JCB Report

Correct spindle elongation at the metaphase/anaphase transition is an APC-dependent event in budding yeast

Fedor Severin,[1] Anthony A. Hyman,[1] and Simonetta Piatti[2]

[1]Max Planck Institute for Cell Biology and Genetics, Dresden 01307, Germany
[2]Dipartimento di Biotecnologie e Bioscienze, Universitá degli Studi di Milano-Bicocca, 20126 Milano, Italy

At the metaphase to anaphase transition, chromosome segregation is initiated by the splitting of sister chromatids. Subsequently, spindles elongate, separating the sister chromosomes into two sets. Here, we investigate the cell cycle requirements for spindle elongation in budding yeast using mutants affecting sister chromatid cohesion or DNA replication. We show that separation of sister chromatids is not sufficient for proper spindle integrity during elongation. Rather, successful spindle elongation and stability require both sister chromatid separation and anaphase-promoting complex activation. Spindle integrity during elongation is dependent on proteolysis of the securin Pds1 but not on the activity of the separase Esp1. Our data suggest that stabilization of the elongating spindle at the metaphase to anaphase transition involves Pds1-dependent targets other than Esp1.

Introduction

At metaphase, a stable spindle is formed with paired sister chromatids attached to opposite poles (Winey and O'Toole, 2001; Wittmann et al., 2001). At the metaphase to anaphase transition, several coordinated events occur: the anaphase-promoting complex (APC)* is activated, sister chromatid cohesion is lost, and the sisters separate to the opposite poles (Biggins and Murray, 1999; Koshland and Guacci, 2000; Pines and Rieder, 2001). One of the defining features of anaphase is an increase in spindle length called anaphase B. (Winey and O'Toole, 2001; Wittmann et al., 2001).

Why do metaphase spindles elongate to a certain length and wait before elongating during anaphase B? One possibility is that the mechanical link established by interactions of sister chromatids with microtubules emanating from opposite spindle poles prevents spindle elongation. Mechanistically, this hypothesis suggests that the forces acting on spindles do not change between metaphase and anaphase; rather it is the loss of sister chromatid cohesion at anaphase onset that removes the barrier to spindle elongation. Another possibility is that cell cycle events at the metaphase to anaphase transition are also required for successful spindle elongation. The metaphase to anaphase transition is controlled by the APC, a multisubunit complex with ubiquitin-ligase (E3) activity, which triggers entry into anaphase, exit from mitosis, and cytokinesis (for reviews see Yanagida et al., 1999; Zachariae and Nasmyth, 1999). APC activity can be inhibited by the spindle checkpoint, which monitors correct bipolar attachment of chromosomes to the spindle, preventing entry into anaphase (for review see Wassmann and Benezra, 2001). The APC triggers degradation of securin (Yanagida et al., 1999; Zachariae and Nasmyth, 1999), resulting in activation of the separase (Amon, 2001), which cleaves the cohesin thereby promoting sister chromatid separation (Uhlmann et al., 2000; Waizenegger et al., 2000). Several different proteins, besides securin, are known to be APC substrates (Peters, 1999), but the physiological relevance of their proteolysis has not been established for all of them.

Relatively little is known about the differential role of cell cycle–regulated events and chromosome-based pole-to-pole links in spindle mechanics at anaphase. Experiments in *Xenopus* (Shamu and Murray, 1992) and yeast (Holm et al., 1985) with inhibition of topoisomerase II have shown that if the link between sister chromatids is not broken at the metaphase-anaphase transition, spindles do not elongate, supporting the mechanical link hypothesis. In contrast, insect spermatocytes from which all chromosomes have been removed maintain metaphase spindles and undergo anaphase spindle elongation with kinetics similar to normal spindles (Zhang and Nicklas, 1996). Furthermore, spindles formed in *Xenopus* egg extracts by plasmid DNA incompetent to assemble kinetochores are the same length as spindles formed by sperm nuclei that assemble kinetochores (Heald

The online version of this article contains supplemental material.

Address correspondence to Simonetta Piatti, Dipartimento di Biotecnologie e Bioscienze, Piazza della Scienza 2, 20126 Milano, Italy. Tel.: 39-02-6448-3547. Fax: 39-02-6448-3565. E-mail: simonetta.piatti@unimib.it

*Abbreviations used in this paper: APC, anaphase-promoting complex.

Key words: anaphase; anaphase-promoting complex; microtubules; securin; spindle

et al., 1996). In *Saccharomyces cerevisiae*, spindle elongation and integrity is affected in cohesion-defective mutants, despite premature separation of sister chromatids (Guacci et al., 1997; Michaelis et al., 1997; Skibbens et al., 1999). Furthermore, the separase Esp1 appears to be required for spindle elongation, besides for separation of sister chromatids (Uhlmann et al., 2000; Jensen et al., 2001). However, in *Schizosaccharomyces pombe* and *S. cerevisiae* mutations affecting the pole-to-pole links result in an increased spindle length at metaphase (Goshima et al., 1999; Skibbens et al., 1999).

