

# The Clp1p/Flp1p phosphatase ensures completion of cytokinesis in response to minor perturbation of the cell division machinery in *Schizosaccharomyces pombe*

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Accepted 5 April 2004

Journal of Cell Science 117, 3897-3910 Published by The Company of Biologists 2004

doi:10.1242/jcs.01244

## Summary

Fission yeast mutants defective in actomyosin ring formation and function exhibit a prolonged G2 delay following cytokinesis failure. This G2 delay depends on the SIN, a signaling network essential for cytokinesis, and the non-essential Cdc14p family phosphatase, Clp1p/Flp1p and has been proposed to signify a cytokinesis checkpoint mechanism. However, the physiological relevance of this proposed Clp1p/Flp1p-dependent checkpoint is unclear because all previous studies were carried out using mutations in essential actomyosin ring components under fully restrictive conditions and thus these cells would have died regardless of the presence of the checkpoint. Here we show that delays in cytokinesis caused by minor perturbations to different components of the cytokinetic machinery, which normally cause only mild defects,

become lethal when Clp1p/Flp1p is inactivated. In addition, we show that Clp1p/Flp1p does not function simply to inhibit further rounds of nuclear division, but also allows damaged actomyosin rings to be maintained to facilitate completion of cell division. Ectopic activation of the SIN significantly bypasses the requirement of Clp1p/Flp1p for G2 delay as well as for completion of cytokinesis. We conclude that the Clp1p/Flp1p-dependent cytokinesis checkpoint provides a previously unrecognized cell survival advantage when the cell division apparatus is mildly perturbed.

Movies available online

Key words: Fission yeast, Cytokinesis, Checkpoint

## Introduction

The production of two viable daughter cells in eukaryotes depends on the execution of DNA synthesis, mitosis and cytokinesis in a strict sequential order. Thus, mitosis is initiated only upon completion of DNA synthesis and cytokinesis is initiated only upon entry into anaphase. In addition, checkpoint mechanisms operate to ensure that DNA is not damaged before entry into mitosis, and that kinetochores have attached to the mitotic spindle before anaphase onset. The mechanisms that ensure alternation between S and M phases, and those that ensure that DNA is not damaged and is attached to the mitotic spindle, operate to produce genetically identical daughter cells that are viable (Kelly and Brown, 2000; Nyberg et al., 2002; Rudner and Murray, 1996). A key question in the regulation of cytokinesis relates to the nature of checkpoint mechanisms that might monitor the completion of this process.

Cytokinesis is the stage in the cell cycle where barriers between divided nuclei are assembled and involves the function of an actomyosin-based contractile ring in several eukaryotes (Field et al., 1999; Gould and Simanis, 1997;

Guertin et al., 2002; Feierbach and Chang, 2001). The fission yeast, *Schizosaccharomyces pombe*, is an attractive organism for the study of cytokinesis, because its cell cycle is well characterized and because this yeast divides using an actomyosin-based ring. In addition, a large bank of mutants defective in cytokinesis have been identified in this yeast (Nurse et al., 1976; Balasubramanian et al., 1998; Chang et al., 1996; Schmidt et al., 1997).

Cytokinesis mutants in fission yeast (Nurse et al., 1976; Balasubramanian et al., 1998; Chang et al., 1996; Schmidt et al., 1997) can be divided into two broad categories (Table 1). Group I comprises those in which mitotic cell cycle progression is delayed upon cytokinetic failure (Liu et al., 1999; Le Goff et al., 1999b; Trautmann et al., 2001; Cuellie et al., 2001; Nurse et al., 1976; Liu et al., 2000). Actomyosin ring assembly and cell wall synthesis mutants fall under this category. Group II comprises those in which the timing of mitotic cell cycle progression is unperturbed following cytokinetic failure (Nurse et al., 1976; Liu et al., 2000; Le Goff et al., 1999b). The SIN (septation initiation network) mutants,

**Table 1. Cytokinesis mutants**

Group I	Group II
<i>cdc3</i>	<i>cdc7</i>
<i>cdc4</i>	<i>cdc11</i>
<i>cdc8</i>	<i>cdc14</i>
<i>cdc12</i>	<i>sid1</i>
<i>cdc15</i>	<i>sid2</i>
<i>act1</i>	<i>sid3/spg1</i>
<i>rng2</i>	<i>sid4</i>
<i>rng3</i>	<i>mob1</i>
<i>rng5/myo2</i>	
<i>cps1</i>	

which define components of the spindle pole body that regulate the timing of cytokinesis (Le Goff et al., 1999a; McCollum and Gould, 2001), belong to this category. Interestingly the mitotic delay following failed cytokinesis in group I mutants is abolished when combined with SIN mutations, suggesting that SIN function is important for mitotic delay, as well as proper cytokinesis (Liu et al., 2000; Le Goff et al., 1999b). Loss of function mutations in the protein phosphatase Clp1p/Flp1p (hereafter referred to as Clp1p) also allow bypass of the mitotic delay in group I mutants (Trautmann et al., 2001; Cuellie et al., 2001). Thus, Clp1p, as with the SIN, appears to play a role in the mitotic delay, but unlike the SIN is not essential for cytokinesis.

The ability of group I mutants (in essential actomyosin ring assembly and cell wall synthetic enzymes) to delay G2/M progression in response to failed cytokinesis implicates a Clp1p-dependent checkpoint mechanism that monitors cytokinesis (Liu et al., 2000; Le Goff et al., 1999b; Trautmann et al., 2001; Cueille et al., 2001). The physiological significance of this Clp1p-dependent G2 delay mechanism, however, was unclear because, unlike in previously described checkpoints, mutant cells died regardless of the presence of the checkpoint.

In this study we show that the Clp1p-dependent cytokinesis checkpoint provides a previously unrecognized cell survival advantage. We show that Clp1p, normally a non-essential protein, becomes critical for maintaining viability upon mild perturbation of the cytokinetic machinery. Furthermore, we provide evidence that Clp1p acts in two ways to aid survival. It is required both for the maintenance of cell division structures upon damage, as well as halting the nuclear cycle. In addition, we demonstrate that ectopic signaling from the SIN compensates for loss of Clp1p in conditions where the cytokinetic machinery is damaged. Given that cell division structures similar to those seen in *S. pombe* are observed in more complex eukaryotes (Guertin et al., 2002), and that Clp1p (as well as some components of the SIN) are conserved (Bembenek and Yu, 2001; Gruneberg et al., 2002; Moreno et al., 2001; Gromley et al., 2003), a similar checkpoint mechanism might operate in other organisms.

## Materials and Methods

*Schizosaccharomyces pombe* strains, media and growth conditions

The *S. pombe* strains used in this study are: *leu1-32 ura4-D18* (wild type), *clp1Δ* (Trautmann et al., 2001; Cueille et al., 2001), *cps1-191* (Liu et al., 1999; Le Goff et al., 1999b), *cdc11-123*, *cdc14-118*,

*cdc7-24*, *cdc15-140*, *cdc4-8*, *cdc25-22*, *cdc3-124*, *cdc12-112* (Nurse et al., 1976), *cdc16-116* (Minet et al., 1979), *myo2-E1* (Balasubramanian et al., 1998), *rng2-D5* (Eng et al., 1998), *myp2::his7+* (Bezanilla et al., 1997; Motegi et al., 1997), *rlc1::ura4+* (Naqvi et al., 2000; Le Goff et al., 2000), *cyk3::ura4+* (W.H.Y. and M.K.B., unpublished), *myo52::ura4+* (Win et al., 2001), *cdc14-118 myo2-E1*, *cdc11-123 myo2-E1*, *cps1-191 clp1Δ*, *cdc4-8 clp1Δ*, *cdc15-140 clp1Δ*, *cdc11-123 clp1Δ*, *cdc3-124 clp1Δ*, *cdc12-112 clp1Δ*, *myo2-E1 clp1Δ*, *rng2-D5 clp1Δ*, *cdc25-22 clp1Δ*, *cps1-191 cdc16-116*, *cdc16-116 clp1Δ*, *cdc16-116 cps1-191 clp1Δ*, *myp2Δ clp1Δ*, *rlc1Δ clp1Δ*, *cyk3Δ clp1Δ*, *myo52Δ clp1Δ*, *rlc1gfp*, *rlc1gfp clp1Δ*, *rlc1gfp sid1-123*, *rlc1gfp sid2-250*, *rlc1gfp cdc7-24*, *rlc1gfp cdc14-118*. Cell culture and maintenance were carried out using standard techniques (Moreno et al., 1991). Vegetative cells were grown in YES medium. Genetic crosses were performed by mixing appropriate strains of opposing mating type on YPD plates and recombinant strains were selected by tetrad dissection carried out on Singer MSM Micromanipulator (Singer instruments, UK). Double mutants were typically isolated from NPD tetrads. Elutriation was performed using a Beckman elutriation chamber (JE 5.0) according to the manufacturer's instructions. Latrunculin A was purchased from Molecular Probes (Eugene, OR). Lat A was used at 0.2 μM (in DMSO).

