

# *S. pombe* FEAR protein orthologs are not required for release of Clp1/Flp1 phosphatase from the nucleolus during mitosis

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## Summary

Cdc14 family phosphatases are highly conserved regulators of cell-cycle progression. Two of the best studied members of this family are budding yeast Cdc14p and its fission yeast homolog Clp1p/Flp1p. The function of both *Saccharomyces cerevisiae* Cdc14p and *Schizosaccharomyces pombe* Clp1p/Flp1p are controlled in part by their regulated sequestration and release from the nucleolus. In the budding yeast *S. cerevisiae* a set of proteins collectively termed the FEAR network promote nucleolar and telomeric DNA segregation by triggering the release of the

conserved Cdc14 phosphatase from the nucleolus. Here we show that FEAR homologs in *S. pombe* do not promote release of the Cdc14 homolog Clp1p/Flp1p from the nucleolus, and that Clp1p/Flp1p is not required for nucleolar and telomeric DNA segregation suggesting that this aspect of Cdc14 regulation and function may not be universally conserved.

Key words: *S. pombe*, Cdc14, Clp1, Flp1, FEAR network

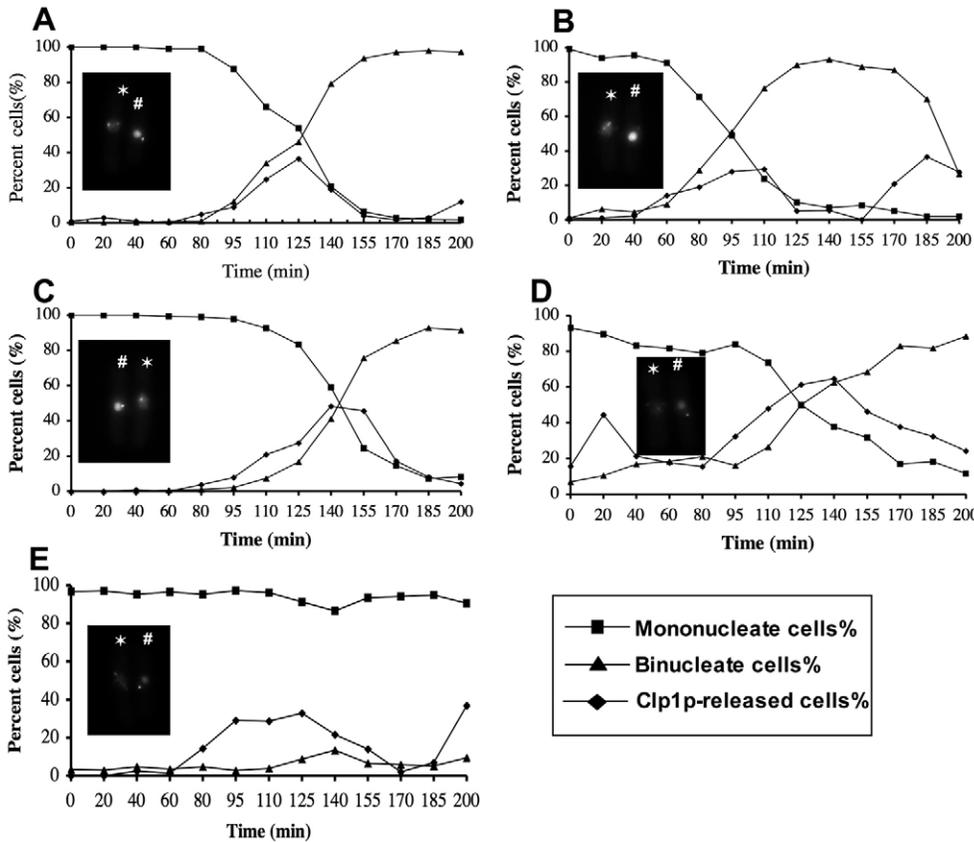
## Introduction

Timely activation and inactivation of cyclin-dependent kinases (CDKs) regulate most cell-cycle transitions. For example, entry into mitosis requires CDK activation and exit from mitosis and cytokinesis requires loss of CDK activity and dephosphorylation of CDK substrates. In the budding yeast *S. cerevisiae*, the phosphatase Cdc14 seems to be the key phosphatase required to dephosphorylate CDK substrates and promote exit from mitosis and cytokinesis (Jaspersen et al., 1998; Visintin et al., 1998). Cdc14 family phosphatases are conserved in all eukaryotes examined, but have been best studied in yeast (for reviews, see D'Amours and Amon, 2004; Krapp et al., 2004). Budding yeast Cdc14 and its fission yeast homolog Clp1p/Flp1p (hereafter referred to as Clp1p) are regulated in part by their localization, with both proteins thought to be sequestered and inactive in the nucleolus in interphase. They are released from the nucleolus in mitosis, and in late mitosis the conserved signaling pathways mitotic exit network (MEN) and septation initiation network (SIN) act to keep Cdc14 and Clp1p, respectively, out of the nucleolus (Cueille et al., 2001; Shou et al., 1999; Trautmann et al., 2001; Visintin et al., 1999). In budding yeast, Cdc14 is released from the nucleolus in early anaphase by a separate pathway known as the FEAR (for Cdc-fourteen early anaphase release) network, consisting of polo kinase (Cdc5p), separase (Esp1p), Slk19p and Spo12p (Pereira et al., 2002; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Yoshida and Toh-e, 2002). FEAR-dependent release of Cdc14 is essential for several mitotic events including proper segregation of rDNA and telomeres (D'Amours et al., 2004; Sullivan et al., 2004; Torres-

