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# The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis

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## SUMMARY

Cytokinesis in budding yeast involves an actomyosin-based ring which assembles in a multistepped fashion during the cell cycle and constricts during cytokinesis. In this report, we have investigated the structural and regulatory events that occur at the onset of cytokinesis. The septins, which form an hour-glass like structure during early stages of the cell cycle, undergo dynamic rearrangements prior to cell division: the hourglass structure splits into two separate rings. The contractile ring, localized between the septin double rings, immediately undergoes contraction. Septin ring splitting is independent of actomyosin ring contraction as it still occurs in mutants where contraction fails. We hypothesize that septin ring splitting may remove a structural barrier for actomyosin ring to contract. Because the Tem1 small GTPase (Tem1p) is required for the completion of mitosis, we investigated its role in regulating septin and actomyosin ring dynamics in the background of the *net1-1* mutation, which bypasses the anaphase cell cycle arrest in Tem1-deficient cells. We show that Tem1p plays a specific role in cytokinesis in addition to its function in cell cycle progression. Tem1p is not required for the assembly of the actomyosin ring but controls actomyosin and septin dynamics during cytokinesis.

Key words: Tem1p, GTPase, Cytokinesis, Budding yeast, Actomyosin, Septin

## INTRODUCTION

Actomyosin-based contractile rings have been implicated in cell division in eukaryotic organisms as diverse as yeast and man (for reviews, see Field et al., 1999; Fishkind and Wang, 1995; Schroeder, 1990). To ensure that the chromosomes are partitioned equally between the daughter cells, contractile ring activity must be coupled spatially and temporally with chromosome segregation. Whereas many studies have indicated that astral microtubules and spindle-associated components are important for the correct positioning of the cleavage furrow (for reviews, see Field et al., 1999; Oegema and Mitchison, 1997), less is known about how the timing of cytokinesis is controlled. Studies in both animal cells and yeast have shown that the onset of cytokinesis requires destruction of mitotic cyclins, which leads to inactivation of the mitotic kinase CDK1 (for reviews, see Balasubramanian et al., 2000; Field et al., 1999). Presumably, phosphate removal from a Cdk1 target is required for the activation of the contractile apparatus. However, the identity of this target is currently unknown. Furthermore, it remains unclear how the timing of Cdk1 inactivation is in turn controlled to ensure temporal coordination between chromosome segregation and cytokinesis.

Model organisms such as yeast are valuable for the elucidation of the structural and regulatory pathways that lead to successful cell division. In the budding yeast *Saccharomyces cerevisiae*, the completion of ana/telophase events is regulated by a number of genes, collectively called the mitotic exit network (MEN), because mutations in these genes cause arrest in telophase with elongated spindles, segregated chromosomes and a high level of the mitotic cyclin, Clb2p (Jaspersen et al., 1998). A key upstream component of this pathway is Tem1p, a GTPase belonging to the Ras superfamily (Shirayama et al., 1994). GTP-bound Tem1p somehow leads to the activation of several MEN kinases such Cdc15p and Dbf2p, which eventually results in the release of the phosphatase Cdc14p from its resident compartment, the nucleolus (Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999). Cdc14p dephosphorylates and thus activates or stabilizes two negative regulators of the Cdk1/Clb kinase complexes (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). These findings strongly suggest that the MEN functions to coordinate Cdk1 inactivation with chromosome segregation.

In budding yeast, cytokinesis involves the function of two ring structures around the bud neck: the septin ring and the actomyosin ring (for a review, see Field et al., 1999). The septins are a family of GTP-binding proteins conserved in eukaryotic organisms (Longtine et al., 1996). The yeast septins localize to a broad ring, made up of 10 nm filaments, around the bud neck through most of the cell cycle (Byers and Goetsch, 1976; Longtine et al., 1996). Morphologically, the septin ring is distinct from the actomyosin ring: the latter covers a narrower region of the bud neck and contracts during cytokinesis whereas the septin ring maintains the same diameter (Lippincott and Li, 1998a). Besides providing a structural scaffold for the localization of many proteins to the bud neck (Longtine et al., 1996), the function of the

septins during cytokinesis is unclear. Cyk2p, a PCH family protein, first associates with the septin ring but moves to the actomyosin ring during cytokinesis, suggesting that structural rearrangements occur within these cytokinetic structures (Lippincott and Li, 1998a; Lippincott and Li, 2000).

The actomyosin ring is required for the cell-cycle-coupled cytokinesis (Rodriguez and Paterson, 1990; Watts et al., 1987). Myosin II, encoded by the *MYO1* gene in yeast, assembles at the bud neck early in the cell cycle, immediately after the appearance of the septin ring, but the recruitment of F-actin to the ring does not occur until telophase, after chromosome segregation (Bi et al., 1998; Lippincott and Li, 1998b). Cyk1p/Iqg1p (here referred to as Cyk1p), an IQGAP-like protein, is required for the assembly of the actin ring as well as for ring contraction (Epp and Chant, 1997; Lippincott and Li, 1998b; Shannon and Li, 1999). Deletion of the COOH-terminal portion of Cyk1p, which contains a GAP-related domain, does not affect the assembly of actin or myosin to the ring but prevents ring contraction, suggesting that the presence of F-actin and myosin II in the ring is not sufficient for contraction (Shannon and Li, 1999). Interestingly, the GAP-related domain in Cyk1p binds to Tem1p but not other small GTPases such Cdc42p and Rho1p, suggesting a putative role for Tem1p in regulating the contraction of the actomyosin ring.

In this study, we further investigated the structural changes and signaling events that occur at the onset of cytokinesis. We first show that the septins undergo a novel structural change concurrent with actomyosin ring contraction. This change may be a structural prerequisite for actomyosin ring contraction. Furthermore, we provide evidence that the Tem1 small GTPase plays a specific role in regulating the dynamics of the septin and actomyosin rings during cytokinesis.