## Fluorescence and time-lapse microscopy

Cell staining with 4',6-diamidino-2-phenylindole (DAPI), aniline blue, and rhodamine-conjugated phalloidin were performed as described previously (Balasubramanian et al., 1997). Aniline blue was used at a final concentration of 0.5 μg/ml. Aniline blue and rhodamine-conjugated phalloidin was purchased from Sigma (St Louis, MO). Images were acquired using a Leica DMLB microscope in conjunction with an Optronics DEI-750T cooled CCD camera and Leica QWIN software. Images were processed and assembled using Adobe Photoshop 5.5. In experiments involving quantification, at least 500 cells were counted. Time-lapse microscopic studies were performed as previously described (Wong et al., 2002). Briefly, *S. pombe* cells were grown in rich medium until mid-exponential phase and then shifted to medium containing 0.2 μM Lat A or DMSO (solvent control) for 30 minutes. 1 μl of cell culture was then mounted on borosilicate glass slide covered with a coverslip (both from Matsunami Trading, Japan). All time lapse microscopy images were obtained using a Leica DMIRE2 microscope equipped with Uniblitz shutter and CoolSnap HQ CCD camera (Photometrics) driven by MetaMorph 4.6r9 software (Universal Imaging). Experiments were carried out at approximately 25°C. In experiments involving temperature-sensitive SIN mutants, cells were grown in rich medium at 25°C and shifted to the restrictive temperature of 36°C before being mounted on a glass slide and imaged using a temperature-controlled stage (MC 60 Linkman Scientific Instruments, UK) maintained at 36°C. At least 10 cells were imaged in all cases. 3D reconstructions were performed by taking Z-series (100 nm sections) of wide-field fluorescent images and de-convolving using the AutoDeblur/AutoVisualize 9.2 package (Autoquant Imaging). Long-term observation of histone-GFP expressing cells was performed using a temperature-controlled flow chamber system (Warner Instrument) filled with YES and maintained at 32°C as previously described (Karagiannis and Young, 2001). Instead of poly-L-lysine, cells were kept immobilized with the use of a small (600 mm diameter) cylindrically shaped 0.8% agarose pad made in YES. The agarose pad was made to the same height as the chamber and thus cells were gently trapped between the agarose surface and the cover-slip forming the bottom of the chamber. Cells were prepared for immunofluorescence as previously described (Balasubramanian et al., 1997). Rabbit anti-GFP primary antibodies (Molecular Probes A-6455, Eugene, OR) and goat anti-rabbit IgG secondary

antibodies (Molecular Probes A-11008), Eugene, OR) were used at 1:1000 and 1:800 dilutions, respectively.

## Results

### Clp1p is essential when the apparatus of cytokinesis is mildly perturbed via mutations or chemical inhibitors

To evaluate the physiological function of Clp1p in coordinating mitosis and cytokinesis, we made double mutants lacking Clp1p and one of several components listed under group I in Table 1. The chosen mutants included myosin light chain *cdc4-8* (McCollum et al., 1995), myosin II heavy chain *myo2-E1* (Balasubramanian et al., 1998), IQGAP-related molecule *rng2-D5* (Eng et al., 1998), PCH and SH3 domain protein *cdc15-140* (Fankhauser et al., 1995) and 1,3- $\beta$ -glucan synthase *cps1-191* (Liu et al., 1999). We also made double mutants between *clp1 $\Delta$*  and four non-essential components of the actomyosin ring, such as the type II myosin heavy chain Myp2p (Bezanilla et al., 1997; Motegi et al., 1997), myosin regulatory light chain Rlc1p (Naqvi et al., 2000; Le Goff et al., 2000), the SH3 domain protein Cyk3p (H.W. and M.K.B., unpublished), and the type V myosin Myo52p (Win et al., 2001; Motegi et al., 2001). Strikingly, we found that under conditions in which single mutants containing wild-type *clp1*<sup>+</sup> were able to divide and form colonies, albeit with altered morphology, the double mutants that lack Clp1p were inviable (Fig. 1A, and data not shown). Single mutants containing *clp1*<sup>+</sup> could assemble functional septa (shown with arrowheads in Fig. 1B), indicating successful cytokinesis, and rarely accumulated more than two nuclei (Fig. 1C). However, when *clp1* was deleted, the double mutants, only infrequently assembled division septa (Fig. 1B and C; data not shown) and a significant proportion of double-mutant cells accumulated 4 or more nuclei (Fig. 1C). Taken together these studies suggest that *clp1 $\Delta$*  cells are supersensitive to a wide variety of perturbations to the cell division machinery, including mutations that affect assembly of the actomyosin ring, division septum and secretion.

The importance of Clp1p in responding to minor damage of cell division structures was further supported by our analysis of wild-type and *clp1 $\Delta$*  cells treated with a low dose of latrunculin A (Lat A), a drug that prevents actin polymerization (Ayscough et al., 1997). Typically, treatment of wild-type cells with 10  $\mu$ M or higher concentrations (high dose), leads to the loss of all detectable F-actin structures and lethality (Pelham and Chang, 2001). In contrast, 0.2  $\mu$ M Lat A (referred to as 'low dose' in this manuscript) did not cause any detectable defects in the appearance of F-actin structures in wild-type cells although, the effect on F-actin function was detected as a delay in progression through cytokinesis.

Wild-type and *clp1 $\Delta$*  cells were synchronized in early G2 by centrifugal elutriation, treated with low doses of Lat A, and subsequently sampled at 30 minute intervals over a 6 hour time frame. The kinetics of the nuclear cycle progression and septation were then examined by staining with aniline blue and DAPI, to visualize the division septum and nuclei, respectively (Fig. 1D and E). Wild-type and *clp1 $\Delta$*  cells treated with DMSO showed similar degrees of synchrony with respect to mitosis and septation. However, these strains differed dramatically when exposed to low doses of Lat A. Although wild-type and *clp1 $\Delta$*  cells entered mitosis synchronously and with comparable kinetics upon low-dose Lat A treatment, wild-type

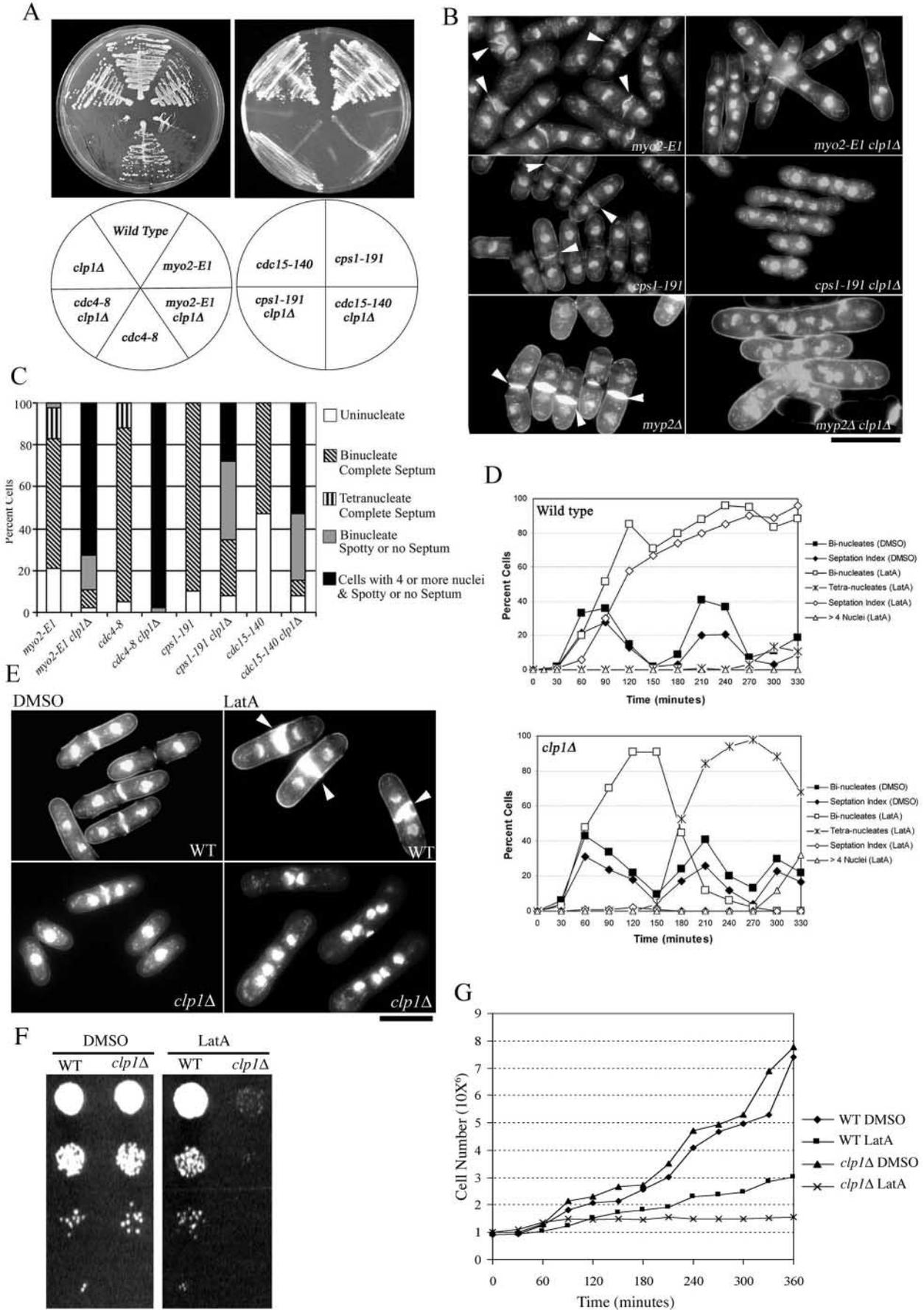
cells persisted as binucleates that slowly completed assembly of the division septum that was abnormal in appearance, but that completely bisected the cell (Fig. 1E; marked with arrowhead). In contrast, *clp1 $\Delta$*  cells treated with Lat A did not assemble detectable division septa, exited mitosis with normal kinetics, and completed another round of mitosis within the time-frame of the experiment leading to the accumulation of tetranucleate cells (Fig. 1D and E). Furthermore, wild-type cells treated with Lat A were capable of colony formation (Fig. 1F) and showed a modest, but convincing, increase in cell number, compared with DMSO-treated wild-type cells (Fig. 1G). However, *clp1 $\Delta$*  cells were unable to form colonies on Lat A-containing plates and did not show a significant increase in cell number upon Lat A treatment (Fig. 1F and G).