Rosell et al., 2004). In contrast to *S. cerevisiae* Cdc14, both *S. pombe* Clp1p (Cueille et al., 2001; Trautmann et al., 2001) and mammalian Cdc14B (Cho et al., 2005; Mailand et al., 2002; Nalepa and Harper, 2004) are released from the nucleolus upon entry into mitosis, although it is not known how this is regulated. FEAR pathway components (separase-Esp1p, polo-kinase-Cdc5p, Slk19 and Spo12) are conserved in *S. pombe* and other species. Here, we examine whether FEAR pathway components function to promote Clp1p release in *S. pombe*, and whether Clp1p is required for segregation of the nucleolus and telomeric DNA.

## Results

To examine the role of FEAR homologs in the early release of Clp1p from the nucleolus in *S. pombe*, we tested whether Clp1p could be released from the nucleolus in mutants defective for homologs of FEAR components. In each case, the *sin* mutant *sid2-250* was also present in each strain, to rule out any influence of the SIN in promoting release of Clp1p from the nucleolus. Cells were synchronized by elutriation, then shifted to 36°C to inactivate *sid2-250* as well as *ts* alleles of FEAR mutants where used. Release of Clp1p from the nucleolus was monitored over time. We first examined whether the *S. pombe* Polo kinase Plo1p is required for release of Clp1p from the nucleolus. Using the *plo1-25* allele (Fig. 1C), we found that Clp1p was released normally in cells going through mitosis similar to *sid2-250* control cells (Fig. 1A). We also tested the *plo1-24C* allele and found similar results (data not shown). We performed similar experiments using cells deleted for *spo12* (Samuel et al., 2000) (Fig. 1B), *alp7/mial1* (a putative

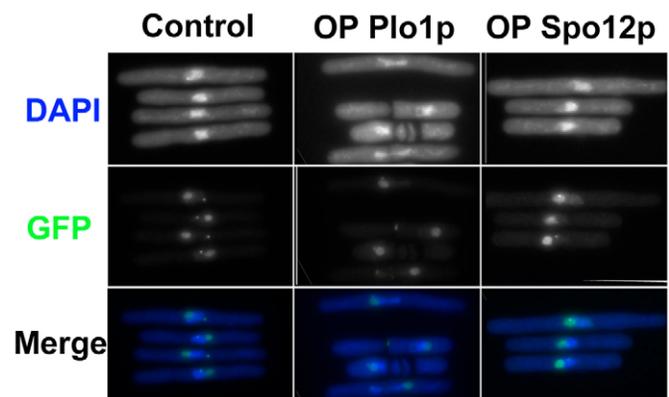


**Fig. 1.** Clp1p nucleolar release in cells carrying mutations in FEAR pathway homologs. *sid2-250 clp1-GFP* cells (A) or *sid2-250 clp1-GFP* cells carrying the *spo12Δ* (B), *plo1-25* (C), *alp7Δ* (D) or *cut1-205* (E) mutations were grown at 25°C, then synchronized by centrifugal elutriation. Cells were then shifted to 36°C. Samples were fixed every 20 minutes in methanol, stained with DAPI, and scored for number of nuclei and Clp1p-GFP localization. At least 100 cells were scored for each time point. Inset images show Clp1p-GFP signal in cells of each strain with Clp1p-GFP released (\*) or not released (#).