## MATERIALS AND METHODS

### Media and genetic manipulations

Yeast cell culture and genetic techniques were carried out by methods described elsewhere (Sherman et al., 1986). Yeast extract, peptone, dextrose (YPD) contained 2% glucose, 1% yeast extract and 2% Bactopeptone (Difco Laboratories Inc., Detroit, MI, USA). YPGR contained 2% galactose, 2% raffinose, 1% yeast extract and 2% Bactopeptone. SC medium was prepared by the method described elsewhere (Kaiser et al., 1994).

### Plasmids and strains

To construct pLP71 (Cyk2-YFP under the control of the *CYK2* promoter on a centromeric plasmid bearing the *URA3* gene), YFP was amplified by PCR using an $NH_2$-terminal primer, which contained an in-frame *Bam*HI site, and a C-terminal primer, which contained an *Xba*I site. This PCR product was cloned into pRS313 (Sikorski and Hieter, 1989) to generate pLP66. Cyk2 was cloned into pLP66 at the *Sal*I and *Bam*HI sites using the *CYK2*-containing *Sal*I-*Bam*HI fragment from pLP2 (Lippincott and Li, 1998a) to generate pLP70. Cyk2-YFP was then cut out from pLP70 using *Sal*I and *Not*I. This fragment was ligated into pRS316 to generate pLP71. pLP41 (Cdc12-CFP) was constructed with the same set of primers used for YFP. This PCR product was then directly ligated into the *Bam*HI and *Xba*I sites of pLP17 (Lippincott and Li, 1998a) to generate pLP41.

All strains used in this study are listed in Table 1.

### Staining of yeast cells to visualize actin

Cells grown in YPD were fixed directly in growth medium by addition of 37% formaldehyde to 5% final concentration and incubation at room temperature for 1 hour with gentle agitation. Cells grown in synthetic medium were shifted to growth in YPD for 1 hour before fixation because the low pH of the synthetic medium resulted in poor fixation. Phalloidin staining was performed on fixed cells treated with zymolyase as described (Lippincott and Li, 1998b). For phalloidin staining of RLY 635, cells were grown overnight in YPGR, then arrested for 3 hours with 0.05 µg/ml α-factor with the addition of glucose to 2% to deplete Tem1p. Cells were washed 3× with sterile water, then grown in YPD for approximately 2 hours, then fixed and stained as described above. Cells were visualized using a Zeiss Axiophot microscope with a HB 100 W/Z high pressure mercury lamp and a Zeiss 100×/1.40 oil objective. Image acquisition was carried out using a cooled RTE/CCD 782Y Interline camera (Princeton Instruments).

### Fluorescence microscopy

Cells were mounted and observed by time-lapse video microscopy on either a DeltaVision or a Nikon E600 microscope system as previously described (Lippincott and Li, 1998a). To generate the flattened three-dimensional view of the cells, multiple *Z*-sections (nine 350 nm sections or thirteen 200 nm sections) were deconvolved and compressed for each time point using DeltaVision software.

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

| Name | Genotype | Source |
|---|---|---|
| RLY370 | MATa ura3-52 his3-Δ200 leu2-3,112 lys2-801 Δ bar1 pCDC12-GFP (pLP17) | Lippincott and Li, 1998b |
| RLY635 | MATa ΔTem1::GAL-UPL-TEM1(TRP1) Δbar1::hisG ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 | Shou, 1999 |
| RLY636 | MATa net1-1 ΔTem1::GAL-UPL-TEM1(TRP1) Δbar1::Leu2, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 | Shou, 1999 |
| RLY637 | MATa net1-1 Δbar1::Leu2, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 | Shou, 1999 |
| RLY638 | MATa Δbar1::hisG, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 | Shou, 1999 |
| RLY652 | MATa net1-1 ΔTem1::GAL-UPL-TEM1(TRP1) Δbar1::Leu2, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 Myo1-GFP:URA3 (pLP12) | This study |
| RLY653 | MATa net1-1 Δbar1::Leu2, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 Myo1-GFP:URA3 (pLP12) | This study |
| RLY769 | MATa ΔTem1::GAL-UPL-TEM1(TRP1) Δbar1::hisG, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1 Myo1-GFP:URA3 (pLP12) | This study |
| RLY773 | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 Δbar1::HIS3 Δtem1::GAL-ubi-pro-Lac-TEM1(TRP1) pCDC12-GFP (pLP17) | This study |
| RLY898 | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 Δbar1::LEU2 Δtem1::GAL-ubi-pro-Lac-TEM1(TRP1) net1-1 pCDC12-GFP (pLP29) | This study |
| RLY1055 | MATa ura3-52 his3-Δ200 leu2-3,112 lys2-801 Δbar1 Δcyk2::HIS3 pCYK2-YFP (pLP71) pCDC12-CFP (pLP41) | This study |
| RLY1157 | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 Δbar1::LEU2 net1-1 pMYO1-GFP (pKT36) | This study |
| RLY1334 | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 Δbar1 Δcyk1::LEU2 GAL1-Cyk1-myc::URA3 pCDC12-GFP | This study |

All strains are in the W303 background.

To examine the effect of Tem1p shutoff on actomyosin ring contraction, RLY652 (Myo1-GFP *net1-1 GAL1-UPL-TEM1*) cells cultured overnight in medium containing 2% galactose were arrested for 3 hours in the presence of 0.05 µg/ml α-factor and 2% glucose, then washed 3× with sterile water and resuspended in glucose-containing media. Cells were placed on an agarose pad as described (Waddle et al., 1996). Living cells were imaged at room temperature using a Nikon Eclipse E600 microscope with a 100×/1.40 oil DIC objective (Nikon Inc.) Images were collected every 2 minutes with 0.1 second exposure to fluorescence using a cooled RTE/CCD 782Y Interline camera (Princeton Instruments). The shutter was controlled automatically using a D122 shutter driver (UniBlitz) and WinView 1.6.2 software (Princeton Instruments) with custom software (courtesy of Aneil Mallavarapu, Harvard Medical School).