Taken together, these results clearly demonstrate that upon low-dose Lat A treatment, wild-type cells are competent to form septa as well as inhibited in progression through further nuclear cycles. In contrast, *clp1 $\Delta$*  mutants are unable to form septa and proceed through subsequent nuclear cycles without delay. The ability of wild-type, but not *clp1 $\Delta$*  cells, to delay nuclear division upon Lat A treatment following failed cytokinesis was also established by imaging individual cells expressing a histone-GFP fusion (see Movies 1-4, <http://jcs.biologists.org/supplemental/>).

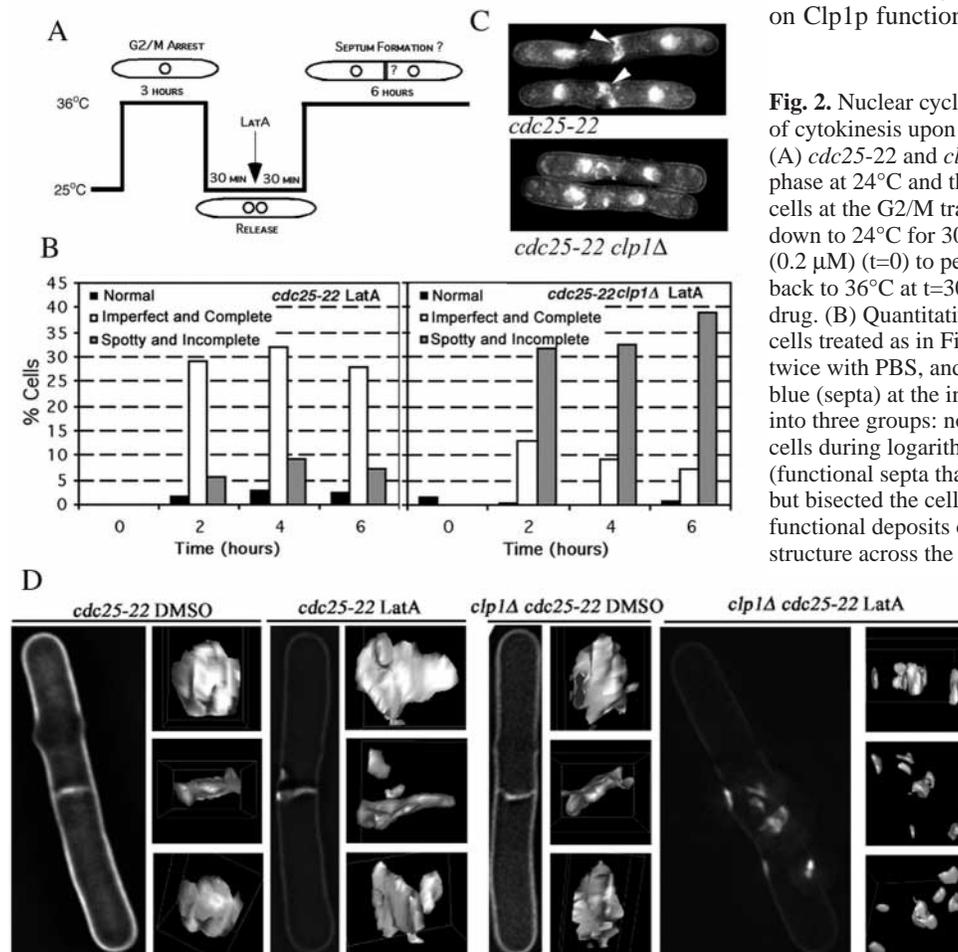
Because *clp1 $\Delta$*  mutants are sensitive to perturbations to F-actin, myosin II heavy and light chains, the PCH domain protein Cdc15p, cell wall enzymes and potential regulators of secretion, it is unlikely that Clp1p acts in a redundant cytokinetic pathway with all these components. We concluded that an important physiological role for Clp1p was in promoting cell viability upon delays in cytokinesis caused by a variety of perturbations that affected the cytokinetic machinery.

Clp1p has a well-defined role in regulating mitotic entry (Trautmann et al., 2001; Cueille et al., 2001) (Fig. 1C). It was thus possible that Clp1p promoted the completion of cytokinesis indirectly through mediating an interphase (G2) arrest. If this were the case (i.e. if Clp1p was required solely for nuclear cycle delay and not for the physical assembly of division septa) then an artificially induced cell cycle block should be sufficient to allow completion of cytokinesis in low dose Lat A treated *clp1 $\Delta$*  cells. Conversely, if Clp1p possessed additional function(s) required for division septum assembly, then an artificially induced cell cycle block would be insufficient to allow completion of cytokinesis.

To distinguish between these two possibilities we utilized a *cdc25-22* mutant to arrest cell cycle progression in G2 independently of Clp1p (Berry and Gould, 1996). Logarithmically growing *cdc25-22* and *cdc25-22 clp1 $\Delta$*  cells were shifted to the restrictive temperature of 36°C to synchronize cells in late G2. Cells were then released to the permissive temperature of 25°C to allow entry into the first mitosis. During the shift-down, cells were treated with a low dose of Lat A to perturb the actomyosin ring and then shifted back to 36°C in order to block entry into the subsequent mitosis (Fig. 2A). Using this strategy we were able to assay the septum-forming ability of *clp1 $\Delta$*  mutants in the presence of low doses of Lat A under conditions where nuclear cycle progression was blocked by a means that did not require Clp1p itself. Intriguingly, even though the majority of *cdc25-22* cells were able to form improper but complete septa, *cdc25-22*



**Fig. 1.** Clp1p is essential when cell division structures are mildly perturbed and ensures the completion of cytokinesis. (A) Cells of the indicated genotype were streaked to YES plates and assayed for colony formation after 3 days at 30°C (left) and 32°C (right). (B) Cells of indicated genotype were cultured to exponential growth phase at 24°C and then shifted to 32°C for 5 hours, fixed and then stained with aniline blue and DAPI to visualize cell wall/septa and nuclei respectively. Septa are indicated with arrowheads. Scale bar 10  $\mu$ m. (C) Septum formation in group I cytokinesis mutants at semi-permissive temperatures in the presence or absence of Clp1p. Cells of the indicated genotype were cultured to exponential growth phase at 24°C, shifted to 32°C for 5 hours, fixed and then stained with aniline blue and DAPI to visualize cell wall/septa and nuclei respectively. (D) Wild-type and *clp1* $\Delta$  cells were cultured to exponential growth phase at 24°C, synchronized in early G2 by centrifugal elutriation and then treated with a low dose (0.2  $\mu$ M) of Lat A or DMSO (solvent control) and cultured at 32°C. Cells were subsequently fixed at 30 minute intervals and stained with DAPI (nuclei) and aniline blue (cell wall/septa). (E) Wild-type and *clp1* $\Delta$  cells treated as in Fig. 1D (at t=240 minutes) and stained with both DAPI (nuclei) and aniline blue (cell wall/septa). Imperfect, but functional, septa are indicated with arrowheads. Scale bar 10  $\mu$ m. (F) Tenfold serial dilutions of wild-type and *clp1* $\Delta$  cells grown on YES plates containing either 0.25  $\mu$ M Lat A or DMSO. Images were captured after 3 days growth at 32°C. (G) Wild-type and *clp1* $\Delta$  cells were cultured to exponential growth phase at 24°C, synchronized in early G2 by centrifugal elutriation and then treated with a low dose (0.2  $\mu$ M) of Lat A or DMSO (solvent control) and cultured at 32°C. Cell number was determined using a haemocytometer.