*SLK19* homolog) (Oliferenko and Balasubramanian, 2002; Sato et al., 2004) (Fig. 1D) and a *ts* allele of separase (*cut1-205*) (Fig. 1E). Although there are some differences in the time it takes each strain to enter mitosis because of variation intrinsic to the elutriation synchronization procedure, Clp1p was released normally in each of these mutant backgrounds as the cells entered mitosis (Fig. 1B-D). As another way to test for a role for separase in Clp1p release from the nucleolus, we induced expression of non-degradable securin Cut2p, which inhibits separase, and then scored for Clp1p nucleolar release in cells with separated SPBs. Expression of non-degradable Cut2p in *sid2-250* cells at the restrictive temperature did not significantly interfere with Clp1p release (74% released) when compared with cells with control plasmid (70% released), further demonstrating that separase is not important for release of Clp1p from the nucleolus.

In budding yeast, overexpression of polo kinase and *spo12* will promote release of Cdc14p from the nucleolus (Shou et al., 2002; Sullivan and Uhlmann, 2003; Visintin et al., 2003; Yoshida and Toh-e, 2002). In *S. pombe*, Clp1p is released from the nucleolus coincident with mitotic entry (Cueille et al., 2001; Trautmann et al., 2001). To examine the effects of overexpression of *plo1+* and *spo12+* in *S. pombe*, we arrested cells immediately before mitotic entry using the *cdc25-22* mutation and tested whether overexpression of Plo1p or Spo12p can promote release of Clp1p (Fig. 2). Cells overexpressing Plo1p (92% nucleolar) and Spo12p (96% nucleolar) did not display increased release of Clp1p from the nucleolus compared with control cells (92% nucleolar). Thus overexpression of Plo1p and Spo12p does not promote nucleolar release of Clp1p.

In budding yeast, the FEAR functions to release Cdc14 in early anaphase and this release is required for a number of functions including: M1 exit in meiosis (Buonomo et al., 2003; Marston et al., 2003), nuclear positioning (Ross and Cohen-Fix, 2004), rDNA segregation (D'Amours et al., 2004; Sullivan



**Fig. 2.** Overproduction of Plo1p and Spo12p do not cause release of Clp1p. *cdc25-22 clp1-GFP* cells carrying either a plasmid expressing *plo1+* or *spo12+* from the thiamine-repressible *nmt1* promoter were grown at 25°C in the absence of thiamine for 12 hours. The cells were then shifted to 36°C for an additional 4 hours in the absence of thiamine to inactivate the Cdc25-22 mutant protein and arrest cells in G2 phase. The cells were then fixed, stained with DAPI, and representative DAPI, GFP and merged images are shown. Note that the *nmt1* promoter does not become active until 12 hours after removal of thiamine.

et al., 2004; Torres-Rosell et al., 2004), MEN activation (Pereira et al., 2002; Stegmeier et al., 2002; Tinker-Kulberg and Morgan, 1999; Visintin et al., 2003) and passenger protein localization to the spindle (Pereira and Schiebel, 2003). We have examined whether any of these functions may be conserved in *S. pombe*. We find that nuclear positioning, passenger protein localization to the spindle all seem normal in the *clp1Δ* deleted cells (Trautmann et al., 2001; Cueille et al., 2001; Trautmann et al., 2004) (data not shown). As in budding yeast, Clp1p helps activate the SIN, and *sin clp1Δ* double mutants display negative interactions (Trautmann et al., 2001; Cueille et al., 2001). However, we did not observe any synthetic interactions between FEAR components and the SIN (data not shown). Therefore these proteins may not contribute to the ability of Clp1p to activate the SIN as is observed in *S. cerevisiae*.

