Developmental Cell, Vol. 4, 345–357, March, 2003, Copyright ©2003 by Cell Press

Phosphorylation-Dependent Regulation of Septin Dynamics during the Cell Cycle

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Summary

Septins are GTPases involved in cytokinesis. In yeast, they form a ring at the cleavage site. Using FRAP, we show that septins are mobile within the ring at bud emergence and telophase and are immobile during S, G2, and M phases. Immobilization of the septins is dependent on both Cla4, a PAK-like kinase, and Gin4, a septin-dependent kinase that can phosphorylate the septin Shs1/Sep7. Induction of septin ring dynamics in telophase is triggered by the translocation of Rts1, a kinetochore-associated regulatory subunit of PP2A phosphatase, to the bud neck and correlates with Rts1-dependent dephosphorylation of Shs1. In *rts1*-Δ cells, the actomyosin ring contracts properly but cytokinesis fails. Together our results implicate septins in a late step of cytokinesis and indicate that proper regulation of septin dynamics, possibly through the control of their phosphorylation state, is required for the completion of cytokinesis.

Introduction

In order to give rise to two viable progenies, cytokinesis must be both spatially and temporally coordinated with the partition of sister chromatids. Thus, the spindle must generate signals to "inform" the cleavage apparatus about both its location and functional status. These signals and how they translate into proper assembly and timely activation of the cleavage machinery are poorly understood.

The cleavage apparatus is organized and functions similarly in fungi and animal cells (Bi, 2001; Field et al., 1999). In these organisms, the cytokinetic machinery assembles at the cell cortex and is composed of at least two major elements: septins and an actomyosin contractile ring. The structure and function of the actomyosin ring is best understood. Its contraction during late anaphase triggers furrowing of the cell and finishes with the formation of the midbody. Breakage of this last bridge (abscission) by a mechanism that remains to be elucidated completes cytokinesis. In budding yeast, the type II myosin Myo1 is required for the formation and the contraction of the actomyosin ring at the end of mitosis (Bi et al., 1998; Lippincott and Li, 1998a).

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Although septins and Myo1 arrive early at the bud neck, assembly of the actual cleavage machinery is tightly coordinated with exit of mitosis. First, the septin ring splits into two adjacent rings and the actomyosin ring assembles between them (Bi et al., 1998; Lippincott et al., 2001). Subsequently, the actomyosin ring contracts and septation ensues. Activation of the mitotic exit network (MEN pathway) upon segregation of the sister chromatids triggers both septin ring splitting and actomyosin ring contraction (Bardin and Amon, 2001; Lippincott et al., 2001; Luca et al., 2001; Menssen et al., 2001). However, the MEN pathway is not required for actomyosin ring formation (Lippincott et al., 2001; Luca et al., 2001; Menssen et al., 2001), suggesting that independent signals trigger the assembly of the cleavage apparatus. After full contraction, the actomyosin ring disassembles, most probably without ensuring final closure of the neck, like in animal cells. How this last bridge is closed in yeast is not known.

Septins are GTPases that assemble into filaments in vitro and probably in vivo (Field and Kellogg, 1999; Longtine et al., 1996). They are conserved from yeast to humans and colocalize with the cleavage apparatus in all organisms in which they have been identified (reviewed in Faty et al., 2002; Field et al., 1999; Field and Kellogg, 1999; Longtine et al., 1996). However, their cytokinetic role is poorly understood. In *Saccharomyces cerevisiae*, the septins Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7 assemble into a ring at the bud neck and are essential for cytokinesis (Carroll et al., 1998; Longtine et al., 1996). One function of the septin ring in cytokinesis is to recruit Myo1 and Hof1, a protein involved in septation, to the site of cleavage (Bi, 2001; Bi et al., 1998; Lippincott and Li, 1998a, 1998b). In addition, the yeast septin ring also forms a diffusion barrier in the plane of the plasma membrane during G2 (Barral et al., 2000). Thereby, it also plays an important role in the control of cell polarity and bud growth. Furthermore, septins are involved in the control of secretion and cortical organization in higher eukaryotes. However, little is known about how septins act in both cytokinesis and cell polarity.

Here, we describe the dynamics of the septin ring in yeast cells using FRAP (Fluorescence Recovery After Photobleaching) and a novel septin dissociation assay. We show that the ring oscillates between two states. Short dynamic periods, characterized by rapid subunit turnover ("fluid"), alternate with an immobile state ("frozen"), during which septins do not move within the ring. The frozen state predominates during bud growth and telophase. Fluidity is observed at bud emergence and at the onset of cytokinesis. In contrast, cells lacking Cla4 or Gin4, a PAK-related and a septin-dependent kinase, respectively (Carroll et al., 1998; Cvrckova et al., 1995), show septin ring fluidity also during bud growth. Activation of septin dynamics at cytokinesis involves PP2A and its B′ regulatory subunit, Rts1 (Shu et al., 1997; Zhao et al., 1997). We show that the septin Shs1, a substrate of the Cla4/Gin4 pathway (Mortensen et al., 2002), is involved in the control of septin ring dynamics and that it is dephosphorylated in a Rts1-dependent

manner at the onset of cytokinesis. Rts1 localizes to kinetochores in S and G2 and translocates to the bud neck at telophase. Translocation of Rts1 depends on mitotic exit and is required for the activation of septin dynamics. Thus, Rts1 links septin dynamics to proper exit of mitosis.

We also show that Rts1 is needed for efficient cytokinesis. However, it is not involved in either the formation or the contraction of the actomyosin ring, suggesting that it acts in a later process. Together, these results suggest that phosphorylation-dependent regulation of septin dynamics is required for the proper completion of a late step in cytokinesis, possibly abscission.

Results

Septin Dynamics Vary during the Cell Cycle

To examine septin dynamics, FRAP experiments were performed on strains expressing a GFP-tagged septin. Dynamics of four of the five yeast septins, Cdc3, Cdc10, Cdc12, and Shs1/Sep7, were investigated. Each septin was tagged individually at its N terminus with GFP and expressed under the control of its own promoter. Half of the fluorescently labeled septin ring was irradiated with a laser beam to irreversibly bleach GFP. Fluorescence was then monitored over time. Fluorescence recovery in the bleached area indicated recruitment of nonbleached molecules. Loss of fluorescence in the unbleached region indicated exchange of fluorescent molecules with some that are bleached. This analysis was carried out with cells representative of the different stages of cell division (Figure 1) and produced similar results with the four reporters used.

Unbudded cells with a newly assembled septin ring (late G1, early S; Figure 1A) showed rapid septin turnover. GFP-fluorescence recovered with a half-time of 175 $\pm$ 110 s (N = 7 cells). Loss of fluorescence was observed concomitantly in the nonbleached area until fluorescence levels equilibrated (Figure 1A). When the entire ring was bleached, no recovery was observed (data not shown), suggesting that there was little or no exchange of material with the cytoplasm. Thus, prior to bud emergence septins moved within the ring.

In contrast to unbudded cells, rings of small- and medium-budded cells never recovered fluorescence after photobleaching (N = 10 cells; Figure 1B). Little or no fluorescence loss was observed in the unbleached area. Thus, rapidly after bud emergence septin molecules were immobilized (same results were obtained by L. Kozubowski and K. Tatchell; K. Tatchell, personal communication).

The splitting of the septin ring marks the onset of cytokinesis. All cells undergoing ring splitting during observation showed a sudden and rapid recovery of fluorescence in the irradiated area (Figure 1C; $t_{1/2}$ = 117 $\pm$ 56 s, N = 6). Recovery was preceded by fluorescence loss in the unbleached area. Subsequently, both rings were homogeneously labeled, indicating that again subunits exchanged between bleached and unbleached areas. FRAP experiments on already split rings indicated that they had regained a frozen state (N = 7 cells; Figure 1D). Shortly prior to cell separation, the two rings were again dynamic (N = 6, Figure 1E). Thus, the septin ring is remarkably immobile during most of the cell cycle. Only three short dynamic phases are observed: prior to bud emergence, at the onset of cytokinesis, and at cell separation. Therefore, septin dynamics are regulated during the division cycle.

Septin Subunit Dissociation Is Prominent at Cytokinesis

To investigate septin dynamics further, we sought to follow variations in the rates of subunit association and dissociation. Our assay was based on the observation that the *cdc12-6* septin allele leads to septin ring disassembly upon shift to the restrictive temperature (Barral et al., 2000). We rationalized that changes in the rate of disassembly at specific cell cycle stages would reflect changes in septin dissociation/association rates. *GFP-CDC3 cdc12-6* cells were shifted to 30°C, the lowest restrictive temperature, and the intensity of the GFP signal at the bud neck was monitored over time (Figures 2A and 2C).