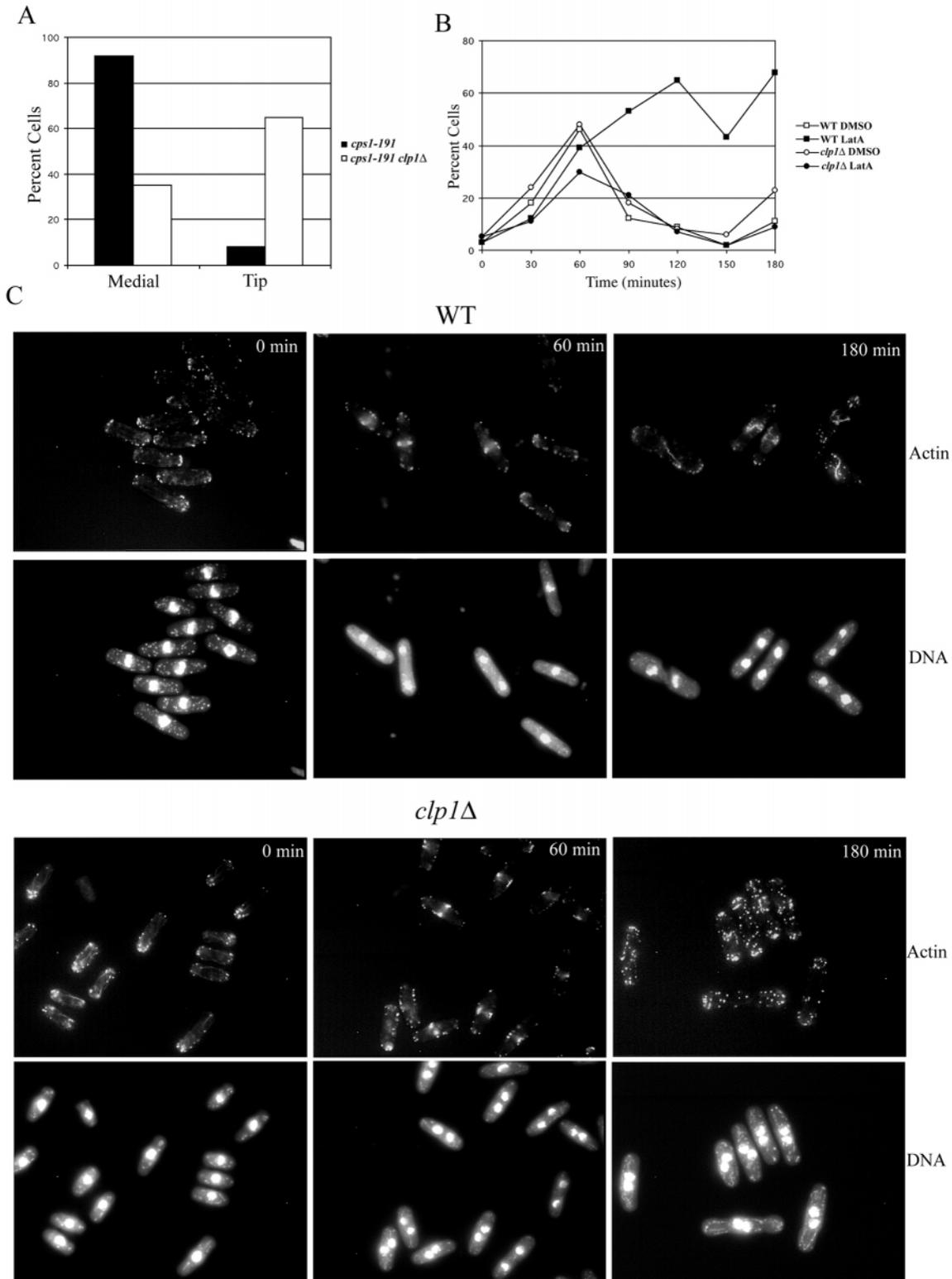


*clp1* $\Delta$  cells were not (Fig. 2B,C). Double mutants generated predominantly binucleate cells with non-functional spot-like deposits of septum material. The functionality of septa in *cdc25-22* versus *clp1* $\Delta$  *cdc25-22* cells was confirmed by 3D reconstructions of de-convolved Z-stack images. While *cdc25-22* cells generally formed mis-shapen, but complete septa that clearly bisected the cell into two independent compartments, *clp1* $\Delta$  *cdc25-22* cells formed fragmented, incomplete septa that were unable to divide the cell into two (Fig. 2D). Thus, in addition to its role in delaying entry into mitosis, Clp1p must also possess other function(s) that contribute to successful cell division when progression through cytokinesis is delayed.

### Clp1p-dependent maintenance of the actomyosin ring upon perturbation of cytokinesis

In fission yeast, F-actin is detected in patch-structures concentrated at the cell tips during interphase and in a ring at the medial region of the cell in mitotic and post-mitotic cells undergoing cytokinesis (Marks and Hyams, 1985). Intriguingly, F-actin is also retained in the medial ring in a high proportion of *cps1-191* single mutants even though arrested *cps1-191* cells contain two G2 nuclei (Liu et al., 2000; Le Goff et al., 1999b). We therefore considered the possibility that Clp1p might also be involved in actively maintaining the actomyosin ring to ensure completion of cytokinesis. Consistent with this possibility, we found that the maintenance of medial actomyosin rings in *cps1-191* mutants depended on Clp1p function (Fig. 3A).

**Fig. 2.** Nuclear cycle arrest is insufficient to allow completion of cytokinesis upon perturbation of the cell division machinery. (A) *cdc25-22* and *clp1* $\Delta$  *cdc25-22* cells were grown to early log phase at 24°C and then shifted to 36°C for three hours to arrest cells at the G2/M transition. Cells were subsequently shifted down to 24°C for 30 minutes, treated with a low dose of Lat A (0.2  $\mu$ M) (t=0) to perturb the actomyosin ring and then shifted back to 36°C at t=30 minutes in the continuing presence of the drug. (B) Quantitative data for *cdc25-22* and *clp1* $\Delta$  *cdc25-22* cells treated as in Fig. 2A and then fixed with methanol, washed twice with PBS, and stained with DAPI (nuclei) and aniline blue (septa) at the indicated time points. Septa were classified into three groups: normal (similar to septa formed in wild type cells during logarithmic growth); imperfect and complete (functional septa that appeared thicker and more disorganized but bisected the cell); and spotty and incomplete (non-functional deposits of septal material that failed to form a linear structure across the width of the cell). (C) *cdc25-22* and *clp1* $\Delta$  *cdc25-22* cells treated as in Fig. 2A (at t=4 hours) and stained with both DAPI (nuclei) and aniline blue (cell wall/septa). Imperfect but functional, septa are indicated with arrowheads. (D) *cdc25-22* and *clp1* $\Delta$  *cdc25-22* cells treated as in Fig. 2A (at t=4 hours) and stained with aniline blue (cell wall/septa). Z-series were obtained and deconvolved as described in Materials and Methods. Max projections of the entire cell are shown to the left of each panel, whereas three alternate views of 3D reconstructions of septa are shown to the right.



**Fig. 3.** Clp1p is important for the maintenance of the actomyosin ring at the medial region of the cell. (A) *cps1-191* and *clp1Δcps1-191* were grown to exponential phase at 24°C, shifted to 36°C for four hours and stained with rhodamine-conjugated phalloidin to determine the distribution of actin. 'Medial' refers to the localization of actin in a medial actomyosin ring structure. 'Tip' refers to the localization of actin patches to the cell ends. (B) Wild-type and *clp1Δ* cells were cultured to exponential growth phase at 24°C, synchronized in early G2 by centrifugal elutriation and then treated with a low dose (0.2 μM) of Lat A or DMSO (solvent control). Cells were subsequently fixed at 30-minute intervals and stained with DAPI (nuclei) and ALEXA-488 conjugated phalloidin (actin). The graph shows the percent cells with actomyosin rings. (C) Images of cells treated as in B.

To address the role of Clp1p in maintenance of actomyosin rings more rigorously, we studied the localization of F-actin in wild-type cells and *clp1Δ* cells treated with a low dose of Lat A, which we have shown leads to a significant delay in cytokinesis. We predicted that F-actin rings would be maintained for a long period of time in wild-type cells treated with a low dose of Lat A, whereas rings would not be maintained in *clp1Δ* cells. Wild-type and *clp1Δ* cells were synchronized in early G2, treated with low doses of Lat A, and were then fixed and stained at 30 minute intervals to determine the proportion of cells with medial F-actin structures. *clp1Δ* and wild-type cells were able to form medial rings upon entry into the first mitosis (~30% and 40% of cells, respectively), suggesting that *clp1Δ* cells were fully capable of actomyosin ring assembly in the presence of low doses of Lat A. The kinetics of ring assembly upon entry into the first mitosis was also similar in cells treated with Lat A or DMSO. Interestingly, wild-type cells could maintain medial F-actin ring structures for the duration of the experiment (Fig. 3B,C) and were able to assemble improper but complete division septa indicative of successful ring constriction (Fig. 1D,E). The appearance of rings ranged from normal to malformed. They contained myosin II (data not shown) and were predominantly in the medial region of the cell as a broader structure. Unlike wild-type cells, medial F-actin rings in *clp1Δ* cells disassembled upon mitotic exit leading to the formation of unseptated cells with two nuclei. Upon failed cytokinesis in the *clp1Δ* mutant nuclei were observed to cluster (Fig. 3C, marked with arrowheads), as typically observed in SIN mutants that fail in cytokinesis (Hagan and Yanagida, 1997). In contrast, the nuclei in wild-type cells treated with Lat A do not cluster (Fig. 3C, marked with arrows), presumably due to the maintenance of the underlying post-anaphase array of microtubules (M.M., unpublished) as has been reported for other group I mutants (Pardo and Nurse, 2003; Liu et al., 2002). The synchronous culture experiment established that the actomyosin ring was maintained for a prolonged period of time in the presence of Clp1p but not in its absence. We concluded that Clp1p function was important to maintain the actomyosin ring at the medial region of the cell until cytokinesis was complete, in particular under adverse conditions.