Because FEAR-dependent release of Cdc14p is essential for progression from meiosis I to meiosis II, we examined whether Clp1p was similarly important for meiotic progression in *S. pombe*. Meiotic progression in wild-type and *clp1Δ* cells was initiated in diploid cells using the *pat1-114* mutation. This experiment showed that homozygous *clp1Δ* cells progressed through meiosis I and II with almost identical kinetics to that of wild-type cells (Fig. 3A,B). In addition, self matings between  $h^+$  and  $h^-$  wild-type or  $h^+$  and  $h^-$  *clp1Δ* cells showed similar numbers of four-spored asci (Fig. 3C,D). The slight decrease in complete asci in *clp1Δ* cells may reflect weakened SIN signaling in the *clp1Δ* cells, since SIN signaling is important for spore formation (Krapp et al., 2006). In both experiments, *clp1Δ* cells showed a slight but reproducible increase in the number of asci with two or three nuclei (Fig. 3A,B,D), suggesting that similarly to mitosis, Clp1p has a role in the fidelity of the process. In addition, examination of Clp1p localization in meiosis, showed no difference between wild-type and *spo12Δ* cells (data not shown). Together, these and previous results (Samuel et al., 2000) show that Clp1p and

**Table 1. Segregation of nucleolar markers in *clp1Δ* and wild-type cells**

	Time of Nuc1p-GFP separation (minutes)	SPB separation at Nuc1-GFP separation ( $\mu$ m)
wt	13.97 $\pm$ 0.36 (18)	4.49 $\pm$ 0.05
<i>clp1Δ</i>	13.76 $\pm$ 0.76 (28)	4.45 $\pm$ 0.06

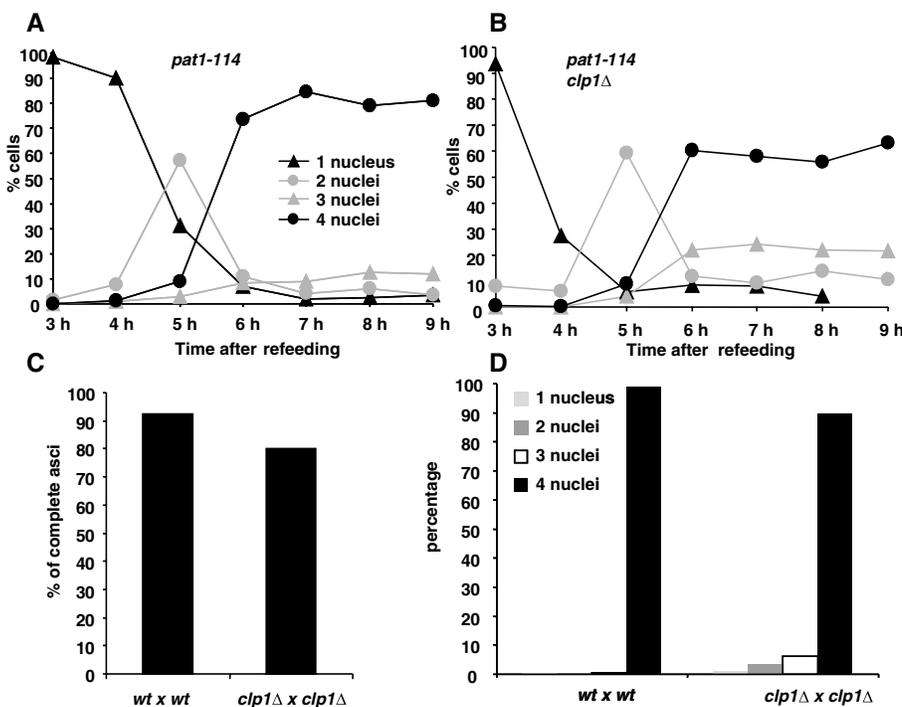
Cells expressing Cdc14p-GFP to label spindle pole bodies, and Nuc1p-GFP to label the nucleolus, were grown at 24°C, synchronized by centrifugal elutriation and examined using time-lapse microscopy. The time of Nuc1p-GFP separation was measured from the time of SPB (Cdc14p-GFP) separation. The distance between SPBs, in micrometers, at the time of nucleolar separation was also measured. The number of cells analyzed is shown in parentheses. The s.e.m. is shown for each measurement.

Spo12p do not play an essential role in meiotic progression in fission yeast.