Cells with a single ring showed a linear decrease of the intensity of the ring (N = 27 cells), which disappeared within 50 min (Figures 2A and 2B). The rate of disassembly did not vary with the size of the bud, suggesting that the balance between septin association and dissociation did not vary during bud growth. In wild-type cells expressing GFP-Cdc3, unsplit rings were fully stable over the entire experiment (75 min; Figures 2A and 2B).

In *cdc12-6* cells undergoing ring splitting, the GFP-Cdc3 signal dropped suddenly and disappeared within 15 $\pm$ 2.5 min (N = 24 cells; Figures 2C and 2D). A loss of signal was also observed concomitantly with the onset of cytokinesis in wild-type. However, in these cells the fluorescence intensity of the separated rings stabilized rapidly (less than 5 min) to about 50% of the original level. These and the FRAP results consistently indicate that septin dynamics are transiently enhanced at cytokinesis.

Loss of PP2A^{Rts1} Suppresses *cdc12-6* Growth Defect at 30°C

To identify factors controlling septin dynamics, we screened for second site mutations that suppress *cdc12-6* at 30°C. Our rationale was that inactivation of genes that facilitate or activate septin dissociation might sufficiently stabilize the ring to allow growth of *cdc12-6* cells at this temperature. Thus, *cdc12-6* cells were mutagenized with EMS and plated at 30°C. At least five complementation groups were identified. This report focuses on *STS13* (suppressor of *t*welve-*s*ix), which is represented by at least two temperature sensitive alleles (see *rts1-13* in Figure 3A). The strongest allele, *sts13-1*, was used for further studies.

The temperature sensitivity of *sts13-1* strictly cosegregated with the suppression of *cdc12-6* (>80 tetrads analyzed), indicating that the two phenotypes were probably due to the same mutation. Thus, we cloned *STS13* by complementation of the temperature sensitive phenotype. Out of three plasmids that restored growth of *sts13-1* at 37°C, all contained the *RTS1* gene. In two cases, *RTS1* was the only open reading frame in the insert. Moreover, *RTS1* and *STS13* were tightly linked (no disjunction in more than 30 tetrads), and disruption of *RTS1* suppressed the growth defect of the *cdc12-6* strain at 30°C (Figure 3A). Thus, we concluded that the

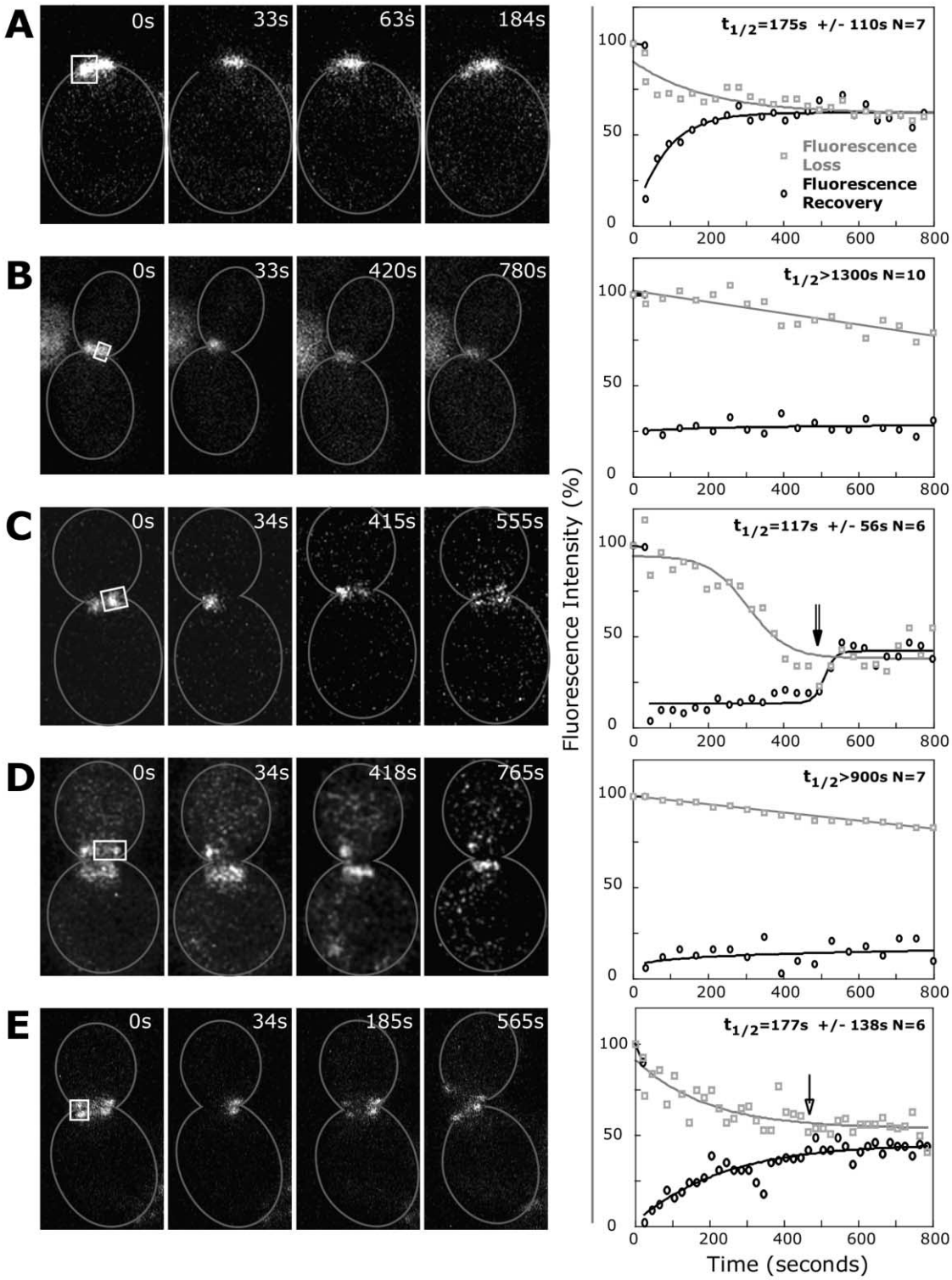

Figure 1. Septin Dynamics during the Cell Cycle

GFP-labeled septin rings were subjected to FRAP. The small box in the first frame of each movie indicates the bleached region. The kinetics and average half time of recovery are shown for each cell stage. No difference was observed whether we used GFP-Cdc12, GFP-Cdc3 YFP-Cdc10, or GFP-Shs1 as a reporter protein.

(A) Unbudded cell.

(B) Budded cell with a single ring.

(C) Large-budded cell undergoing ring splitting. The double arrow indicates the first frame where splitting is visible.

(D) Large-budded cells with a split ring.

(E) Separating cells. The arrow shows the first frame were the two daughter cells move away from each other.

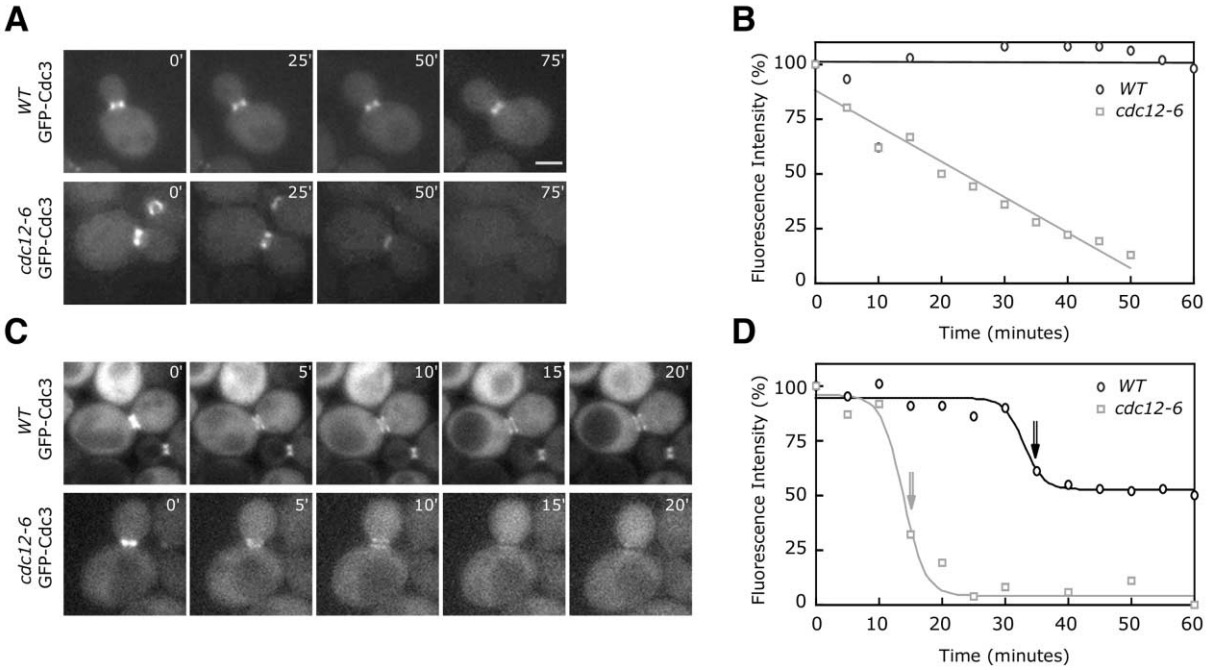

Figure 2. Analysis of Septin Ring Stability in *cdc12-6* Cells in G2 and at Cytokinesis