### Analysis of cell morphology

For analysis of the morphology of Tem1-deficient cells, RLY635, 636, 637 and 638 were grown overnight in YPGR. Cells were then arrested with 0.05 µg/ml α factor and glucose was added to 2%. After 3 hours, cells were released from arrest by washing 3× in sterile water, then resuspended in YPD and grown for 4 or 6 hours. Cells were fixed by addition of formaldehyde to 5% with gentle shaking at room temperature for 1 hour. Cells were treated with zymolyase in sorbitol buffer as previsouly described (Lippincott and Li, 1998a) before counting.

## RESULTS

### Septins localize to an hourglass structure, which resolves into two distinct rings

We first characterized the dynamics of the septin ring during the cell cycle using a DeltaVision 3-dimensional deconvolution microscope system. When observed under conventional fluorescence microscopes, especially by immunofluorescence staining, the septin-containing structure often appears as a double ring around the bud neck. However, when observed using wide-field deconvolusion three-dimensional microscopy (Agard et al., 1989), the septin ring is in fact a continuous hourglass shaped structure around the bud neck, consistent with the images obtained by electron microscopy (Byers and Goetsch, 1976). To observe the dynamics of this structure, we performed time-lapse optical sectioning microscopy on cells expressing GFP-tagged Cdc12p, a subunit of the septins. The septin structure maintains the hourglass shape through most of the cell cycle; however, this structure splits abruptly (within 1-2 minutes, between 2′ to 4′ time points for the top cell, 31′ to 33′ for the bottom cell) from the middle in large budded cells, resulting in two distinct rings (Fig. 1). This splitting event is specific to the septins because Cyk2p, which colocalizes with the septins during most of the cell cycle, actually tightens into a narrow ring around the center of the bud neck and contracts within 2-3 minutes thereafter in a myosin II-dependent manner (Lippincott and Li, 1998a; Lippincott and Li, 2000).

### Septin rearrangement occurs at the onset of actomyosin ring contraction

The change in septin structure described above occurs around the time of cytokinesis because the new bud emerges about 30 to 40 minutes thereafter, and this is roughly the same length of time between actomyosin ring contraction and new bud emergence (Lippincott and Li, 1998a; Lippincott and Li, 1998b). To further compare the timing of septin rearrangement and actomyosin ring contraction, we constructed a yeast strain that simultaneously expresses cyan fluorescent protein-tagged Cdc12p (Cdc12-CFP) and yellow fluorescent protein-tagged Cyk2p (Cyk2-YFP), which was used as a marker for the contractile ring. Since the absorption and emission
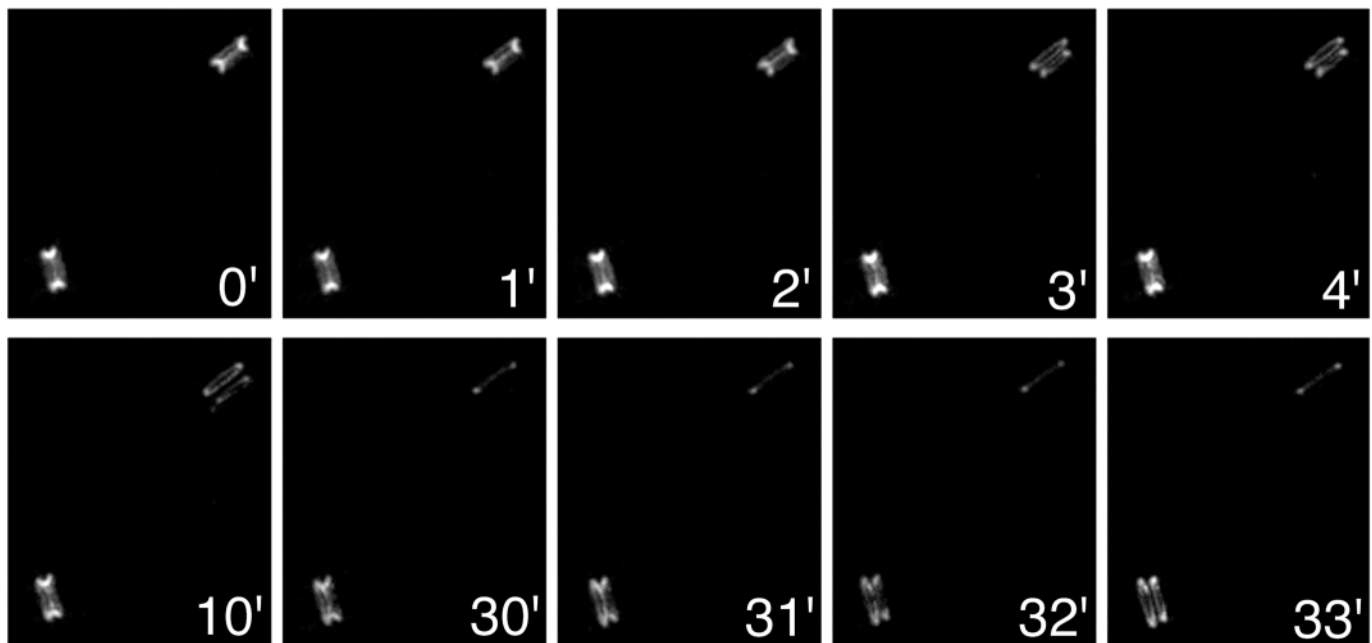