We have shown that the actomyosin ring is not maintained in *clp1Δ* cells upon perturbation of the cytokinetic machinery. We therefore investigated the dynamics of the actomyosin ring upon low dose Lat A treatment to gain insight into the mechanism of actomyosin ring maintenance in wild-type cells and its collapse in *clp1Δ* cells. We utilized wild-type or *clp1Δ* mutant cells expressing a GFP-tagged version of the actomyosin ring component Rlc1p (Naqvi et al., 2000; Le Goff et al., 2000) and performed time-lapse imaging of these cells treated with either DMSO (control) or a low dose of Lat A. At least 10 cells with medial rings with well-separated nuclei were typically imaged for each experiment. Dynamics of actomyosin ring constriction and progression through cytokinesis in DMSO treated *clp1Δ* cells was comparable to that in wild-type cells and the process of ring constriction and septum assembly typically lasted between 25-35 minutes (Fig. 4). As shown earlier (Fig. 3B,C), *clp1Δ* cells treated with a low dose of Lat A were capable of assembling actomyosin rings. However, these rings did not constrict upon completion of mitosis but fragmented leading to the loss of the actomyosin

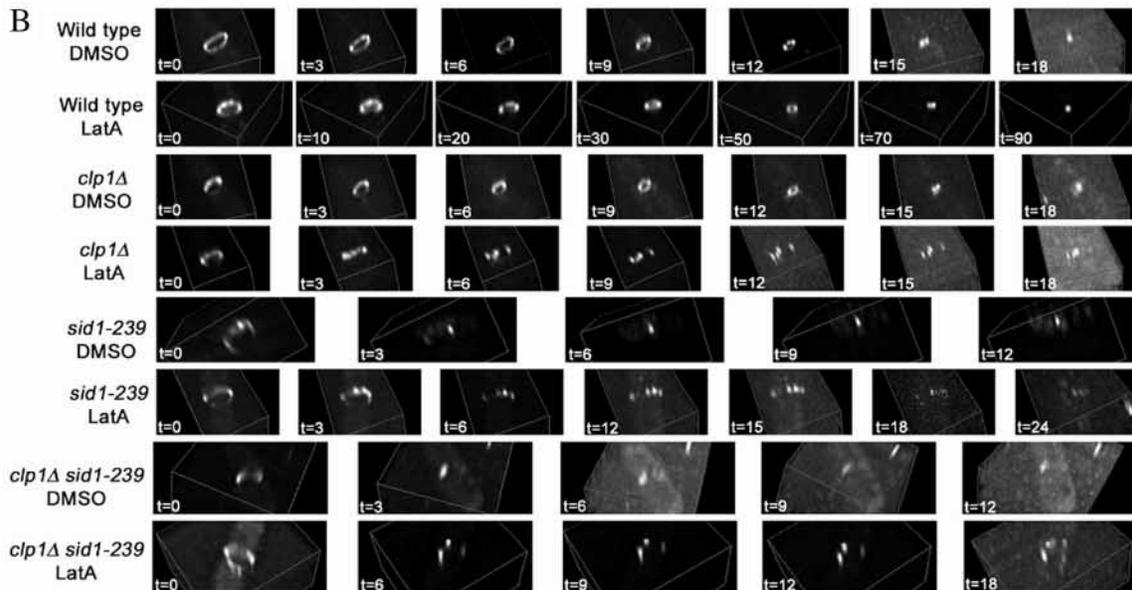
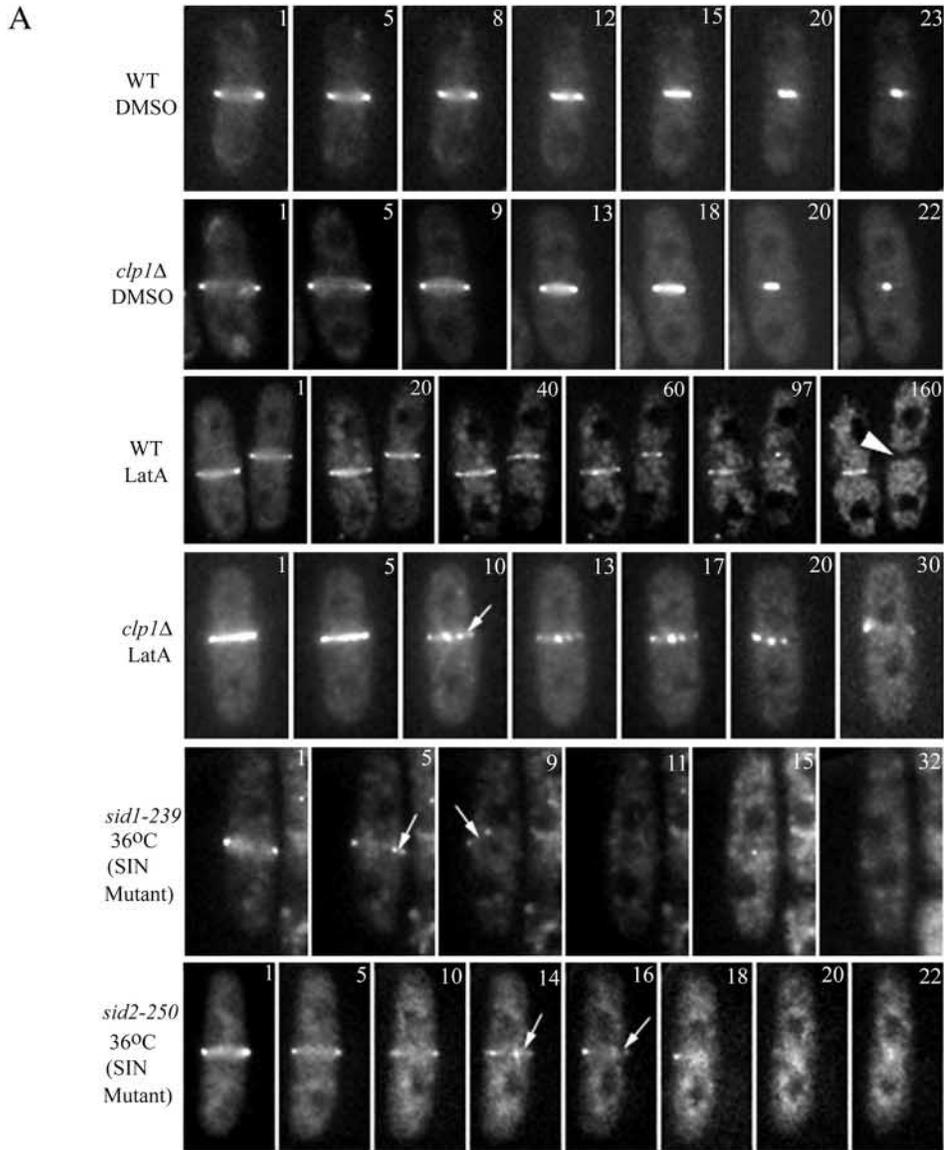
ring (Fig. 4A; marked with an arrow). We then imaged wild-type cells treated with a low dose of Lat A to study actomyosin ring dynamics in these cells (Fig. 4A). Interestingly, the actomyosin ring was maintained for prolonged periods between 40-80 minutes and eventually underwent slow constriction (60 and 97 minutes in Fig. 4A) and division septum assembly leading to cell division (arrowhead in Fig. 4A).

To observe ring structure more closely we performed similar experiments in which 3D reconstructions of de-convolved Z-stack images were examined. These results were similar to our previous time lapse microscopy results and clearly showed that rings in *clp1Δ* cells fragmented in response to Lat A whereas rings in wild-type cells were stabilized to a sufficient degree to allow constriction, albeit over a much longer time frame than DMSO controls (Fig. 4B). Thus, whereas in wild-type cells, where the actomyosin ring is maintained for a long period of time and its constriction is slowed down but completed upon exposure to a low dose of Lat A, the rings disassemble under a similar regime in *clp1Δ* cells.

Previous studies have shown that SIN mutants assemble actomyosin rings that are not maintained upon completion of mitosis, leading to defects in septation (Gould and Simanis, 1997; Guertin et al., 2002; Balasubramanian et al., 1998). We considered the possibility that the dynamics of the actomyosin ring in SIN mutants might phenocopy the actomyosin ring loss phenotype of *clp1Δ* cells treated with a low dose of Lat A. To address this question, *sid1-239* and *sid2-250* cells (Fig. 4; data not shown for *spg1-106* and *cdc14-118*) expressing Rlc1p-GFP were shifted to the restrictive temperature and the dynamics of the actomyosin ring monitored. SIN mutants were capable of assembling medial actomyosin rings as previously shown in several studies (Gould and Simanis, 1997; Guertin et al., 2002; Balasubramanian et al., 1998). Interestingly, these actomyosin rings disassembled upon completion of mitosis (marked with arrows in Fig. 4A) in a manner similar to that observed in low-dose Lat A treated *clp1Δ* cells. Furthermore, *sid1-239 clp1Δ* cells shifted to semi-permissive temperatures in the presence of DMSO or Lat A showed a ring disassembly phenotype similar to that shown by *sid1-239* single mutants at the restrictive temperature, suggesting that Sid1p and Clp1p function in a common pathway rather than in overlapping additive pathways (Fig. 4B). Based on these studies we conclude that the SIN is important for actomyosin ring maintenance during every cell cycle, whereas Clp1p is important for actomyosin ring maintenance only if the cell division machinery is perturbed.