(A) Cells with a medium bud. Wild-type (upper row) and *cdc12-6* (lower row) cells expressing GFP-Cdc3 grown at 22°C and shifted to 30°C were monitored by time-lapse microscopy.

(B) Quantification of the GFP-fluorescence intensity of the septin ring in G2 cells (*WT*, N = 21; *cdc12-6*, N = 27).

(C) Cells in cytokinesis, undergoing ring splitting.

(D) Quantification of fluorescence intensity at the bud neck during ring splitting (*WT*, N = 16; *cdc12-6*, N = 24). The first image on which splitting is observed is indicated by an arrow in each case. All images are at the same magnification.

Scale bar in (A) represents 2 μm.

sts13 mutations are novel alleles of the *RTS1* gene. The *sts13-1* mutation was renamed *rts1-13*.

Rts1 is one of the two alternate regulatory subunits of protein phosphatase 2A (PP2A) in yeast (Shu et al., 1997; Zhao et al., 1997). These subunits control the subcellular distribution of PP2A catalytic subunits, Pph21 and Pph22 (Gentry and Hallberg, 2002). Thus, we asked whether loss of PP2A activity also suppressed the growth defect of the *cdc12-6* strain at 30°C. The strains *cdc12-6 pph21*-Δ and *cdc12-6 pph22*-Δ were constructed and tested for growth at 30°C. All *pph21*-Δ *cdc12-6* segregants formed colonies at 30°C, indicating that *pph21*-Δ suppressed the *cdc12-6* mutation (Figure 3A). Suppression of *cdc12-6* by *pph22*-Δ was less penetrant. Nevertheless, the observation that dosage reduction of PP2A catalytic subunits suppresses the *cdc12-6* growth defect is consistent with $PP2A^{Rts1}$ regulating septin dynamics or organization.

Suppression of septin defects by *rts1*-Δ depended on the septin allele. Unlike *cdc12-6*, the *cdc12-1* mutation affects new septin ring formation at the end of G1 but not the stability of previously existing rings (Y. Barral, unpublished data). None of our *rts1* alleles could suppress the growth defects due to the *cdc12-1* mutation (Figure 3A). Therefore, $PP2A^{Rts1}$ may specifically regulate septin ring stability.

Cells Lacking RTS1 Fail to Properly Regulate Septin Dynamics Specifically at Cytokinesis

We next investigated the effect of *RTS1* deletion on septin organization and function. *RTS1* and *rts1*-Δ cells expressing GFP-Cdc12 were shifted to 37°C and septin rings were monitored microscopically for several hours (Figure 3B). A GFP-Nop1 reporter was used to stage the cells in the cell cycle. GFP-Nop1 labels the nucleolus, which allows visualization of nuclear division.

In small- and medium-budded cells, unsplit septin rings were morphologically indistinguishable between mutant and wild-type cells (Figure 3B). Also ring splitting occurred normally in *rts1*-Δ cells. However, split rings were misshapen in these cells. They also failed to disassemble properly upon emergence of new buds. However, newly assembled rings were normal, indicating that Rts1 is not involved in septin ring formation. Thus, Rts1 is required to maintain proper septin organization during cytokinesis, and to allow proper ring dissociation in G1.

Next, we used our dissociation assay to determine which aspect of septin dynamics was affected by the *rts1*-Δ mutation. Prior to ring splitting, the rate of septin ring disappearance was not affected in the *cdc12-6 rts1*-Δ double mutant compared to *cdc12-6* (Figure 3C). However, the *rts1*-Δ mutation clearly stabilized split rings. Moreover, FRAP experiments also failed to reveal a reactivation of septin dynamics at telophase in *rts1*-Δ (data not shown). Altogether, our results indicate that Rts1 activates septin dynamics specifically at cytokinesis.

Rts1 Accumulates at the Bud Neck upon Exit of Mitosis

We next characterized the localization of Rts1 during the cell cycle. As already described (Gentry and Hallberg,

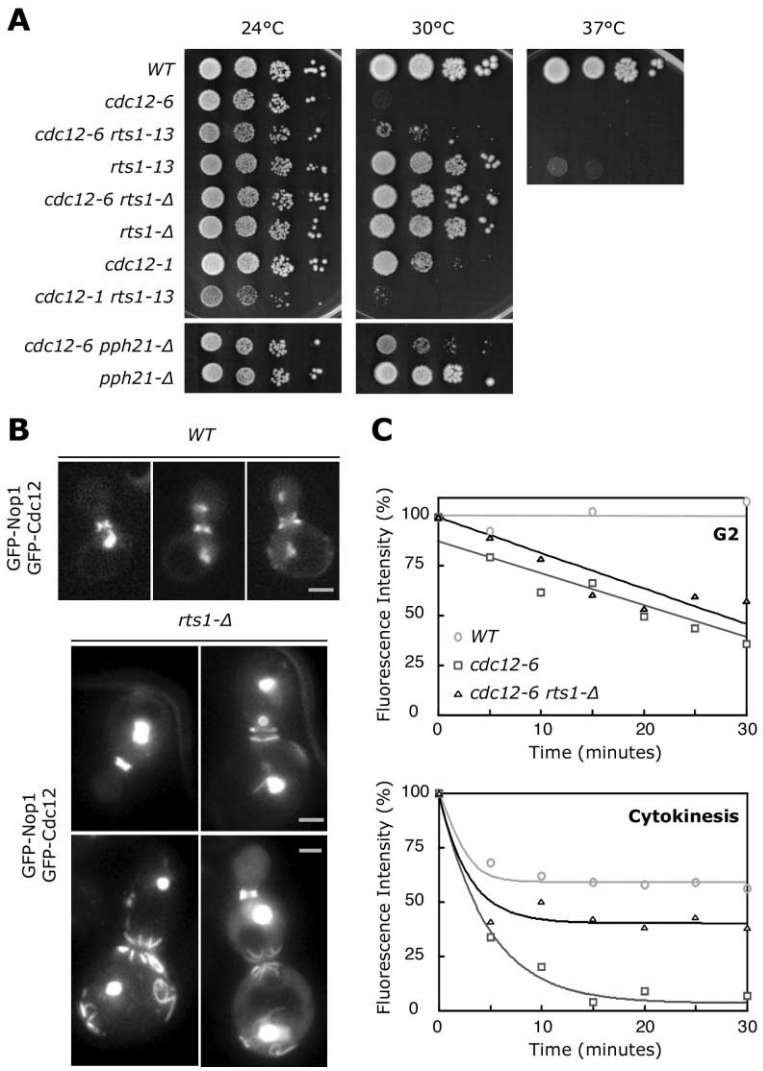

Figure 3. Rts1 Is Involved in Septin Dynamics at Cytokinesis

(A) Disruption or mutation of the *RTS1* gene leads to suppression of the *cdc12-6* growth defect at 30°C. Cells with the indicated genotype were spotted on plates after serial dilution and incubated for 4 days at the indicated temperature.

(B) Septin organization in wild-type and *rts1*-Δ cells. Cells expressing GFP-Cdc12 and GFP-Nop1, as a nuclear marker, were grown at 25°C and shifted to 37°C for 3 hr. Scale bars are 2 μm.

(C) The stability of the septin ring in cells of the indicated genotype was determined as in Figure 2 for cells in G2 and at cytokinesis (*rts1*-Δ *cdc12-6* G2, N = 12; cytokinesis, N = 4).