**Fig. 1.** The septins undergo dramatic structural rearrangement late in the cell cycle. Three-dimensional time-lapse analysis of cells expressing Cdc12-GFP. All images were collected and deconvolved on a DeltaVision microscope system as described in Materials and Methods. To generate the flattened three-dimensional view of the cells, thirteen 200-nm sections were deconvolved and compressed for each time point using Delta Vision software. Bar, 10 µm.
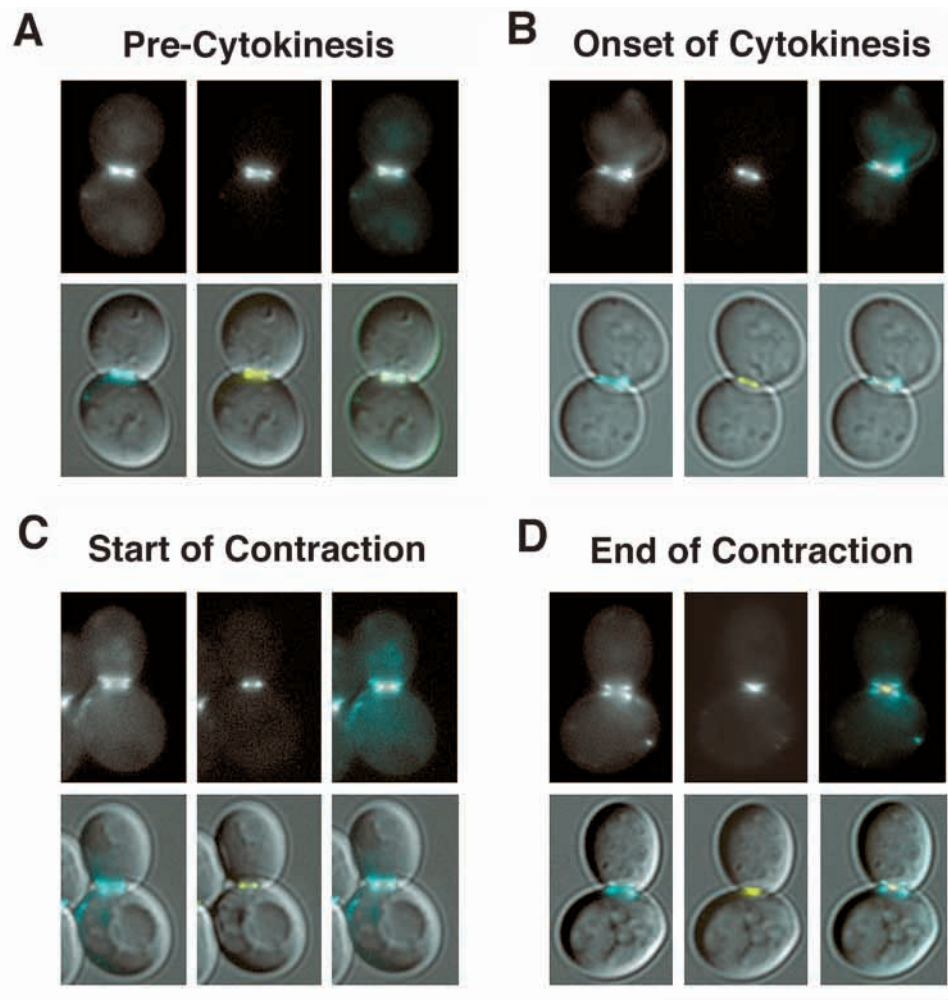
## A  Pre-Cytokinesis



## B  Onset of Cytokinesis



## C  Start of Contraction



## D  End of Contraction



**Fig. 2.** Septin ring splitting occurs concurrent with actomyosin ring contraction. Cells expressing both Cdc12-CFP and Cyk2-YFP (RLY1055) were imaged as described in Materials and Methods and representative cells at different stages of cytokinesis are shown. Left panels, septin-CFP; middle panels, Cyk2-YFP; right panels, merged CFP and YFP images. (A) A large-budded cell prior to cytokinesis. Cyk2-YFP colocalizes with Cdc12-CFP hourglass structure. (B) A cell where the Cyk2-YFP ring had converged to a tight single ring in the center of the bud neck just prior to contraction. At this point the Cdc12-CFP hourglass had not split. (C) A cell where the Cyk2-YFP ring had just begun contracting. The Cdc12-CFP hourglass structure had resolved to two distinct rings which flank the contractile ring. (D) The Cyk2-YFP ring had contracted to nearly a dot, which is flanked by the Cdc12-CFP double rings. Bar, 10 μm.

wavelengths of these two GFP variants are separable, it is possible to examine the dynamics of the two proteins in the same cell using different filter sets. We were unable to successfully capture a time-lapse series of these cells because the CFP signal photobleaches extremely rapidly. However, we were able to compare the morphology of these structures in individual cells during various stages of mitosis and cytokinesis. Fig. 2 shows representative cells at different stages. We observed that in more than 100 cells where Cyk2-YFP had merged into a single ring, the septins were still in an un-split hourglass structure (Fig. 2B). Because we did not observe cells (among hundreds of cells observed) where the septin hourglass had split but Cyk2p remained in a broad ring, we conclude that tightening of the Cyk2 ring occurs before septin ring splitting. In 23 cells where where Cyk2-YFP had

merged into a single ring (Fig. 2C) or had started contraction (Fig. 2D), the septin hourglass had resolved into two separate rings, demonstrating that septin splitting occurs at or slightly before the onset of actomyosin ring contraction. Furthermore, the contractile ring, as marked by the tightened Cyk2 ring, was located exactly in the space between the two septin rings (Fig. 2C,D).