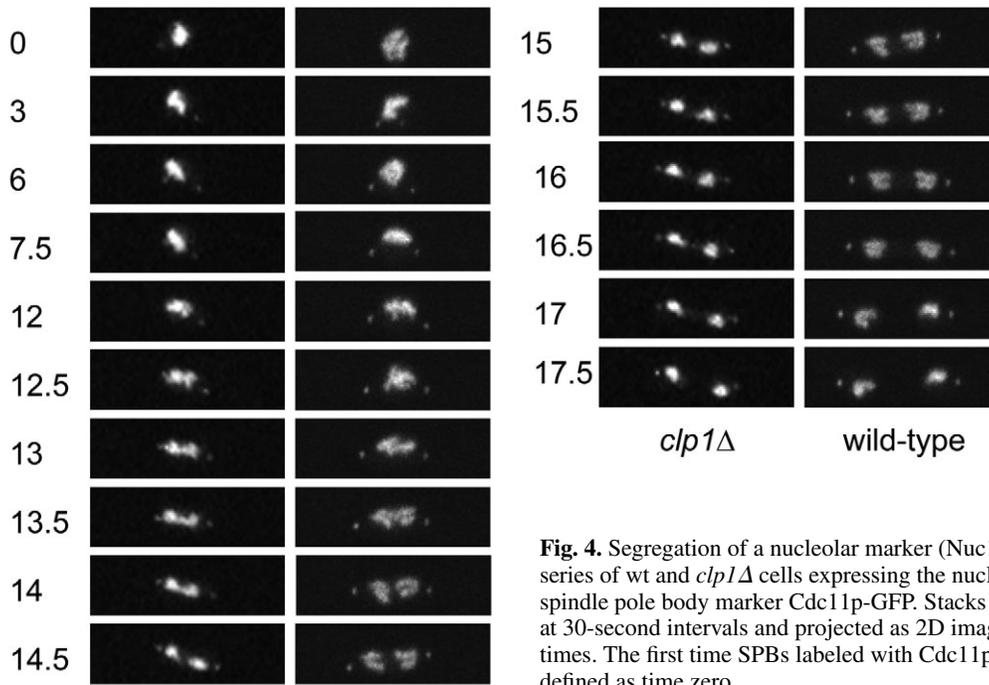
We next examined whether Clp1p functioned in rDNA and telomere segregation. Nucleolar and telomere segregation was monitored using Nuc1p-GFP to label the nucleolus and a LacO array integrated at the *sod2* locus near the telomere in cells expressing LacI-GFP (Ding et al., 2004). The separation of each signal was analyzed by comparing the amount of time after SPB separation (mitotic entry) before separation of the GFP signals, as well as the distance between SPBs when the nucleolar or telomere GFP signals separate. Interestingly, Nuc1-GFP signals separated at almost the same time post SPB separation. In addition, when the SPBs were separated, the same overall distance was measured in wild-type and *clp1Δ* cells (Fig. 4, Table 1). Similarly, telomere separation was not delayed relative to wild-type cells in *clp1Δ* cells (Table 2). These results show that Clp1p does not play a significant role in segregation of the nucleolus and telomeres.

## Discussion

Studies in yeast suggest that a key mechanism for Cdc14 phosphatase regulation is through regulated nucleolar



**Fig. 3.** Meiosis and spore formation in *clp1Δ* cells. (A,B) Diploid cells of the genotype  $h^+h^+ ade6\text{-}M210/lade6M\text{-}216 pat1\text{-}114/lpat1\text{-}114$  and  $h^+h^+ ade6\text{-}M210/lade6M\text{-}216 pat1\text{-}114/lpat1\text{-}114 clp1::kanMX6/clp1::kanMX6$  were grown to mid-exponential phase and then transferred to minimal medium without ammonium chloride to starve cells in G1. Cells were inoculated into complete medium at 33°C to induce meiosis. Samples were fixed at intervals and the number of nuclei per cell was determined. The key shown in A also applies to B. (C,D) Wild-type  $h^+$  and  $h^-$  cells were mated on minimal medium lacking ammonium chloride. Cells were taken from the mating mixture and the percentage of complete asci (C) and number of nuclei per meiotic cell were determined (D).