2002), the fully functional Rts1-GFP fusion protein localized to different places during the cell cycle (Figure 4A). In unbudded cells, it predominantly accumulated in the nucleus (Figure 4A, a). This diffuse nuclear staining was observed throughout the cell cycle. Upon bud emergence, Rts1 was enriched at kinetochores (Gentry and Hallberg, 2002; see Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/4/3/345/DC1]), which was manifested by the formation of an intense, intranuclear dot (Figure 4A, b) that occasionally separated into two distinct spots (Figure 4A, c). This dot(s) faded away in pre-anaphase cells and only diffuse nuclear staining was observed during anaphase (Figure 4A, d). In about 75% of the cells with divided nuclei, Rts1 was also seen at the bud neck (Figures 4A, e, f, g, 4B, and 4C). Thus, Rts1 appeared at the bud neck some time during mitosis. These results are consistent with the report that PP2A catalytic subunits localize to the bud neck in a Rts1-dependent manner during mitosis (Gentry and Hallberg, 2002).

To clarify the timing of Rts1 localization to the bud neck, we analyzed microtubule organization in cells with Rts1-GFP at the bud neck (Figure 4B). Only 3% (2/65) of these cells still contained a spindle. By contrast, cells with an elongated spindle (late anaphase) rarely showed Rts1 staining at the bud neck (3%; 2/78). Similarly, faint Rts1 neck staining was observed in only a few large-budded cells with an unsplit septin ring (4%; 1/27). In these cells, Rts1 and septin staining overlapped extensively. In all other cells with Rts1 at the neck, ring splitting had already occurred (Figure 4C). Also, Rts1 was not observed at the bud neck of cells arrested in late anaphase by virtue of the *tem1-1*, *cdc15-1*, and *cdc14-1* mutations (data not shown). Thus, appearance of Rts1 at the bud neck took place after spindle breakdown and septin ring splitting and required activation of the MEN pathway. Therefore, Rts1 required mitotic exit to localize to the site of cleavage.

Rts1 Relocalization to the Bud Neck Is Required for Activation of Septin Dynamics

To test whether Rts1 acted directly on septin dynamics, we determined whether Rts1 translocation was required for proper septin organization and dynamics. The N-terminal 250 amino acids of Rts1 are conserved in all budding yeasts. Truncation of the first 216 amino acids leads to the Rts1-Δ3 protein, which is fully functional for its nuclear roles, such as stress response and growth on

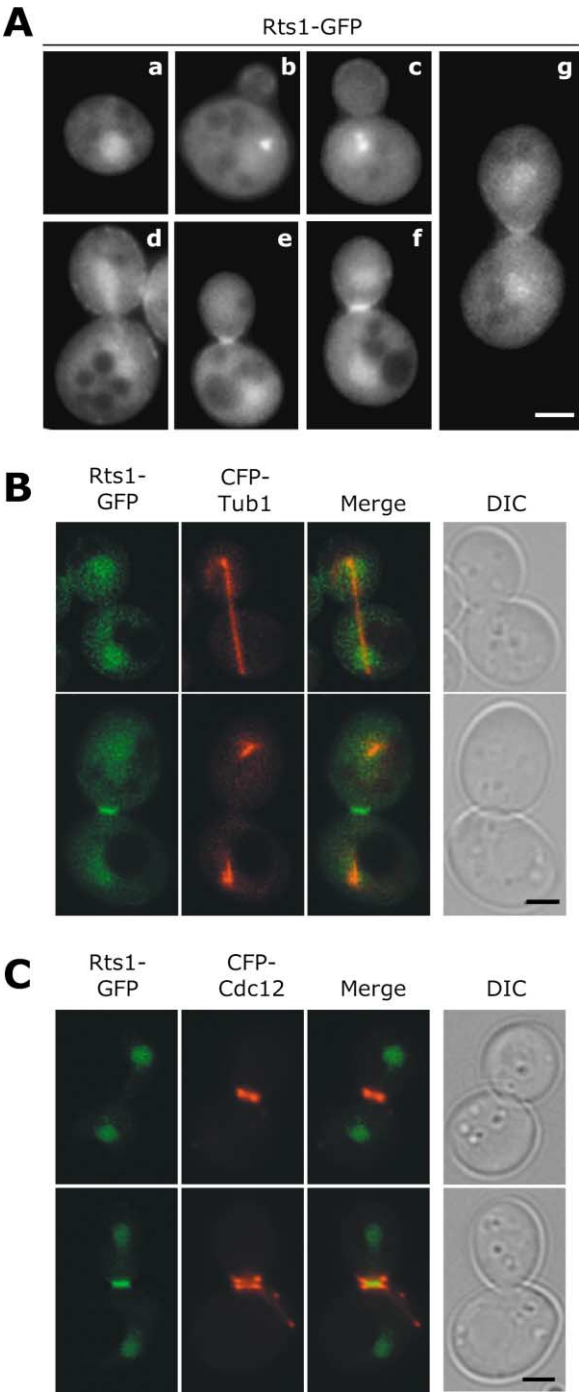

Figure 4. Rts1 Translocates to the Bud Neck upon Exit from Mitosis

(A) Cells expressing Rts1-GFP at endogenous level. Cells representative of the different stages of the cell cycle are shown.

(B) Localization of Rts1 and microtubules at the end of mitosis. Wild-type cells expressing CFP-Tub1 and Rts1-GFP.

(C) Localization of Rts1 and septins in late mitotic cells. Wild-type cells expressing CFP-Cdc12 and Rts1-GFP. Scale bars represent 2 μm.

glycerol medium (Shu et al., 1997). Rts1-Δ3 also localized to kinetochores properly (see Supplemental Figure S1D [http://www.developmentalcell.com/cgi/content/full/4/3/345/DC1]) and co-isolated with centromeric DNA in ChIP experiments (Figure 5A). However, Rts1-Δ3 failed to localize to the bud neck upon exit of mitosis at room temperature (Figure 5B). This defect was not due to a block in cell cycle progression, since *rts1-Δ3* proliferated normally at this temperature.

Thus, we asked whether Rts1-Δ3 was still able to stimulate septin dynamics. It was not the case. Although it was not as defective as in the *rts1-Δ* strain, septin organization was not restored in the *rts1-Δ3* cells shifted to 37°C (Figure 5D). In addition, the *cdc12-6 rts1-Δ3* strain still survived at 30°C (Figure 5C), while the *cdc12-6 RTS1* strain was unable to grow at this temperature. Thus Rts1-Δ3 was unable to replace wild-type Rts1 for its septin-related function. Therefore, our results are consistent with translocation of Rts1 to the bud neck being required to activate septin dynamics. Altogether, our data indicate that Rts1 links septin dynamics with exit of mitosis.

Cla4 and Gin4 Antagonize Rts1

Activation of septin dynamics by $PP2A^{Rts1}$ indicates that phosphorylation events control the rigidity of the septin ring. Three kinases have been shown to influence septin organization: Elm1, Cla4, and Gin4 (Bouquin et al., 2000; Cvrckova et al., 1995; Longtine et al., 1998). Two additional kinases, Hsl1 and Kcc4, also localize to the bud neck (Barral et al., 1999). If one of them acts to stabilize the septin ring, disruption of the corresponding gene should be synthetic lethal with the *cdc12-6* allele. Tetrad analysis indicated that neither *elm1-Δ*, *kcc4-Δ*, nor *hsl1-Δ* exacerbates the *cdc12-6* phenotype. Only *cla4-Δ* and *gin4-Δ* were synthetically lethal with *cdc12*-6, suggesting that Cla4 and Gin4 specifically regulate septin dynamics.

Therefore, FRAP experiments were performed in *gin4-Δ* and *cla4-K594A* cells expressing GFP-Cdc12 to characterize the effect of these mutations on septin dynamics. The *cla4-K594A* allele encodes a kinase inactive form of Cla4 (Tjandra et al., 1998). In the *cla4-K594A* cells, non-split septin rings were dynamic (Figure 6A) independently of the size of the bud. Recovery was also observed in the *gin4-Δ* strain (Figure 6B), but it was restricted to large budded cells. Thus, Cla4 and Gin4 are required to establish and/or maintain the frozen state of the ring prior to cytokinesis. Cla4 may act earlier than Gin4.