### Splitting of the septin structure is independent of actomyosin ring contraction

One possible explanation for the temporal correlation between septin hourglass splitting and actomyosin ring contraction is that the former event is a consequence of the latter. To test this possibility, we examined Cdc12-GFP dynamics in a *cyk1* mutant strain where actomyosin ring contraction and

**Fig. 3.** Septin ring splitting is unaffected in Cyk1-deficient cells. RLY 1334 cells (expressing Cdc12-GFP with the sole source of Cyk1p under the control of the Gal1 promotor) were depleted of Cyk1p by arresting with α-factor for 3 hours in the presence of 2% glucose to repress Gal1-Cyk1p expression. The cells were then released into glucose-containing medium and observed on a Deltavision microscope at various time points as described in Materials and Methods. Bar, 1 μm.
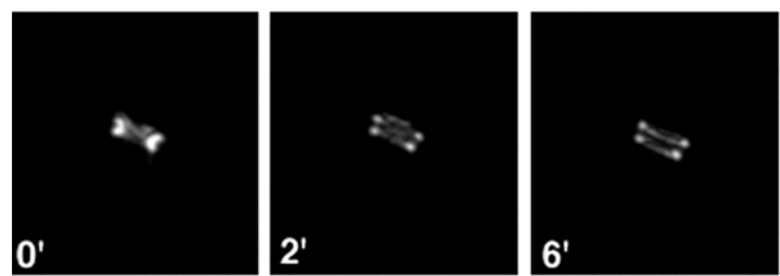
cytokinesis does not occur. Cyk1p is a component of the contractile ring required for both the assembly and contraction of the actomyosin ring (Lippincott and Li, 1998b; Shannon and Li, 1999). We showed previously that the Myo1 ring does not contract in cells lacking Cyk1p (Shannon and Li, 1999). As shown in Fig. 3A, the septin hourglass splits with normal dynamics in Cyk1-deficient cells (splitting occurred abruptly approx. 30 minutes before the cell budded again). Conversion of Cyk2-GFP to a tight single ring also occurs with normal timing in Cyk1-deficient cells and, as expected, this single ring never contracted and remained localized at the bud neck (data not shown). This result suggests that septin hourglass splitting is not simply a consequence of actomyosin ring contraction and cytokinesis.

## The Tem1 small GTPase plays a specific role in cytokinesis

To understand how septin and actomyosin structural changes are initiated at the onset of cytokinesis, we sought to identify regulatory proteins that are important for these changes. A key regulator of post-anaphase events is Tem1p, a Ras family small GTPase (Morgan, 1999). The observation that Tem1p binds the GAP-related domain of Cyk1p first led us to speculate that Tem1p may directly regulate the onset of cytokinesis. However, in order to know whether Tem1p has a specific role in cytokinesis, it was necessary to bypass the requirement for Tem1p in cell cycle progression. To do this we took advantage of the *net1-1* allele (Shou et al., 1999). In wild-type cells, Net1p binds and sequesters the Cdc14 protein phosphatase in the nucleolus, and Tem1p is required for Cdc14p to be released from the nucleolus and trigger mitotic exit (Shou et al., 1999; Visintin et al., 1999). However, *net1-1* has a reduced affinity for Cdc14p, so in this mutant background Tem1p is no longer required for Cdc14p release and mitotic exit (Shou et al., 1999). It was noticed in the same report that the Tem1-deficient cells that had bypassed the cell cycle arrest in this manner appeared as chains. To further test if this indicated a cytokinesis defect and if so, which step of cytokinesis was affected, we used a *net1-1, GAL1-UPL-TEM1* strain. UPL, which stands for ubiquitin-proline-LacI, acts as a destabilization signal so that Tem1p is quickly degraded after transcription is shut off (Shou et al., 1999). This strain, a *net1-1 TEM1*

strain, a *NET1 GAL1-UPL-TEM1* strain, and a wild-type strain were arrested in G$_1$ phase using α-mating factor. Glucose was added during this arrest to deplete Tem1p. Cells were then released from the arrest into glucose-containing medium and at 4 and 6 hours after the release, cells were fixed, cell walls removed with zymolyase, and the number of cells that formed chains (i.e. cells with three or more cell bodies) was scored by light microscopy. Both wild-type and *net1-1* cells showed no cytokinesis defects after this treatment (Fig. 4A). *GAL1-UPL-TEM1* cells were uniformly arrested as large budded cells, as expected (Fig. 4A). However, *net1-1 GAL1-UPL-TEM1* cells showed a dramatic cytokinesis defect. At 4 hours, 29% of cells were in chains of three cell bodies or more. By 6 hours, 51% of cells depleted for Tem1p in the net1-1 background had formed chains (Fig. 4A,B). These results indicate that Tem1p plays an important role in cytokinesis, in addition to its function in cell cycle progression.

## Tem1p is not required for the assembly of the actomyosin ring

Because mutations in several MEN proteins have been shown to affect actin ring assembly (Frenz et al., 2000; Jimenez et al., 1998; Lippincott and Li, 1998b), we performed phalloidin staining of cells after depletion of Tem1p to see if Tem1p is required for actin ring formation. Cells with *GAL1-UPL-TEM1* as the sole source of Tem1p were arrested for 3 hours using α-factor with the addition of glucose. Cells were then released from α-factor arrest, and these cells budded and grew until they arrested in mitosis due to lack of Tem1p. To our surprise, F-actin is localized to a ring at the bud neck in greater than 80%
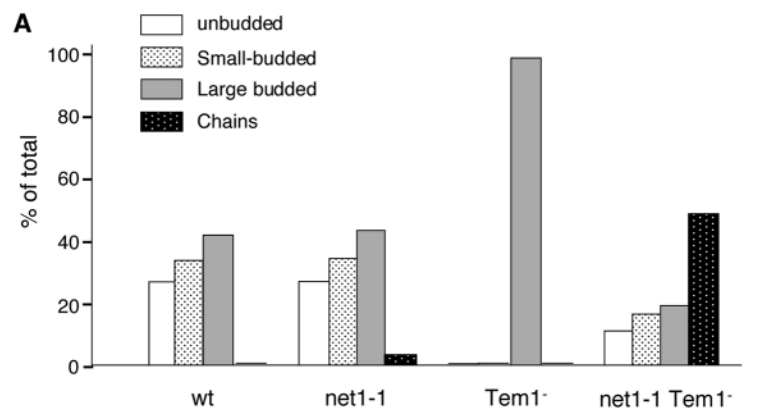