**Fig. 4.** Segregation of a nucleolar marker (Nuc1p-GFP) in *clp1Δ* cells. Time-lapse series of wt and *clp1Δ* cells expressing the nucleolar marker Nuc1p-GFP and the spindle pole body marker Cdc11p-GFP. Stacks of 11 z-sections of 0.5  $\mu\text{m}$  were taken at 30-second intervals and projected as 2D images. Cells are shown at the indicated times. The first time SPBs labeled with Cdc11p-GFP appeared as separate dots was defined as time zero.

sequestration (for reviews, see D'Amours and Amon, 2004; Krapp et al., 2004). Thus it is important to understand how their nucleolar localization is regulated. Two conserved signaling networks in budding and fission yeast, the MEN and SIN, respectively, seem to play a conserved role in maintaining the phosphatase outside the nucleolus in late mitosis. By contrast, initial release of the phosphatase from the nucleolus seems to be governed differently. In budding yeast the FEAR pathway and Cdk1 promotes release of Cdc14 from the nucleolus in early anaphase (Azzam et al., 2004; Pereira et al., 2002; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Yoshida and Toh-e, 2002). However the *S. pombe* Cdc14 homolog Clp1p is released in early mitosis, and as we show here, this release does not depend on homologs of the FEAR network. In budding yeast, FEAR-dependent release of Cdc14 is important to allow Cdc14 to function in segregation of the rDNA, nucleolus and telomeres (D'Amours et al., 2004; Sullivan et al., 2004; Torres-Rosell et al., 2004). However, we found that Clp1p is not essential for these functions in *S. pombe*. One reason for the additional functions of Cdc14 in

anaphase might be that budding yeast maintains high Cdk activity throughout anaphase unlike most other eukaryotes, which lose Cdk activity upon anaphase onset. Therefore dephosphorylation of mitotic CDK substrates by Cdc14 might be especially important for anaphase events in budding yeast. Although the FEAR pathway does not play a conserved role in regulating the Cdc14 homolog in *S. pombe*, it remains a possibility that Cdk activity might play a conserved role in promoting release of the phosphatase from the nucleolus in both organisms. Given the similar timing of release from the nucleolus of *S. pombe* Clp1p (Cueille et al., 2001; Trautmann et al., 2001) and human Cdc14B (Cho et al., 2005; Mailand et al., 2002; Nalepa and Harper, 2004) it seems likely that they may be regulated through a conserved, FEAR-independent pathway.

## Materials and Methods

### Microscopy and data analysis

For time-lapse experiments, cells were pre-grown to early log phase in YE with supplements (YES) at 25°C. G2 cells obtained by centrifugal elutriation were concentrated and resuspended in 500  $\mu\text{l}$  medium (approximately  $5 \times 10^6$  per ml). Cells were allowed to recover for 75 minutes at 25°C before recording. 2  $\mu\text{l}$  of concentrated cells were mounted on a thin layer of YES containing 1–2% agarose, and sealed under a coverslip with nail polish. Strains were imaged at 22°C–25°C using a Zeiss axiovert 200 microscope equipped with a confocal scanner unit model CSU10 (Yokogawa Electric Corporation), a coolSNAP HQ camera (Photometrics), and 63 $\times$  1.4 NA plan-apo or 100 $\times$  1.4 NA plan-apo objective. Images were collected using Metamorph software (Universal Imaging, version 4.5) with 1 $\times$ 1 binning at intervals of 0.5–1 minute using exposures of 0.3 second for *nuc1-GFP cdc11-GFP* and 1 second for *sod2-LacO Lac1-GFP cdc11-GFP*. The same software was used for image processing and quantification. Analysis of meiosis in *clp1Δ* cells was done as previously described (Krapp et al., 2006).

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**Table 2. Segregation of telomeres in *clp1Δ* and wild-type cells**

	Time of telomere separation (minutes)	SPB separation at telomere separation ( $\mu\text{m}$ )
wt	31.3 $\pm$ 0.85 (20)	4.5 $\pm$ 0.1
<i>clp1Δ</i>	26.2 $\pm$ 1.3 (11)	4.4 $\pm$ 0.59

Cells expressing Cdc11p-GFP to label spindle pole bodies and Lac1-GFP in cells containing a LacO array integrated at the *sod2* locus near the telomere were grown at 21.5°C, synchronized by centrifugal elutriation and examined using time-lapse microscopy. The time of telomere (Lac1-GFP) separation was measured from the time of SPB (Cdc11p-GFP) separation. The distance between SPBs, in micrometers, at the time of telomere separation is also measured. The number of cells analyzed is shown in parentheses. The s.e.m. is shown for each measurement.

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