Epistasis analyses were performed to determine whether *RTS1* acted antagonistically to *CLA4* and *GIN4* genes. Since $PP2A^{Rts1}$ is thought to reverse a phosphorylation event, it should have no function left if the corresponding phosphate(s) is missing due to the lack of the kinase. Thus, *rts1-Δ* should not suppress septin phenotypes in a strain lacking such kinase(s). Supporting the idea that Rts1 may reverse Cla4 function, the *rts1-Δ* mutation was unable to suppress the lethality of the *cla4 cdc12-6* double mutant. Likewise, *cdc12-6 gin4-Δ rts1-Δ* cells were barely viable. In contrast, the *rts1-Δ* mutation still fully suppressed the *cdc12-6* phenotype in the *elm1-Δ* context. Altogether, these results are consistent with Rts1 counteracting Cla4 and Gin4.

The Septin Shs1 Is a Likely Substrate of Rts1

Shs1/Sep7 is phosphorylated in a Cla4- and Gin4-dependent manner in vivo (Mortensen et al., 2002), and

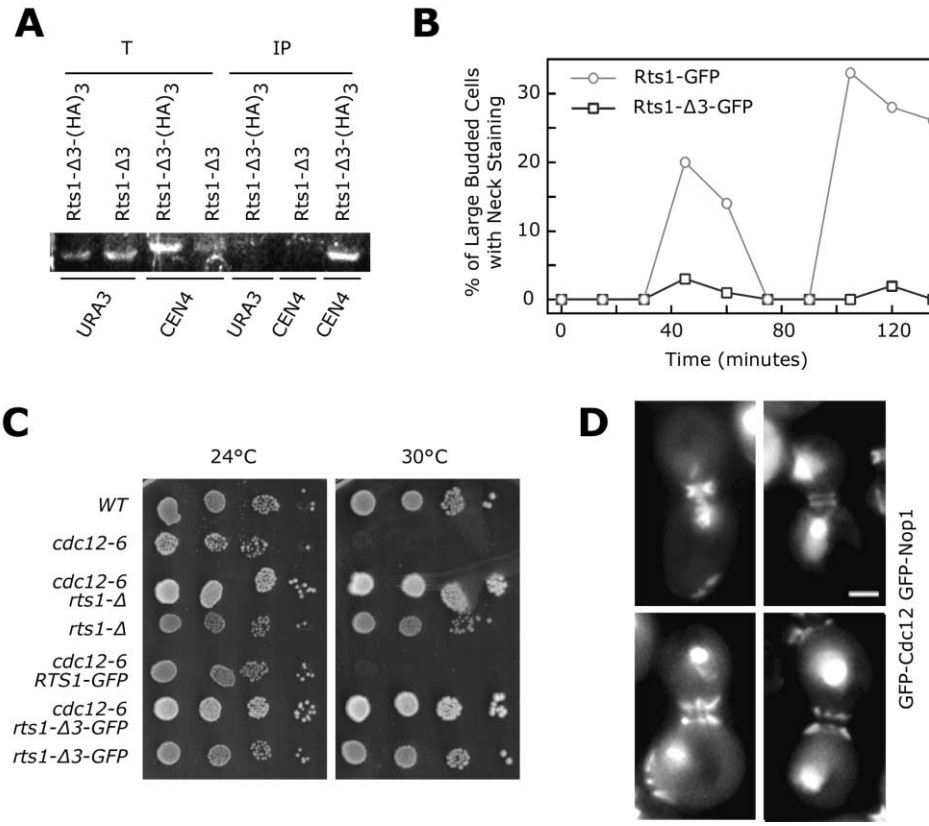

Figure 5. Rts1 Translocation to the Bud Neck Is Required for Proper Regulation of Septin Dynamics

(A) Rts1-Δ3 associates normally with kinetochores. Anti-HA ChIP assays were performed as in Supplemental Figure 2B [http://www.developmentalcell.com/cgi/content/full/4/3/345/DC1] on *rts1-Δ3-(HA)$_3$* and *rts1-Δ3* cells.

(B) Rts1-Δ3 does not relocate to the bud neck upon exit from mitosis. Rts1-GFP and Rts1-Δ3-GFP cells were arrested as in Figure 4B and released in fresh medium. The percentage of large budded cells with GFP at the bud neck is represented at regular time intervals after release.

(C) Rts1-Δ3 cannot replace Rts1 for the activation of septin dynamics. Cells carrying the *cdc12-6* allele and expressing the indicated forms of Rts1 were spotted and incubated as in Figure 3A. The strains were as follows: wt, (wt, *pLEU2*), *cdc12-6* (*cdc12-6 pLEU2*), *cdc12-6 rts1-Δ* (*cdc12-6 rts1-Δ pLEU2*), *rts1-Δ* (*rts1-Δ pLEU2*), *cdc12-6 RTS1-GFP* (*cdc12-6 rts1-Δ pLEU2-RTS1-GFP*), *cdc16-6 rts1-Δ3-GFP* (*cdc12-6 rts1-Δ pLEU2-rts1-Δ3-GFP*), and *rts1-Δ3-GFP* (*rts1-Δ pLEU2-rts1-Δ3-GFP*).

(D) Expression of Rts1-Δ3 cannot suppress the septin organization defects due to the *rts1-Δ* mutation. *rts1-Δ* cells expressing GFP-Cdc12, GFP-Nop1, and Rts1-Δ3 were grown at 22°C and shifted to 37°C for 3 hr. Scale bars are 2 μm.

it is a good target candidate for the Cla4/Gin4/Rts1 pathway. Thus, we asked whether Shs1 plays any special role in septin dynamics. As already reported, Shs1 was not required for cell viability and hence for septin ring formation (Carroll et al., 1998). Disruption of the *SHS1* gene also had no effect on the viability of the *cdc12-1* mutant. However, it was colethal with the *cdc12-6* allele (no viable double mutants in 21 tetrads). Furthermore, septin rings were more dynamic in the *SHS1* deleted strain than in wild-type (Figure 6C). Thus, Shs1 plays some specific role in septin dynamics.

Shs1 phosphorylation state during the cell cycle was also consistent with Shs1 being a substrate of Rts1. In synchronized cells, Shs1 became hypophosphorylated shortly after the cells exited mitosis and during bud emergence (Figure 6D). We were unable to analyze the phosphorylation state of Shs1 in *rts1-Δ* cells at 37°C because of difficulties in synchronizing this strain at this temperature. However, *rts1-Δ* cells failed to fully dephosphorylate Shs1 upon exit of mitosis at 30°C. At 30°C *rts1-Δ* cells have no strong phenotype. Another phosphatase may be able to partially substitute for PP2A^{Rts1} at this temperature. Altogether, our results indicate that the septin Shs1 is one of the targets of the Cla4/Gin4/Rts1 pathway and suggest that its phosphorylation state(s) regulate(s) the dynamic behavior of the septin ring.

Cells Lacking Rts1 Are Defective in a Late Stage of Cytokinesis

We next investigated the physiological relevance of the regulation of septin dynamics at cytokinesis. Therefore, the phenotype of *rts1-Δ* cells was analyzed in more detail. Interestingly, exponentially growing *rts1-Δ* cells shifted to 37°C for 3 hr accumulated into clusters of unseparated cells (Figure 7A). Cells also accumulated as large budded with a single nucleus (Shu et al., 1997), suggesting that they were also delayed for the entry into mitosis. These results were globally confirmed when we analyzed the progression of synchronized cells (Supplemental Figure S2C [http://www.developmentalcell.com/cgi/content/full/4/3/345/DC1]). The cell clusters were