**Fig. 4.** Tem1p plays a specific role in cytokinesis. (A) Bypass of the requirement for Tem1p in cell cycle progression reveals a role for Tem1p in cytokinesis. RLY635 (Tem1⁻), 636 (Tem1⁻*net1-1*), 637 (*net1-1*) and 638 (wt) cells (see Table 1) were synchronized with α-factor and release into the cell cycle as described in Materials and Methods. The numbers of cells that were unbudded, small budded, large budded, or in chains of three or more cell bodies were counted after zymolyase treatment (*n*≥100 for each strain). Their percentages of the total cell population were calculated and presented as histograms. The experiment was repeated three times with similar results. (B) Morphology of Tem1⁻ (RLY636; a) and Tem1⁻ *net1-1* (RLY637; b) cells 6 hours after release from the G$_1$ arrest in the above experiment. (C) Phalloidin staining of large-budded RLY636 (Tem1⁻) cells after Tem1p depletion. Arrows point to the actin ring. Bar, 10 μm.
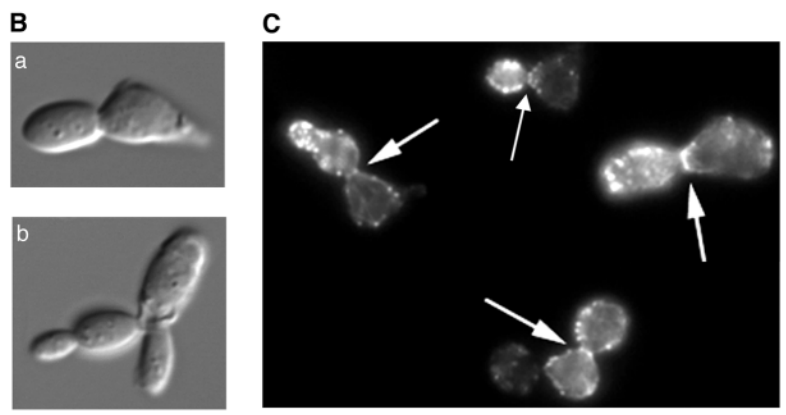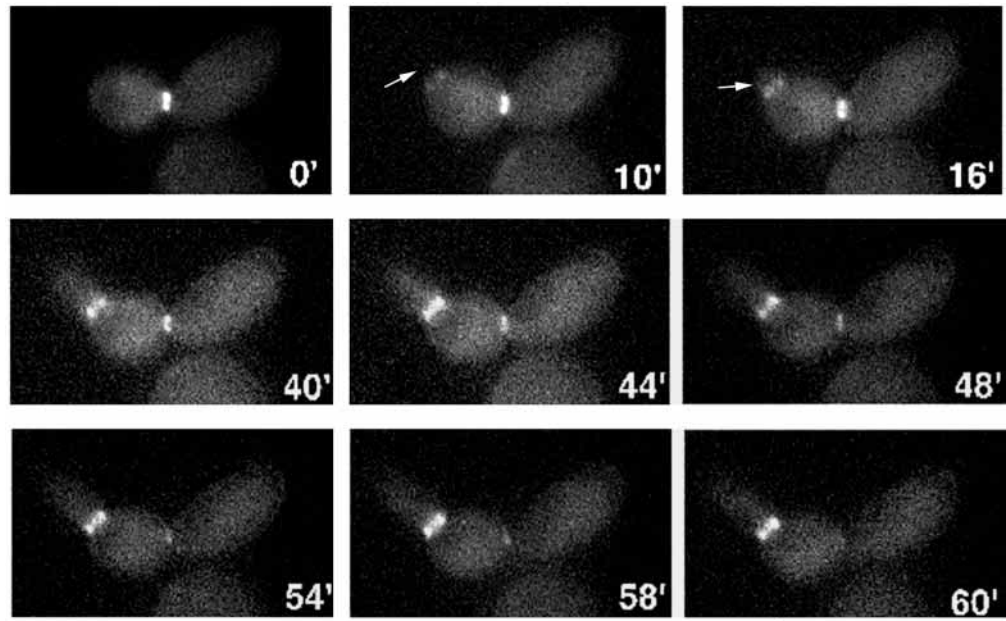
**Fig. 5.** Failure of actomyosin ring contraction in Tem1⁻ *net1-1* cells. Images are from a time-lapse sequence of a Myo1-GFP movie in a *net1-1 GAL1-UPL-TEM1* strain (RLY652) after Tem1p depletion, as described in Materials and Methods. Notice that the myosin ring had not contracted when the new bud emerged at the 10 minute time point (arrows in 10′ and 16′ panels), and as the Myo1-GFP ring at the new bud grew brighter, the Myo1-GFP ring at the old bud neck faded. Bar, 10 μm.

of Tem1p-deficient cells arrested in telophase (Fig. 4C), suggesting that Tem1p is not required for recruitment of F-actin to the ring. Similarly, myosin II localization was also unaffected in Tem1p-deficient cells as visualized using GFP-tagged Myo1p (Fig. 5).

## Tem1p regulates septin and actomyosin ring dynamics during cytokinesis

Since Tem1p does not appear to have a defect in actomyosin ring formation, we went on to determine if Tem1p had a role in ring contraction. To do this we introduced Myo1-GFP into the *net1-1 GAL1-UPL-TEM1* strain. Analysis of live cells after Tem1p depletion using time-lapse microscopy showed that although Myo1p was able to form rings at the bud neck, these rings maintained the same diameter until the new bud had emerged. At this time, a new Myo1-GFP ring had formed at the new bud neck, while the previous Myo1-GFP ring gradually faded (Fig. 5). The same contraction defect was also observed when the ring was marked with Cyk2-GFP (data not shown). Next we examined whether septin rearrangement is also affected in Tem1p-depleted *net1-1* cells by time-lapse analysis. Septin ring splitting did not occur in a majority (13 out of 15 observed by time-lapse microscopy) of Tem1p-depleted *net1-1* cells that had initiated a new round of budding (Fig. 6A, showing a frame where the septins remained in a single ring at the old bud neck after new bud emergence). In a small fraction (2 of 15 observed by time-lapse microscopy) of the cells, the septin hourglass structure did appear to split right before or around the time of new bud emergence. Fig. 6B shows an example of such cells. The septin structure in the upper cell resolved into two separate rings at the 15 minute time point, only three minutes before the new bud emerged at time 18 minutes. In the same strain background, tightening of the Cyk2 ring occurred 30-40 minutes before new bud emergence, but contraction, which normally follows immediately, did not happen due to the *tem1* mutation (data not shown). This suggests that the septin splitting event observed in these cell is much delayed compared to the wild type. Septin and actomyosin ring dynamics appeared to be

normal in net1-1 cells (data not shown). These results suggest that Tem1p activity is required for actomyosin and septin dynamics during cytokinesis.