#### Active SIN signaling greatly bypasses the need for Clp1p function upon perturbation of the cell division machinery

The SIN plays an important role in the G2 delay following cytokinesis failure (Gould and Simanis, 1997; Feierbach and Chang, 2001). Thus, we wanted to test whether the SIN, like Clp1p, was essential for cell viability when cytokinesis was slowed down. Because the SIN is essential we used a SIN mutant, *cdc14-118*, that was partially active, but viable, at 30°C. We found that *myo2-E1 cdc14-118* cells resembled *myo2-E1 clp1Δ* mutants (Fig. 1A,B) in that these cells were inviable at 30°C, whereas both single mutants were viable at



**Fig. 4.** Dynamics of Clp1p-dependent actomyosin ring maintenance upon perturbation of cytokinetic machinery. (A) Cells of the indicated genotypes expressing Rlc1p-GFP were imaged under conditions shown on the left-hand side. The numbers on the top right of each frame indicates the time in minutes. Note that whereas the Rlc1p-GFP rings are maintained for a prolonged period of time, leading to the accomplishment of division septum assembly in wild-type cells upon treatment with a low dose of Lat A (0.2  $\mu$ M), these rings fragment in similarly treated *clp1* $\Delta$  cells. Note also that SIN mutants display a phenotype similar to that observed in *clp1* $\Delta$  cells treated with a low dose of Lat A. (B) 3D-reconstructions of Rlc1p-GFP expressing cells of the indicated genotype treated with DMSO or 0.2  $\mu$ M Lat A. Time (t) is indicated in minutes. Wild-type, *clp1* $\Delta$ , and *clp1* $\Delta$  *sid1-239* cells were imaged at 32°C. *sid1-239* cells were imaged at 36°C.

this temperature. Examination of the double mutants showed that they were multinucleate and were incapable of assembling division septa at 30°C, whereas the single mutants were largely unaffected (Fig. 5A,B). Furthermore, all SIN single mutants treated with low doses of Lat A under permissive and semi-permissive conditions accumulated multiple nuclei and failed to septate similar to *clp1* $\Delta$  mutants (data not shown). Given that SIN mutants display a phenotype similar to that displayed by *clp1* $\Delta$  mutants upon mild damage to the cell division apparatus, and that ectopic SIN activation leads to septation in interphase cells, we reasoned that SIN hyper-activation might rescue the checkpoint defect in *clp1* $\Delta$  cells. We therefore examined if ectopic activation of the SIN by mutation of *cdc16* (encodes a GAP for the Spg1p GTPase) (Minet et al., 1979; Fankhauser et al., 1993; Furge et al., 1998) allowed *clp1* $\Delta$  cells treated with low doses of Lat A to complete division septum assembly and arrest with two nuclei. Cells of the genotypes *cdc16-116*, *clp1* $\Delta$ , and *cdc16-116 clp1* $\Delta$  were treated with a low dose of Lat A and shifted to the restrictive temperature of 36°C for four hours to allow inactivation of the temperature-sensitive Cdc16-116p (Fig. 5C,D). Interestingly, we observed a high proportion of *clp1* $\Delta$  *cdc16-116* cells with two nuclei and an improper but complete division septum (marked with arrowheads in Fig. 5C). This was in contrast to *clp1* $\Delta$  single mutants where only 3% of cells displayed such a phenotype. These observations suggested that ectopic activation of the SIN cascade significantly rescues the G2 arrest and septation defects of *clp1* $\Delta$  cells upon perturbation of the cytokinetic machinery and suggests that a normal role for Clp1p is to prolong SIN signaling to allow completion of cytokinesis. Similar results were obtained when the cytokinesis checkpoint was activated using other means, such as using the *cps1-191* mutant (data not shown).

To further understand the mechanism by which Clp1p influenced SIN function, we assayed the localization of SIN components in wild-type and *clp1* $\Delta$  cells exposed to low doses of Lat A. The SIN is composed of a group of spindle pole body localized proteins. Of these, Cdc7p, Sid1p, and Cdc14p localize to one SPB in late mitosis and remain there until the completion of cytokinesis. Previous studies have proposed that the localization of these proteins to a single SPB signifies active SIN signaling (reviewed in McCollum and Gould, 2001). We therefore assessed the kinetics of localization of Cdc7p and Sid1p in wild-type and *clp1* $\Delta$  cells treated with Lat A. Synchronous populations of wild-type and *clp1* $\Delta$  cells

expressing GFP-Sid1p or Cdc7p-GFP were generated by centrifugal elutriation and released into growth medium containing Lat A. As in previous experiments, wild-type and *clp1* $\Delta$  cells entered mitosis with roughly comparable kinetics. Sid1p-GFP and Cdc7p-GFP were also detected on one spindle pole body with relatively similar kinetics (Fig. 6A). However, whereas Sid1p-GFP and Cdc7p-GFP were retained at the SPB for prolonged periods in wild type cells, until completion of cytokinesis, Sid1p-GFP and Cdc7p-GFP were undetectable in *clp1* $\Delta$  cells treated with Lat A (Fig. 6A). Thus, we conclude that Clp1p is not required for the initial localization of Sid1p and Cdc7p to one SPB during mitosis, but is required to maintain them at the SPB upon cytokinesis delay in the presence of Lat A, to allow completion of cell division.

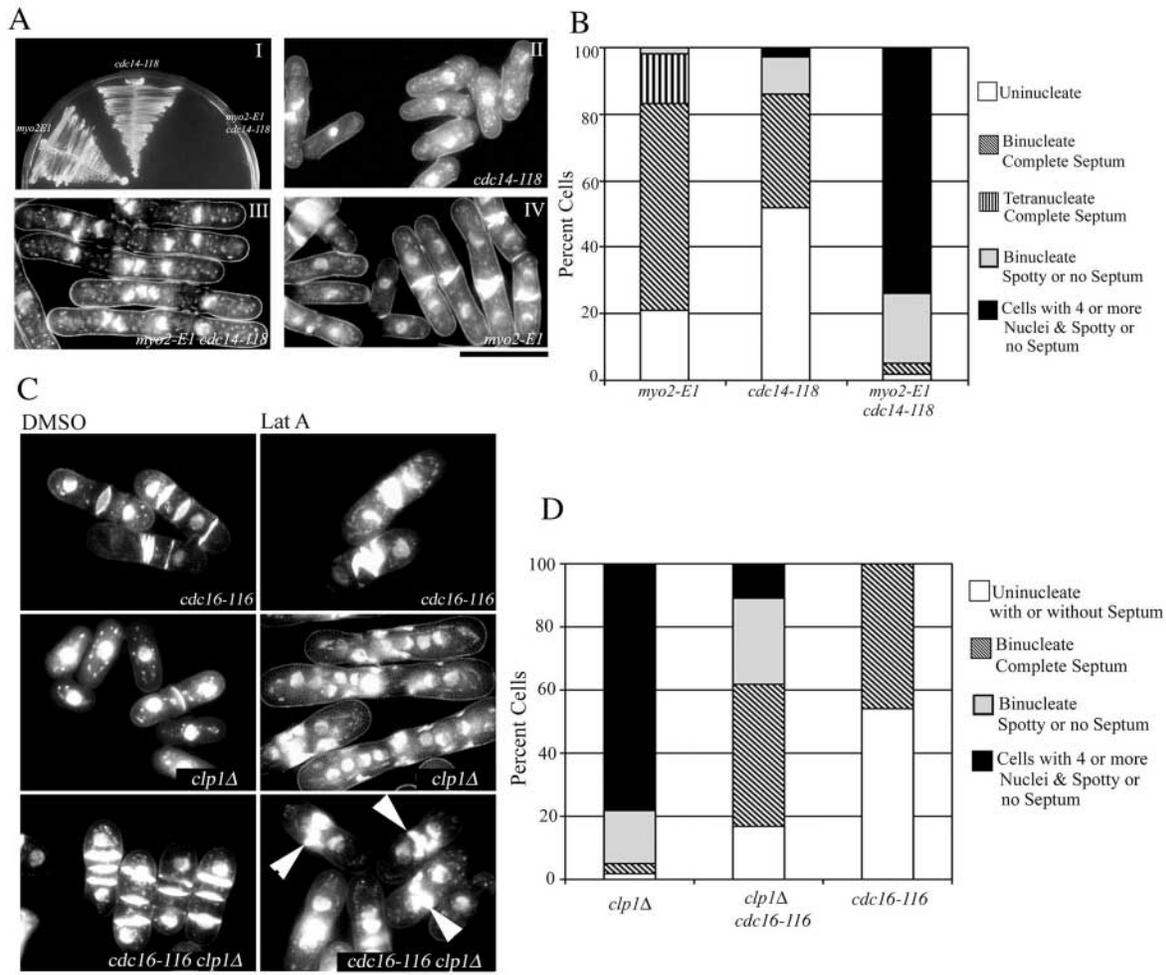
We then studied the role of SIN in Clp1p function. Previous studies have shown that Clp1p is present in the nucleolus in interphase cells and is released from the nucleolus to the cytoplasm in cells undergoing cytokinesis (Trautmann et al., 2001; Cueille et al., 2001). In addition, Clp1p is also detected at the SPB, mitotic spindle, and the actomyosin ring. We have shown that weak cytokinetic defects caused by faults in various components of the cell division apparatus result in lethality to SIN mutants even under permissive temperature conditions, suggesting a role for SIN in 'cytokinesis checkpoint' function. We therefore studied the localization of Clp1p-GFP in wild-type and *cdc14-118* mutants (grown at permissive temperature) in the presence and absence of Lat A. In wild-type and *cdc14-118* mutants treated with DMSO, Clp1p-GFP was in the nucleolus of interphase cells and in the cytoplasm of mitotic and cytokinetic cells. Interestingly, whereas Clp1p-GFP was retained in the cytoplasm of wild-type cells treated with Lat A, Clp1p-GFP readily relocalized to the nucleolus in *cdc14-118* cells treated with Lat A (Fig. 6B). We therefore conclude that Clp1p-GFP maintenance in the cytoplasm in response to cytokinesis failure requires functional SIN.