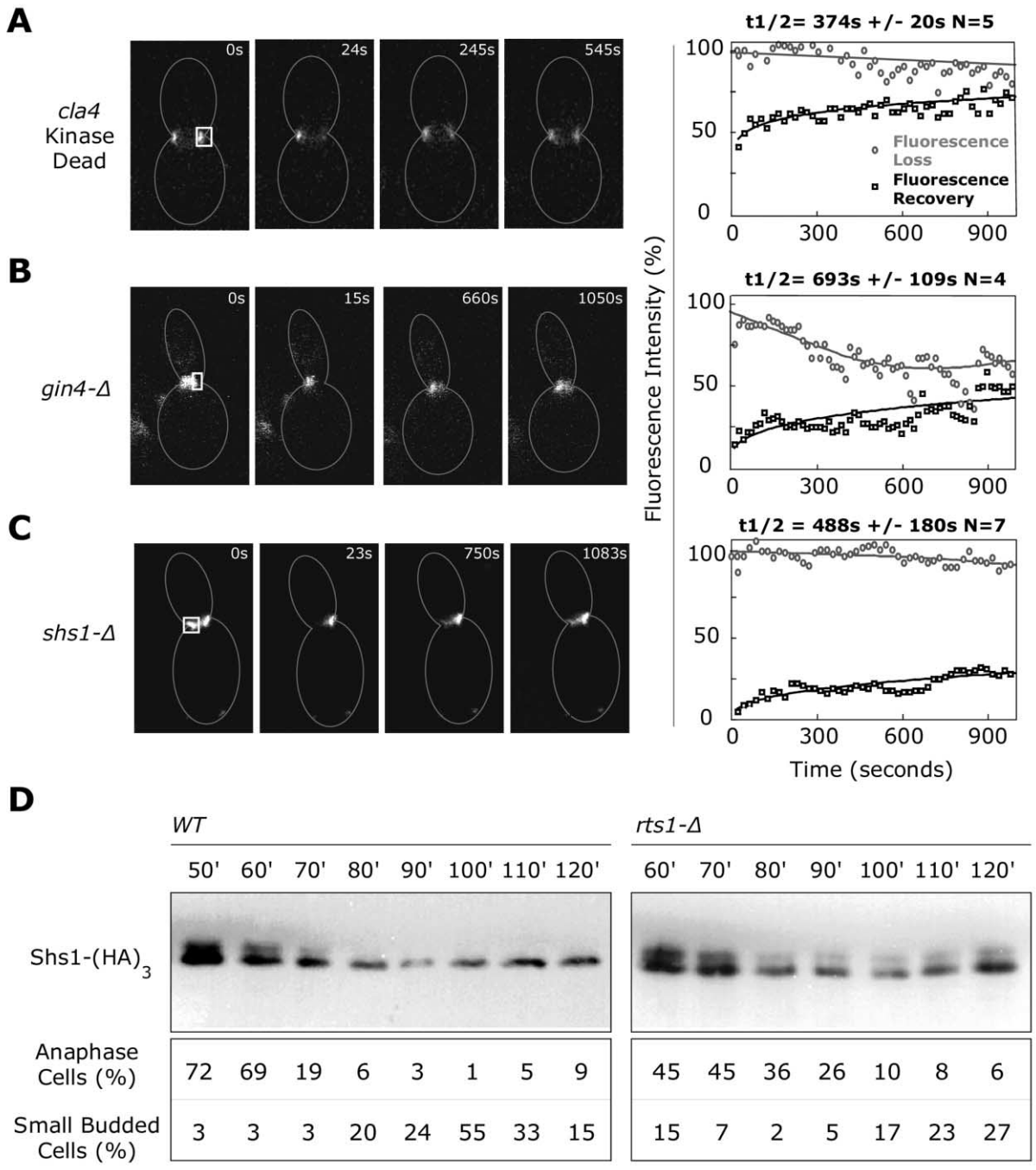

Figure 6. Cla4 and Gin4 Regulate Septin Dynamics Prior to Mitosis, Possibly in Part through Shs1 Phosphorylation

(A) *cla4K594A* (kinase dead).

(B) *gin4*-Δ.

(C) *shs1*-Δ. Septin rings were labeled with GFP-Cdc12 and FRAP was carried out as in Figure 1. Kinetics and average half recovery times are shown.

(D) Shs1 is dephosphorylated upon exit of mitosis. *RTS1*-deleted cells fail to dephosphorylate Shs1 properly. Cells of the indicated genotype and expressing Shs1-$(HA)_3$, where Shs1 is C-terminally tagged with a triple HA epitope, were arrested with HU at 24°C for 2 hr, shifted to 30°C for 1 hr, and released in fresh medium at 30°C. Samples were taken every 10 min and cell extracts were analyzed by Western using anti-HA antibodies. The proportion of anaphase and small-budded cells is indicated for each sample.

not resolved by treatment with zymolyase (Figure 7B), indicating that unseparated cells were still connected through their plasma membrane. In addition, tubulin staining indicated that *rts1*-Δ cells were not defective for exit of mitosis and spindle breakdown (Figure 7C). Therefore, the cluster phenotype was due to a failure in

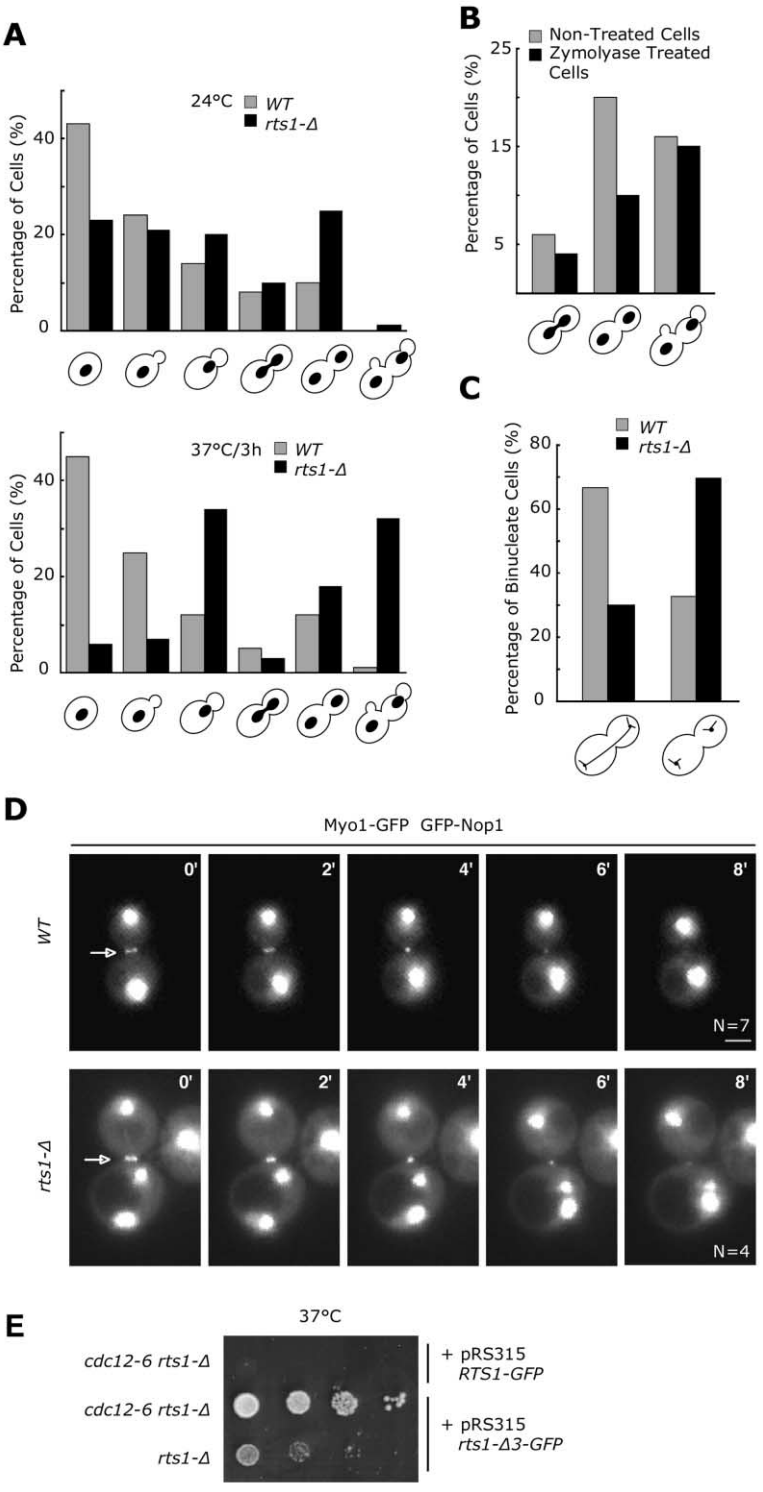

Figure 7. Cytokinetic Defects in *rts1*-Δ

(A) Disruption of *RTS1* leads to a cytokinetic defect at 37°C. Wild-type and *rts1*-Δ cells grown at 24°C were shifted to 37°C for 3 hr. The fraction of premitotic and postanaphase cells and of cells undergoing new bud emergence without proper separation is shown ($N > 600$).

(B) Zymolyase treatment of *rts1*-Δ cells. The cell wall of cells grown at 37°C for 3 hr was digested with zymolyase. The proportion of anaphase, binucleate cells, and clusters before and after zymolyase treatment are shown ($N > 300$).

