1.14 ± 0.15 (32)	1.7 ± 0.2 (38)
DHY103-9B	<i>cdc3-1</i>	4	26	0.96 ± 0.16 (36)	1.3 ± 0.2 (33)
DHY103-9B	<i>cdc3-1</i>	4	30	1.1 ± 0.15 (51)	1.6 ± 0.2 (55)
DHY103-9B	<i>cdc3-1</i>	5.5	30	1.16 ± 0.18 (61)	1.9 ± 0.4 (50)

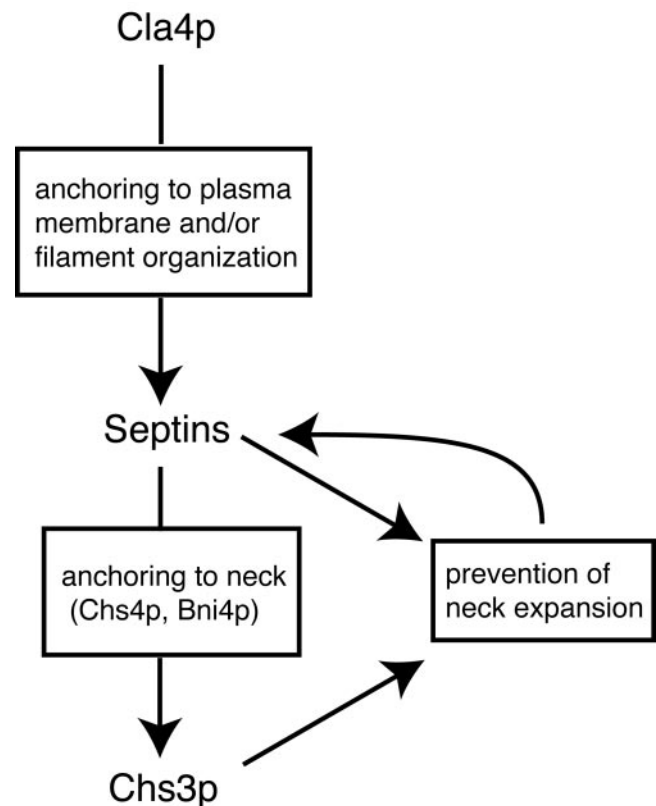
^a Data are given as averages plus/minus the standard deviation, which indicates the variability of the neck diameter. Values in parentheses indicate the number of necks measured. The final concentration of nikkomycin Z, when added, was 1 mM. The difference between the values with or without nikkomycin Z, as measured by the z score, was at a significance level of <0.001 in all cases, except for the wild-type strain.

^b Only the neck diameters of small buds were measured here.

A reasonable explanation is suggested by what we know about cell wall structure and the order of assembly of cell wall components. As previously reported (Kollár *et al.*, 1995, 1997), chitin is bound to the cell wall structure in two types of linkages: to side chains of $\beta(1\rightarrow6)$ glucan and to the non-reducing end of $\beta(1\rightarrow3)$ glucan. The latter position is the same where, in other chains, $\beta(1\rightarrow6)$ glucan is attached to $\beta(1\rightarrow3)$ glucan. Mannoproteins, in turn, are linked to $\beta(1\rightarrow6)$ glucan. The order of addition *in vivo* is $\beta(1\rightarrow3)$ glucan, $\beta(1\rightarrow6)$ glucan, mannoprotein (Roh *et al.*, 2002a). Because of its high concentration in the neck region, chitin may cap most of the $\beta(1\rightarrow3)$ glucan nonreducing ends, making them unavailable for addition of $\beta(1\rightarrow6)$ glucan, hence of mannoprotein. Thus, cell wall growth and consequent neck expansion would be prevented in that area.

Our conclusions are summarized in Scheme 1. Cla4p has a role in septin localization and/or assembly. This scheme does not postulate a mechanism for Cla4p action and it would be valid even if Cla4p acted through Cdc42p, as recently postulated by Gladfelter *et al.* (2002), so long as the ultimate targets are the septins. Septins, in turn, determine the localization of the chitin synthase III system, which is necessary for the formation of a chitin ring at the neck, at bud emergence (Shaw *et al.*, 1991). The chitin ring prevents growth at the neck, probably also blocked by the presence of the septins, which appear to act as a barrier between mother cell and bud, impeding the movement of proteins on the plasma membrane (Gladfelter *et al.*, 2001). When either the septins or the chitin synthase system are functional, relatively little change is seen at the neck. When both are defective, growth in that region is not controlled and the neck enlarges. Thus, in the same way that septins and Chs3p normally cooperate with each other, a defect in one aggravates that of the other. A septin defect leads to partial delocalization of chitin synthesis, whereas a Chs3p defect causes enlargement of the neck with reduced binding of the already abnormal septins.

These results show that two stabilizing systems, the septins and the chitin ring, are required to ensure the constancy of diameter and structure of the bud neck. When both fail,



Scheme 1. Proposed effect of Cla4p and Chs3p on septin anchoring and organization. Cla4p helps anchoring of the septin ring at the neck. The septin ring, in turn, localizes Chs3p at the neck through binding of Bni4p and Chs4p as intermediaries (DeMarini *et al.*, 1997). Both septins and the chitin ring, dependent on Chs3p activity, cooperate in preventing growth at the neck. When either the septins or Chs3p are defective, not much change in neck growth is noted. If both are faulty, however, neck growth becomes unrestrained, which contributes further to septin disorganization (curved arrow).

the neck enlarges and the cell dies, an outcome that underlines the importance of neck integrity.

It is interesting to note that, although the existence of the chitin ring has been known for 30 years (Hayashibe and Katohda, 1973), its function remained unknown until now. Retrospectively, this is understandable, because a chitin ring defect has relatively little consequence (Shaw *et al.*, 1991) in the absence of a concomitant septin abnormality.

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