## Discussion

In this study we have provided evidence for, and described the physiological role of Clp1p in ensuring cell viability upon mild perturbation of the cell division machinery. Based on this evidence we propose a model in which Clp1p, together with its effector, the SIN, form an integral part of a cytokinesis checkpoint that ensures damaged cell division structures are maintained and/or reformed in order to allow the completion of cytokinesis.

### Clp1p, a bona fide checkpoint protein, and its physiological role

We propose that aspects of cytokinesis, such as actomyosin ring function, cell wall assembly, and possibly secretion are monitored by the cytokinesis checkpoint, through a mechanism requiring Clp1p and the SIN. This is demonstrated by the dramatic cytokinetic failure of *clp1* and SIN mutants (under semi-permissive conditions) when the cell division machinery is perturbed through the use of a variety of cytokinesis mutants as well as through the use of cytoskeletal inhibitors. The mutants studied include those that are defective in actomyosin ring assembly, division septum assembly, as well as a type V myosin mutant that is likely involved in vesicle targeting. The fact that



**Fig. 5.** SIN mutants are hypersensitive to mild perturbations of the cell division machinery whereas ectopic activation of the SIN compensates for the loss of Clp1p. (AI) *cdc14-118*, *myo2-E1*, and *myo2-E1 cdc14-118* mutants were streaked to YES plates and assayed for colony formation after 3 days at the semi-permissive temperature of 30°C. (AII, III and IV) *cdc14-118*, *myo2-E1* and *myo2-E1 cdc14-118* cells were grown at 24°C to early-log phase, shifted to 30°C for six hours, and then fixed and stained with DAPI and aniline blue to visualize nuclei and division septa, respectively. Scale bar 10  $\mu$ m. (B) Quantitative data for cells in AII-IV. (C) Cells of the indicated genotypes were grown to early-log phase at 24°C, shifted to 36°C, and then treated with 0.2  $\mu$ M of Lat A or DMSO (solvent control) for 5 hours. Cells were subsequently fixed and stained with DAPI and aniline blue to visualize nuclei and division septa respectively. Scale bar 10  $\mu$ m. (D) Quantitative data for cells treated with Lat A in C.

Clp1p is a component of the actomyosin ring (Trautmann et al., 2001; Cueille et al., 2001) makes it an attractive candidate for monitoring the progression/completion of cytokinesis. However, it is equally likely that Clp1p is a downstream effector that responds to cues initiated upon a failure of cytokinesis.

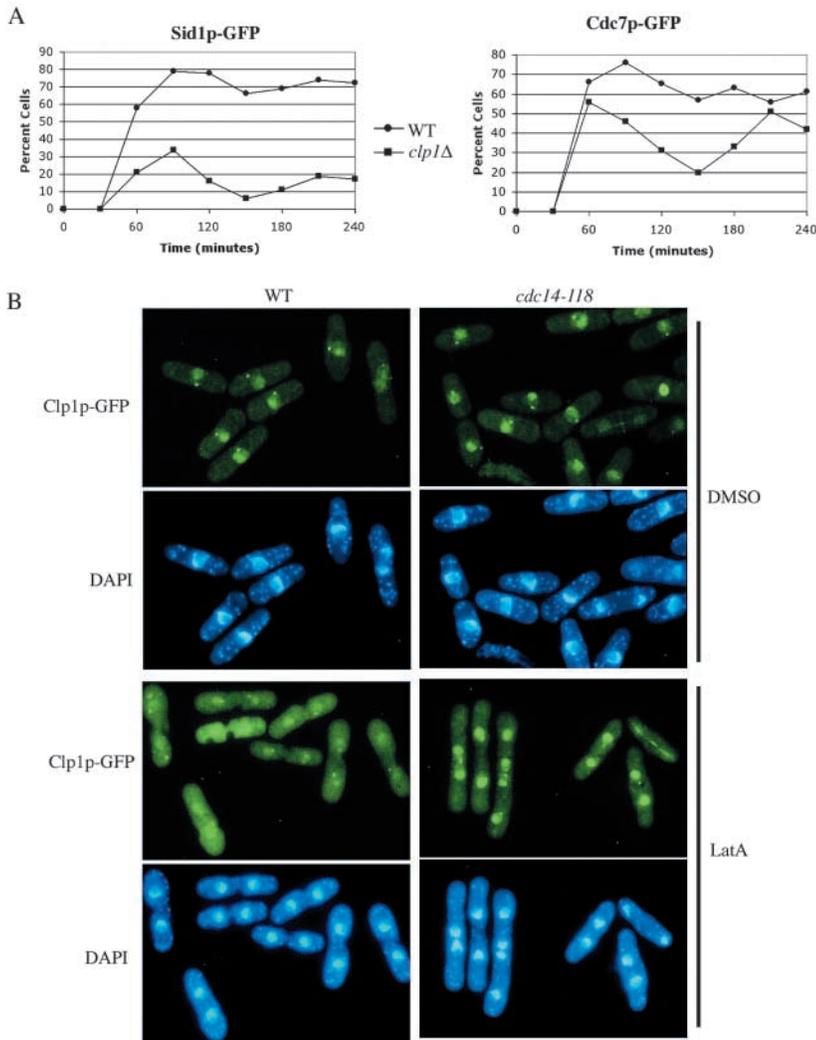
It is also interesting to note the parallels between Clp1p and other previously described checkpoint proteins, such as Mad2p and the budding yeast Rad9p (He et al., 1997; Weinert and Hartwell, 1988). All three of these proteins are normally non-essential, but do play a critical role in ensuring viability upon perturbation of actin/cell wall, microtubular machinery and DNA integrity, respectively. The fact that cells defective for Clp1p are sensitive to a variety of perturbations to the cell division machinery is also consistent with a role for Clp1p in monitoring several aspects of cytokinesis. This is akin to the sensitivity of, for example, *rad9* mutants to a variety of DNA damaging agents.

What is the normal function of this checkpoint? The checkpoint might be involved in allowing a period of time to

correct minor defects in the assembly of the cell division apparatus due to temperature shifts, or possibly toxins (equivalent to cytochalasin A, latrunculin A, pneumocandins and echinocandins) (Cooper, 1987; Denning, 1997; Georgopadakou, 1998; Spector et al., 1989; Ayscough et al., 1997) secreted by other organisms that destroy the actomyosin ring, cell wall biosynthetic machinery, or secretory machinery. The fact that a low percentage of *clp1<sup>-</sup>* cells display cytokinesis defects (Cueille et al., 2001; Trautmann et al., 2001) indicates that processes, such as actomyosin ring assembly and cell wall secretion in normal wild-type cells are also prone to errors that might be corrected by the cytokinesis checkpoint.

Repair of the cell division apparatus and completion of division septum assembly in response to activation of the cytokinesis checkpoint

What are the responses to cytokinesis checkpoint activation?



**Fig. 6.** (A) Wild-type and *clp1*Δ cells carrying integrated copies of Sid1p-GFP and Cdc7p-GFP were cultured to exponential growth phase at 24°C, synchronized in early G2 by centrifugal elutriation and then treated with a low dose (0.2 μM) of Lat A or DMSO (solvent control) and cultured at 32°C. Cells were subsequently fixed at 30 minute intervals, stained with antibodies against GFP (see Materials and Methods), and scored for localization of Sid1p-GFP (left) and Cdc7p-GFP (right) to the SPB. (B) Wild-type and *cdc14-118* cells carrying an integrated copy of Clp1p-GFP were treated with 0.2 μM Lat A or DMSO, shifted to 32°C for 3.5 hours, fixed and then stained with DAPI. Clp1p-GFP localization was monitored using GFP autofluorescence.