(C) Percentage of large-budded cells with an elongated versus a broken spindle after shift to 37°C for 3 hr. Spindles of wild-type and *rts1*-Δ cells were visualized by immunofluorescence with an anti-tubulin antibody (YOL1/34, SeraLab). $N > 450$.

(D) Effect of *RTS1* disruption on actomyosin ring contraction. Arrows indicate the Myo1 rings (Myo1-GFP). $T = 0$ corresponds to the first frame at which the spindle reaches maximal elongation, as judged by nucleoli segregation (GFP-Nop1). Genotypes of the cell are indicated. The elapsed time is indicated in each frame (in min).

(E) Suppression of *rts1*-Δ*3* by *cdc12-6*.

Scale bar is 2 μm.

cytokinesis and not in either cell separation (primary septum degradation) or exit of mitosis.

The cytokinetic defect was not due to problems in actin ring organization. Actin rings could be observed in the *rts1*-Δ cells at roughly the same frequency (3/120) as in wild-type cells (2/135), even after 3h at 37°C. Analysis of actomyosin ring contraction in *MYO1-GFP GFP-NOP1* and *rts1*-Δ *MYO1-GFP GFP-NOP1* by time-lapse microscopy indicated that this process was also not affected at 37°C. In both strains, contraction rapidly followed the full separation of the GFP-Nop1 masses and took a similar amount of time to reach completion (Figure 7D). In addition, the *rts1*-Δ *hof1*-Δ double mutant was not sicker than either of the single mutants. Since *hof1*-Δ cells require Myo1 function to complete cytokinesis and remain viable (Vallen et al., 2000), this result indicates

that the Myo1 pathway is functional in *rts1*-Δ cells. Similar data also demonstrated that Rts1 did not affect the Hof1 pathway either. First, *myo1*-Δ *rts1*-Δ double mutants were not much sicker than either single mutant, indicating that the Hof1 pathway is functional in *rts1*-Δ cells. Second, Hof1 localized normally in *rts1*-Δ cells. Finally, *rts1*-Δ did not show any genetic interaction with *iqg1-1*, a mutation in the yeast IQGAP gene. Thus, Rts1 defines a novel step in cytokinesis that is distinct from and most likely subsequent to the stage involving Myo1 and Hof1. It may therefore be involved in the yeast equivalent to the abscission event described in animal cells.

Importantly, Rts1 acted in a septin-dependent process. This was indicated by the observation that the *cdc12-6 rts1-Δ3* cells grew better at 37°C than the strains carrying either of these mutations alone (Figure 7E). Thus, not only did *rts1-Δ3* suppress the growth defect due to *cdc12-6*, but the *cdc12-6* allele was also able to suppress the growth defect caused by *rts1-Δ3*. Suppression was clearly due to the *cdc12-6* allele since the *rts1*-Δ growth defect was restored upon introduction of the wild-type *CDC12* gene (data not shown). Therefore, our genetic analysis implicates Rts1 in a septin-specific pathway in cytokinesis. This is the first evidence that septins have Hof1- and Myo1-independent roles in cell division.

Discussion

The Septin Ring Oscillates between a Frozen and a Fluid State

We used FRAP analysis to characterize the dynamics of the yeast septin ring during the division cycle. Our study reveals that the septin ring is dynamic in late G1 and late M but not during the rest of the cycle. Septin dynamics were characterized by subunit exchange between the different parts of the ring. At least in late M phase, it also correlated with an increase in the rate of septin dissociation. Altogether, our results indicate that the septin ring exists in a dynamic ("fluid") and an immobile ("frozen") state. In the fluid state, subunits move rapidly inside the ring, possibly via the cytoplasm. In the frozen state, little or no subunit movement is observed. Transitions between these states are strictly regulated in time and take place at bud emergence and cytokinesis.

The observation that the septin ring spends most of its time in the frozen state suggests that this is its functional conformation. This is in contrast to tubulin- and actin-dependent structures, which need to be dynamic to be functional. Together with the findings that septins are able to form filaments in vitro and are part of the 10 nm neck filaments observed in vivo, our results are consistent with septins forming stable filaments in vivo. Furthermore, our FRAP results indicate that there is no substantial pool of free septin in the cytoplasm. Together, our data suggest that polymerization is a central aspect of septin function. In yeast the main role of the septin ring is to form a spatial landmark at the bud neck (Faty et al., 2002; Longtine et al., 1996). There, it acts as a scaffold for the recruitment of other neck components and as a diffusion barrier to prevent mixing of bud- and mother-specific membrane and membrane-associated factors. We propose that its ability to freeze is crucial for the maintenance of its position over an extended period of time. Probably, freezing is also crucial for the barrier and scaffold functions of the septin ring, although further studies will be required to clarify this point.

Role of Septin Dynamics during Cytokinesis

The fluid state of the ring may have several functions. It is likely that it is important to loosen the septin ring in late G1 for disassembly and reformation purposes. Our data indicate that activation of septin dynamics is also required at cytokinesis, although not for ring splitting. Thus, another function of septin dynamics may be to permit reorganization of the ring as the topology of the plasma membrane changes. Prior to bud emergence, the plasma membrane at the budding site has a low inward curvature. Upon bud emergence, the curvature of the membrane inverts to form the bud neck. Therefore, the septin ring, which is tightly associated with the plasma membrane, must change conformation upon bud emergence (Gladfelter et al., 2001). Closure of the neck at cytokinesis brings the plasma membrane back to its topology prior to bud emergence. In consequence, the split rings must reshape as well. In support of this idea, three-dimensional reconstruction of the septin ring by deconvolution microscopy (Lippincott et al., 2001) is consistent with the septin ring adopting an hourglass shape during bud growth and a disk-shape upon splitting. Failure to reshape may lead the ring to break as the plasma membrane changes curvature. This last possibility is in fact what we observe in cells lacking Rts1. Thus, we propose that one function of septin dynamics is to adapt the septin ring morphology to changes in plasma membrane topology.

The current model for the cytokinetic role of the septin ring is that it serves to recruit the cleavage apparatus (reviewed in Bi, 2001; Faty et al., 2002; Longtine et al., 1996). Rts1 function defines a novel septin-dependent step in cytokinesis. Indeed, the *rts1*-Δ mutation leads to a clear defect in cytokinesis. It does not interact, though, with *myo1*-Δ, *hof1*-Δ, or *iqg1-1*. In fact, the only genetic interaction observed between *rts1*-Δ and another cytokinesis mutant is the suppression of *rts1-Δ3* by *cdc12-6*. Therefore, the main function of Rts1 at cytokinesis appears to be the activation of septin dynamics. Furthermore, our data show that this event impinges on cytokinesis without affecting actomyosin ring contraction or the Hof1 pathway. Thus, septins may have additional roles in cytokinesis besides recruiting the cleavage apparatus. Since Rts1 cells have no defects in the Myo1 and Hof1 pathways, we suggest that septins are required for a later step in cytokinesis. One possibility is that upon splitting it serves as a double diffusion barrier in the plane of the membrane much like during bud growth (Barral et al., 2000). In this manner, the split rings could delineate a new plasma membrane compartment between them, dedicated to division. This compartment may then acquire specific competences for vesicle fusion and plasma membrane fission. In that sense, it would be essential for the vesicle fusion events probably required for the final closure of the bud neck after actomyosin ring contraction, i.e., for abscission. In *rts1*-Δ mutants, the discontinuous ring would no longer

maintain their barrier function, providing a possible explanation for the cytokinetic defect observed.

Coordination of Septin Dynamics with Cell Cycle Events

Here, we show that activation of septin dynamics at the onset of cytokinesis depends on PP2A and Rts1. Furthermore, translocation of Rts1 to the bud neck was required for normal activation of septin dynamics. PP2A catalytic subunits have been shown to relocalize to the bud neck in a septin and Rts1-dependent manner in late mitosis (Gentry and Hallberg, 2002). Thus, our results are consistent with $PP2A^{Rts1}$ inducing septin dynamics by dephosphorylating some factor(s) at the bud neck. Consequently, phosphorylation of the same factor(s) may be required to establish the frozen state. Cla4 and Gin4 clearly meet the criteria for being involved in the control of septin dynamics. Disruption of either of them was colethal with *cdc12-6*, both mutations were epistatic over *rts1-*Δ, and prevented proper freezing of the septin ring. One of the substrates of Gin4 is the septin Shs1/Sep7 (Mortensen et al., 2002). In summary, our and previous results are consistent with the following working model (Supplemental Figure S3 [http://www.developmentalcell.com/cgi/content/full/4/3/345/DC1]). We propose that septin phosphorylation regulates their dynamics. It would presumably do so by stabilizing interactions between septin complexes. Supporting this idea, genetic data suggest that Shs1 acts specifically in septin ring stabilization and not in septin ring formation. Moreover, the septin ring failed to properly freeze in cells lacking Shs1. However, it was by far not as fluid as during bud emergence or cytokinesis. Therefore, Shs1 is a relevant but not the only substrate of the Cla4/Gin4 pathway. Other septins, such as Cdc3 and Cdc11, are also phosphorylated in vivo (Ficarro et al., 2002; Tang and Reed, 2002), although their modification is not well characterized. Also, several mammalian septins are phosphorylated in vivo (Xue et al., 2000). Therefore, phosphorylation-dependent modulation of septin dynamics may be a general phenomenon.