## DISCUSSION

### Septin dynamics during cytokinesis

We have described experiments that were aimed at understanding the structural and regulatory events required for the initiation of cytokinesis. First, we found that prior to cytokinesis, the septins abruptly split into two separate rings, leaving a space that is occupied predominantly by the actomyosin ring. This change in septin distribution is immediately followed by and occurs independently of actomyosin ring contraction and cytokinesis. Electron microscopy studies have shown that the septins form 10 nm filaments around the bud neck during most of the cell cycle (Byers and Goetsch, 1976), and these filaments provide scaffolding for the localization of many neck components, most notably Myo1p, the budding yeast myosin II (Bi et al., 1998; Lippincott and Li, 1998b). Time-lapse microscopy studies revealed that the septin structure does not constrict during cytokinesis, unlike the actomyosin ring (Lippincott and Li, 1998a), and this could be due to the rigidity of the septin filaments. If this is true, then the actomyosin ring must free itself from the septins in order to undergo contraction. Thus, septin ring splitting may be an important structural change that enables the actomyosin ring to contract (Fig. 7). This is an appealing possibility as these two events are coupled both temporally and spatially. However, without a means of specifically inhibit septin ring splitting, we cannot directly test the significance of this structural change.

What might cause the septins to disappear from the middle? One possibility is filament disassembly, as it was reported that the septin filaments were not observed by electron microscopy in late anaphase cells undergoing cytokinesis (Byers and Goetsch, 1976). If the actomyosin ring, which occupies the
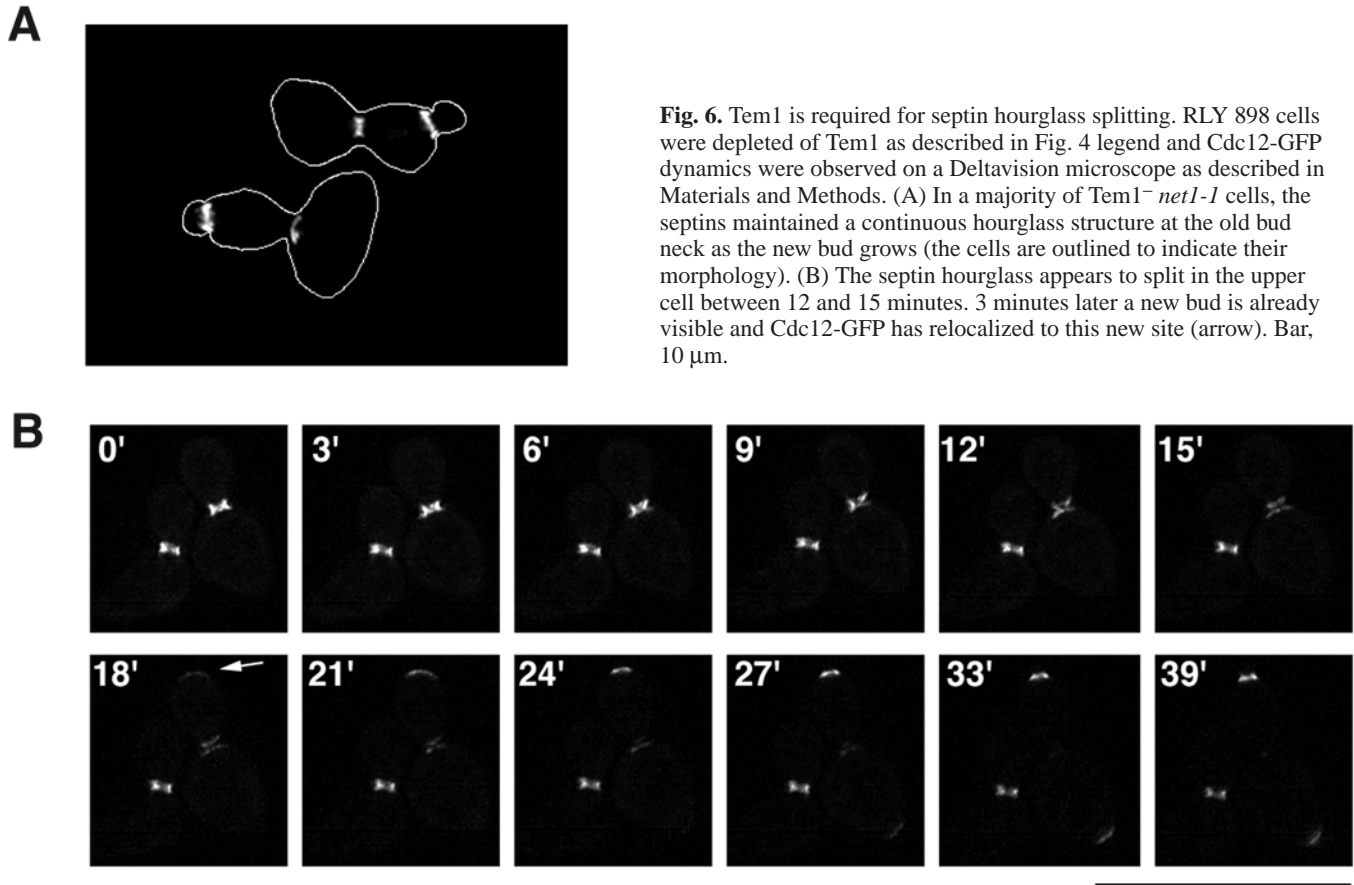
## A