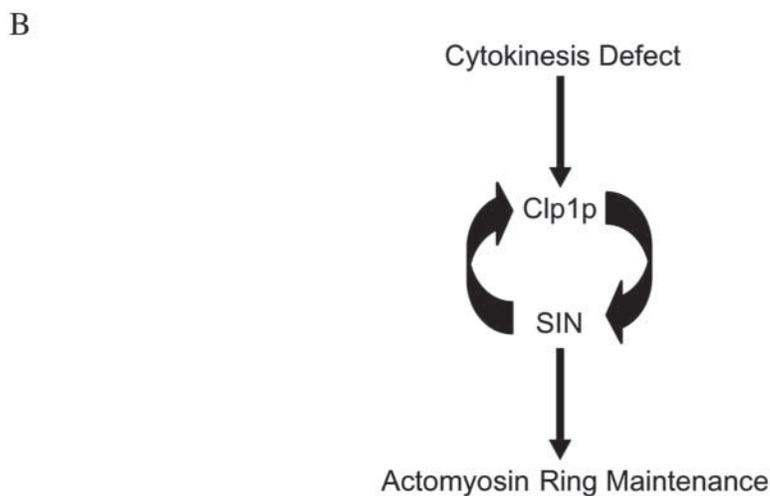
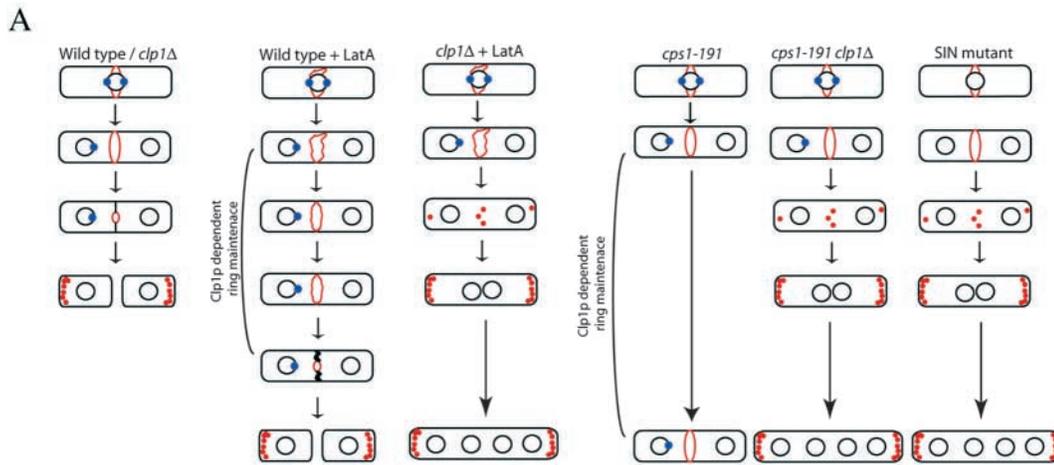
and in *cps1-191* mutants at the restrictive temperature (Trautmann et al., 2001) suggesting a role for Clp1p in prolonging the duration of SIN signaling to effect completion of cytokinesis. The suggestion that Clp1p functions by prolonging the duration of SIN signaling is also consistent with the similar phenotypes observed (summarized in Fig. 7A) in SIN mutants at the fully restrictive temperature, as well as *clp1*Δ cells in which the cytokinetic machinery has been perturbed through treatment with Lat A. Interestingly, the localization of Clp1p itself appears to depend on SIN function, given that Clp1p is not maintained in the cytoplasm in *cdc14-118* (a SIN mutant) cells treated with Lat A under semi-permissive conditions, whereas Clp1p is retained in the cytoplasm of wild-type cells treated with Lat A (Fig. 6). Thus, we propose that Clp1p and SIN function in a positive-feedback loop in which the localization SIN depends on Clp1p and the maintenance of Clp1p in the cytoplasm depends on SIN function (Fig. 7B). We have also shown

In this and previous studies, it has been shown that the checkpoint prevents the two interphase nuclei following failed cytokinesis from entering mitosis (Trautmann et al., 2001; Cueille et al., 2001). We have shown the activation of the checkpoint actively maintains physical structures important for cell division until cytokinesis is completed (Figs 3, 4). We have also shown that in cells lacking Clp1p, a G2 delay provided using a *cdc25-22* mutation is insufficient to allow completion of division septum assembly. This result establishes that Clp1p has independent roles in the physical maintenance of the actomyosin ring and in providing a G2 delay upon perturbation of the cell division apparatus. These data also establish that negative regulation of Cdc2p kinase function is not the sole function of Clp1p, but that Clp1p also functions (via SIN, discussed in a later section) to maintain the actomyosin ring in a Cdc2p-independent manner.

While the molecular role of Clp1p in re-establishing cell division structures is unclear, it appears that a major function of Clp1p is to keep the SIN active in order to effect G2 arrest and the reassembly of actomyosin rings and completion of assembly of division septa. This and previous studies have shown that Cdc7p and Sid1p are retained at one SPB in a Clp1p-dependent manner in wild-type cells treated with Lat A

that the requirement for Clp1p in G2 delay and in re-establishment and maintenance of cell division structures is significantly bypassed by ectopic activation of the SIN (Fig. 5). Thus, although SIN and Clp1p function in a positive feedback loop, active SIN alone is largely sufficient to allow completion of cytokinesis and maintain G2 arrest. Previous studies have shown that ectopic activation of the SIN results in repeated rounds of septation in interphase arrested cells (Schmidt et al., 1997; Fankhauser et al., 1993; Balasubramanian et al., 1998; Minet et al., 1979). The ability of cells to complete septation in a SIN and Clp1p-dependent manner from interphase, upon perturbation of the cell division apparatus, might provide a physiological explanation for the observed ability of ectopic SIN signaling to promote septation in interphase arrested cells (Minet et al., 1979; Fankhauser et al., 1993; Schmidt et al., 1997).

It is currently unclear whether Clp1p acts at the level of Byr4p-Cdc16p complex, Plo1p, or at the level of Spg1p, all of which can be modulated to maintain an active SIN cascade (Minet et al., 1979; Furge et al., 1998; Schmidt et al., 1997; Balasubramanian et al., 1998). Given that the Cdc14p family of phosphatases has been shown to reverse Cdc2p/Cdk1p-phosphorylation on several of its substrates (Visintin et al., 1998;



**Fig. 7. Summary and Model.** (A) Summary of cellular behavior in differing genotypes and growth conditions. Actin patches and rings are shown in red. Nuclei are shown as black circles. Cdc7p localization to the spindle pole body (a marker of active SIN) is shown schematically as a blue circle. Wild-type or *clp1Δ* cells under normal growth conditions (column 1); wild-type or *clp1Δ* cells upon treatment with low doses of Lat A (columns 2 and 3, respectively); *cps1-191* and *clp1Δ cps1-191* cells at the restrictive temperature of 36°C (columns 4 and 5, respectively); a SIN mutant at the restrictive temperature of 36°C (column 6). (B) Model. Upon perturbation of the cytokinesis machinery Clp1p and the SIN function in a positive feedback loop to maintain the integrity of the actomyosin ring (see Discussion).

Ubersax et al., 2003), Clp1p might also regulate SIN through other mechanisms not involving known components of the SIN.

#### SIN components: essential proteins important for the cytokinesis checkpoint

Of particular interest is the similarity between the phenotype of SIN mutants at the fully restrictive temperature, and low dose Lat A treated *clp1Δ* cells (Fig. 4). In both cases, the primary defect is a failure to maintain the integrity of the ring leading ultimately to its disassembly. Because SIN genes are essential, and *clp1* non-essential, the simplest interpretation taking all data together is that Clp1p function is required for ring maintenance only upon perturbation of the cell division machinery while SIN function is required for maintenance of the actomyosin ring at the end of every cell cycle during ring constriction and septation. It is also possible that SIN has other essential functions in cytokinesis, because delivery and assembly of Cps1p, an integral membrane protein important for division septum assembly is abrogated in SIN mutants (Liu et al., 2002; Cortes et al., 2002).

#### Does a similar checkpoint mechanism operate in other organisms?

Several lines of evidence suggest the existence of similar arrest

mechanisms in mammalian cells upon perturbations to the cytokinetic machinery. Animal cells that exit mitosis while cytokinesis is blocked remain arrested in a telophase like state with persistent cell division structures. Furthermore, these cells can initiate cytokinesis when the cytokinesis block is removed (Martineau et al., 1995; Canman et al., 2000). In addition, mammalian cells impaired for synthesis of phosphotidylethanolamine are unable to carry out cytokinesis and arrest with a stable actomyosin ring and interphase nuclei (Emoto and Umeda, 2000). Finally, mammalian cells treated with a myosin II ATPase inhibitor arrest as binucleate cells with intact actomyosin rings and the underlying microtubular network as in fission yeast cells (Straight et al., 2003; Pardo and Nurse, 2003). Presently it is unclear how this arrest is mediated in metazoan cells. However, proteins similar to Clp1p and SIN components (such as Mob1p and Cdc11p) have been identified in several eukaryotes including animal cells (Bardin and Amon, 2001; Pereira and Schiebel, 2001; Bembenek and Yu, 2001; Gruneberg et al., 2002; Moreno et al., 2001; Gromley et al., 2003), raising the possibility that similar molecules might function in cytokinesis checkpoint-like processes in a variety of eukaryotic cell types.

In summary, we have demonstrated that the cytokinesis checkpoint mechanism in fission yeast is essential for cell viability in conditions where the cell division machinery is

mildly perturbed. The cytokinesis checkpoint responds by providing an interphase arrest, and by promoting the reassembly of cell division structures, such as the actomyosin ring and the division septum. These two responses thereby allow the completion of cytokinesis before the subsequent mitosis. These mechanisms operate to ensure that a mother cell divides to produce two viable daughter cells whose ploidy is identical to that of the mother. In future it will be important to identify the nature of the signal(s) that are monitored by the cytokinesis checkpoint, the detailed mechanism by which actomyosin rings and possibly other cell division structures are reassembled, and the molecular mechanism leading to G2 arrest.

We wish to thank all members of the yeast and fungal biology laboratories at the Temasek Life Sciences Laboratories for their encouragement, advice and critical reading of the manuscript and Michael Glotzer and Uttam Surana for critical comments and discussion. This work was supported by research funds from the Temasek Life Sciences Laboratory. J.K. is a recipient of the Singapore Millennium Foundation Post-Doctoral Fellowship. D.M. is supported by grants from the NIH, USA.

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