Possibly, Cla4 first establishes ring freezing upon bud emergence. Subsequent recruitment and activation of Gin4 by septins (Carroll et al., 1998) would reinforce this signal and help to maintain the frozen state when Cdc42, and hence Cla4, are being shut down at the apical to isotropic growth transition. In the absence of Cla4, Gin4 may be able to reduce septin dynamics, although not as efficiently as in the presence of Cla4. Thus, this model would have the advantage to explain why *cla4* and *gin4* mutants have partial defects in septin dynamics and why the double mutant *cla4-*Δ *gin4-*Δ has a much stronger phenotype than either single mutant (Tjandra et al., 1998). It would also explain why *gin4-*Δ affected the dynamics of the ring only in large-budded cells. Activation of septin dynamics at the onset of cytokinesis depends on the relocalization of $PP2A^{Rts1}$ to the bud neck. We propose that Rts1 acts twice at cytokinesis: first to induce a transient increase in dynamics at ring splitting and second during ring disassembly. The first event is difficult to characterize at the biochemical level, presumably because of its transient nature. However, both microscopy and genetic data strongly argue for Rts1 acting already at this stage on septin dynamics. During cytokinesis, the rings were found to freeze again. Interestingly, Cla4 is found to relocalize briefly to the bud neck after ring splitting (M. Peter, personal communication). Hence, Cla4 may trigger refreezing of the ring during cytokinesis. Three observations argue for Rts1 acting in ring disassembly in early G1. First, the septin ring is found to be again dynamic upon cell separation, indicating that septin dephosphorylation occur again at the end of cytokinesis. Second, *rts1-*Δ cells showed defects in septin ring disassembly. Third, Shs1 dephosphorylation prior to bud emergence was at least in part dependent on Rts1 function. Thus, Rts1 may act to induce septin dynamics at both the onset and the completion of cytokinesis.

Finally, the translocation of Rts1 to the bud neck depends on the completion of mitosis and activation of the MEN pathway. Thus, Rts1 links septin dynamics to exit of mitosis. In this regard, it is interesting to notice that Rts1 localized to kinetochores very much like chromosomal passenger proteins in higher eukaryotes. Thus, it may serve to integrate septin dynamics with proper spindle function. Alternatively, the function of Rts1 at the kinetochore may be independent of its function at the bud neck. In order to distinguish between these two possibilities, further studies will have to establish the mechanism controlling Rts1 function.

Experimental Procedures

Strains, Plasmids, and Growth Conditions

All strains used were isogenic or congenic with S288c (YYB384). Yeast media and standard genetic techniques were performed as described (Guthrie and Fink, 1991). The original *rts1-13* mutation and all other mutations were purified before use by backcrossing three times with wild-type. Strains disrupted for *RTS1*, *PPH21 PPH22*, and *SHS1* were obtained from Euroscarf; the *gin4*Δ, *hsl1*Δ, *kcc4*Δ (Barral et al., 1999), *tem1-1, cdc15-1*, and *cdc14-1* strains are described. Septin-CFP, -YFP, and -GFP (*GFP-CDC3*, pYB405, *GFP-CDC12*, pYB407, *CFP-CDC12*, pYB458, *YFP-CDC10*, pYB480, and *GFP-SHS1*, pYB481) were constructed by in vivo recombination. S. Gasser kindly provided the GFP-Nop1 plasmid. pRS315 Δ3-3HA is described (Shu et al., 1997).

For immunofluorescence microscopy, cells were fixed with 3.7% formaldehyde for 30 min. Cells stained with anti-Tub1 antibodies and DAPI (Vogel et al., 2001) were scored for cell cycle stages counting at least 600 cells per sample. Zymolyase treatment (20 μg/ml final concentration) was performed for 30 min at 37°C. Cells were then vortexed extensively. For cell cycle analysis, *rts1*Δ cells were arrested with α-factor (5 μg/ml final concentration) for 2.5 hr at room temperature. Arrested cells were then shifted for 1 hr to 37°C. The cells were then resuspended in fresh medium at 37°C. Samples were taken every 10 min and the cells fixed with 3.7% formaldehyde.

For analysis of the role of de novo protein synthesis in Rts1 localization cells expressing Rts1-GFP and CFP-Tub1 were grown overnight in YPD, diluted to $OD_{600} = 0.20$ in fresh medium, and grown for 5 hr to $OD_{600} \approx 0.65$. Nocodazole was added (final concentration of 2 mg/ml), and cells were left shaking for 3 hr at 30°C. Nocodazole was washed away and the culture was split. One half was resuspended in YPD, the other half in YPD + 10 μg/ml cycloheximide. Pictures were taken every 15 min and large-budded cells were scored for elongated spindles and neck localization of Rts1. Rts1-Δ3-GFP localization was determined in the same manner.

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) was performed as described (Meluh and Broach, 1999) with the modifications described in Gentry and Hallberg (2002).

FRAP and Microscopy

FRAP experiments were performed on a Zeiss LSM 510 confocal microscope. Cells expressing GFP or YFP septins were grown on YPD overnight. Cells were resuspended in synthetic complete medium and spread on a 1.6% agarose pad. Half the septin ring was bleached with a sequence of 20 to 25 irradiations at 50% of laser intensity. Pictures were taken at least every 30 s for 30 min. Fluorescence intensities were analyzed with NIH image. In each picture we scored the average values of both the bleached and non-bleached part. To correct for general bleaching, a reference cell was present in each movie.

All other microscopy techniques used have been described (Kusch et al., 2002). To visualize septin dissociation septins were visualized using GFP-CDC3. Cells were grown as above and shifted in temperature under the microscope using a prewarmed heating stage at 30°C. Pictures were taken every 5 min for up to 75 min. Septin ring intensities were the average of the two sides of the ring (the focal plane cuts the ring in the middle, which results in two intense dots at the plasma membrane) in cells with a single ring, and the four sides of the two rings after ring splitting. Background staining was subtracted. No correction was applied for bleaching.

To monitor Rts1-GFP, cells were grown overnight on YPD. Three focal planes separated by 0.3 μm were taken using a piezo motor and projected into a single image. For GFP-CDC12, Nop1p-GFP, CFP-Cdc12p, Spc42p-CFP, and CFP-Tub1p, seven focal planes were taken. Colocalization studies were performed with CFP and YFP filters, which enabled total separation of the CFP and GFP signals.

Shs1 Phosphorylation

WT and *rts1*Δ cells containing Shs1-$(HA)_3$ were arrested with HU (7.6 mg/ml final concentration) for 2 hr at room temperature. Cells were then shifted for 1 hr to 30°C. The cells were released from the arrest at 30°C and samples were frozen every 10 min. Afterward, samples were processed together and loaded on a gel. Western detection was done with an anti-HA-HRP antibody (Santa Cruz).

Acknowledgments

We thank M. Peter and C. Mann for providing strains, plasmids, and useful suggestions on the manuscript; S. Gasser, S. Jensen, N. Bouquin, D. Kellogg, R. Li, F. Luca, and U. Surana for strains and plasmids; and L. Dowse, S. Grava, R. Kroschewski, U. Kutay, C. Lüdeke, and D. Liakopoulos for critical reading of the manuscript. We thank the members of the Hallberg and Barral groups for helpful discussions. We are particularly indebted to K. Tatchell and E. Bi for communicating unpublished results, S. Cotesta (curve fittings), C. Berney and G. Danuser (FRAP Microscopy), and R. Moser and T. Lehman (technical support). J.D. and Y.B. are supported by the Swiss Federal Institute of Technology and a grant from the Swiss National Foundation. M.S.G. and R.L.H. were supported by National Science Foundation grants MCB-9603733 and MCB-0113355 awarded to R.L.H.

Received: July 1, 2002
Revised: February 10, 2003

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