**Fig. 6.** Tem1 is required for septin hourglass splitting. RLY 898 cells were depleted of Tem1 as described in Fig. 4 legend and Cdc12-GFP dynamics were observed on a Deltavision microscope as described in Materials and Methods. (A) In a majority of Tem1⁻ *net1-1* cells, the septins maintained a continuous hourglass structure at the old bud neck as the new bud grows (the cells are outlined to indicate their morphology). (B) The septin hourglass appears to split in the upper cell between 12 and 15 minutes. 3 minutes later a new bud is already visible and Cdc12-GFP has relocalized to this new site (arrow). Bar, 10 μm.

## B



middle region of the bud neck, only interacts with filamentous septin, septin filament disassembly may reduce the interaction with the contractile ring and promote septin subunits to dissociate from the neck region, although this does not explain why the septins remain associated with the cortical regions flanking the contractile ring. Alternatively, septin clearance from the middle of the bud neck may be due to localized protein degradation or modification. The APC-dependent degradation machinery is active during cytokinesis, but there is no data to
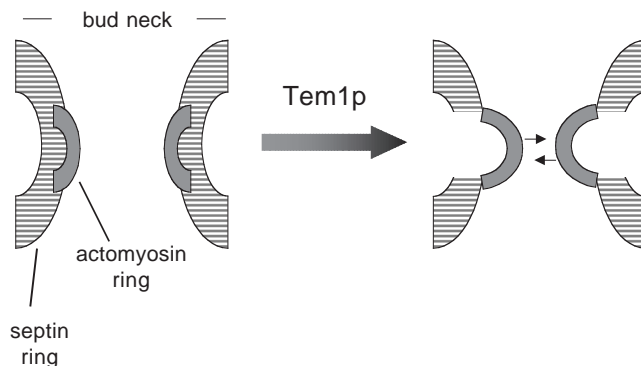
suggest that septin components are degraded by this pathway. The septins are known to be sumoylated late in the cell cycle, however, the importance of septin sumoylation is not yet clear (Johnson and Blobel, 1999; Takahashi et al., 1999).

### The role of Tem1 small GTPase in regulating late cell cycle events

Recent studies reported an exciting role for the Tem1 small GTPase in ensuring that the spindle is correctly oriented prior to cytokinesis (for a review, see Li, 2000). Tem1p is preferentially localized on the spindle pole body that migrates into the daughter cell and is activated following spindle penetration into the bud, presumably due to the presence of Lte1, a putative nucleotide exchange factor for Tem1p. Once activated, Tem1p is thought to activate the mitotic exit network, consisting of Cdc5, Cdc15 and Dbf2/20 kinases and a non-kinase protein, Mob1p, eventually leading to exit from mitosis and cell cycle re-entry (Morgan, 1999). In this study, we showed that Tem1p also controls actomyosin and septin dynamics during cytokinesis, a role that is only unmasked when its requirement in anaphase progression is circumvented. Tem1p is not required for the assembly of actin and myosin to the bud neck but is required for the contraction of the actomyosin ring.

This role is similar to that described for the fission yeast homolog of Tem1p, Spg1p. *spg1* mutant cells do not exhibit any cell cycle defect but are deficient in the final steps of cytokinesis (Schmidt et al., 1997). Our results suggest that Tem1p has dual roles in budding yeast, one in cell cycle



**Fig. 7.** A model depicting septin and actomyosin structural changes during cytokinesis. In this model, the actomyosin ring is stabilized by the septins prior to cytokinesis. At the onset of cytokinesis, as a consequence of Tem1p activation, the septins delocalize from the middle of the hourglass structure, allowing the actomyosin ring to subsequently undergo contraction.

regulation, and the other in the onset of cytokinesis. The latter role is likely to be conserved with that carried out by Spg1p. Still, it remains a question as to whether the mitotic exit network proteins, several of which are homologous to those acting downstream of Spg1p (Balasubramanian et al., 2000), are also involved in the cytokinesis function of Tem1p. Preliminary results suggest that the mitotic exit network proteins are required for cytokinesis when their cell cycle function is circumvented (K. Shannon and R. Li, unpublished). However, several studies have shown that these mutants are defective in actin ring formation at the non-permissive temperature (Frenz et al., 2000; Jimenez et al., 1998; Lippincott and Li, 1998b), whereas most of the Tem1 deficient cells can assemble an F-actin ring. This implies either that Tem1p regulates a step different from that by other MEN proteins, or that the other MEN proteins have a role in actin ring formation in addition to their participation in the Tem1p-dependent function in regulating contraction. However, it is also possible that the cell cycle arrest at high temperature in the MEN mutants causes destabilization of the actin ring. This is likely since we have noticed that the percentage of Tem1p-deficient cells that contained actin rings decreased to approx. 50% if the cells were grown at 37°C, and the actin rings that were present appeared significantly fainter than those in cells grown at room temperature (data not shown).

What might be the ultimate target of Tem1p in the final steps of cytokinesis? We have observed that both septin splitting and actomyosin ring contraction are defective in Tem1-deficient cells. If former event is a prerequisite for the latter, then the key target of Tem1p in cytokinesis may be the septins. However, we previously reported that Tem1p can bind directly to the GRD domain of Cyk1p, and this domain, like Tem1p, is required for actomyosin ring contraction, not assembly (Shannon and Li, 1999). These observations suggest that an interaction between Tem1p and the GRD of Cyk1p may trigger actomyosin ring contraction. If this is true, because septin splitting occurs normally in cells lacking Cyk1p, Tem1p may have a separate target in controlling the dynamics of the septins.

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