One way to address the role of bipolar attachment of chromosomes in spindle elongation would be to prevent establishment of sister chromatid cohesion during S phase and assay the effect on spindle length and structure. In this paper, we disrupt bipolar attachment using mutants in *S. cerevisiae* affecting sister chromatid cohesion (Tanaka et al., 2000) or DNA replication (Piatti et al., 1995) and show that though the spindles elongate eventually they are unable to stabilize their midzones. Our data suggest that in addition to sister chromatid separation, successful anaphase B requires an APC-dependent event that stabilizes the microtubules of the elongating spindle. Stabilization requires destruction of the securin Pds1 but not activity of the separase Esp1, suggesting that Pds1 proteolysis is necessary for stabilization of the central spindle at mitosis independently of Esp1.

Results and discussion

In budding yeast, the cohesin Scc1/Mcd1 is required for successful chromosome cohesion at metaphase (Guacci et al., 1997; Michaelis et al., 1997). Several observations have suggested that in mutants affecting chromatid cohesion, spindles do not elongate properly (Guacci et al., 1997; Michaelis et al., 1997; Skibbens et al., 1999). We compared the kinetics of spindle elongation in a ts *scc1* mutant (*scc1-73*) (Michaelis et al., 1997) with those of wild-type cells. Synchronous cultures were obtained by centrifugal elutriation and released into the cell cycle at 37°C. Comparison of mutant and wild-type profiles shows that spindle elongation in *scc1-73* mutants took place 30 min later than in wild-type cells with respect to the onset of budding (Fig. 1 A). However, when spindles elongated in *scc1-73* mutants they looked fragile and often broken in the middle (Fig. 1 B). We conclude that premature loss of sister chromatid cohesion is not sufficient to trigger proper spindle elongation. Other cell cycle–dependent events might be involved in controlling this process.

A reasonable working hypothesis to explain the lack of spindle stability in the absence of sister chromatid cohesion would be that in wild-type cells the APC coordinately promotes both sister chromatid separation and spindle elongation/stability (Skibbens et al., 1999). In *scc1* mutants, the activation of the spindle checkpoint would result in sister

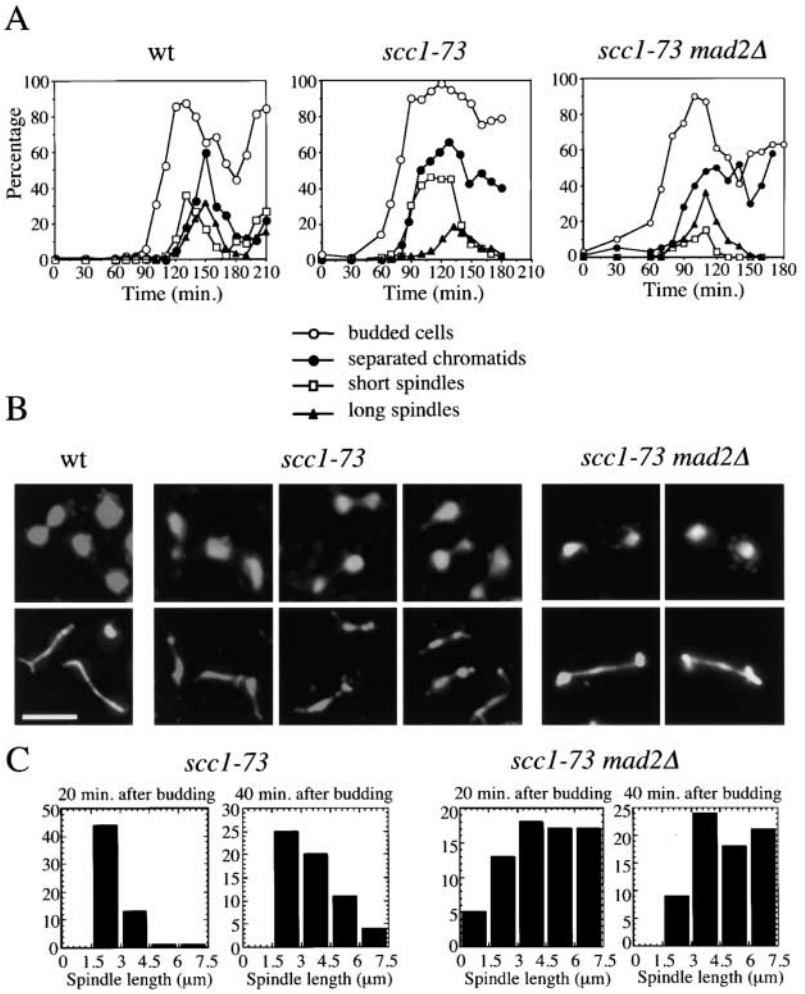

Figure 1. **Cohesin mutants show defects in spindle elongation and stability that depend on spindle checkpoint activation.** Wild-type (TH560), *scc1-73* (TH572), and *scc1-73 mad2Δ* (SP1250) cells, carrying the tetR-tetO system to detect sister chromatid separation, were elutriated to obtain small G1 cells ($t = 0$ min) and released in YEPD at 37°C. At the indicated times, cells were collected to analyze the DNA contents by flow cytometry (unpublished data), spindle structure by in situ immunofluorescence (A and B), and the kinetics of budding and sister chromatid separation (A). Wild-type, *scc1-73,* and *scc1-73 mad2Δ* spindles were photographed at 150, 130, and 120 min after release, respectively. Spindle length distributions (C) were measured in the *scc1-73* and *scc1-73 mad2Δ* mutants with the NIH image software at 20 and 40 min after 50% of the cells have budded. (50–100 cells were scored for each histogram.) Bar, 5 μm.

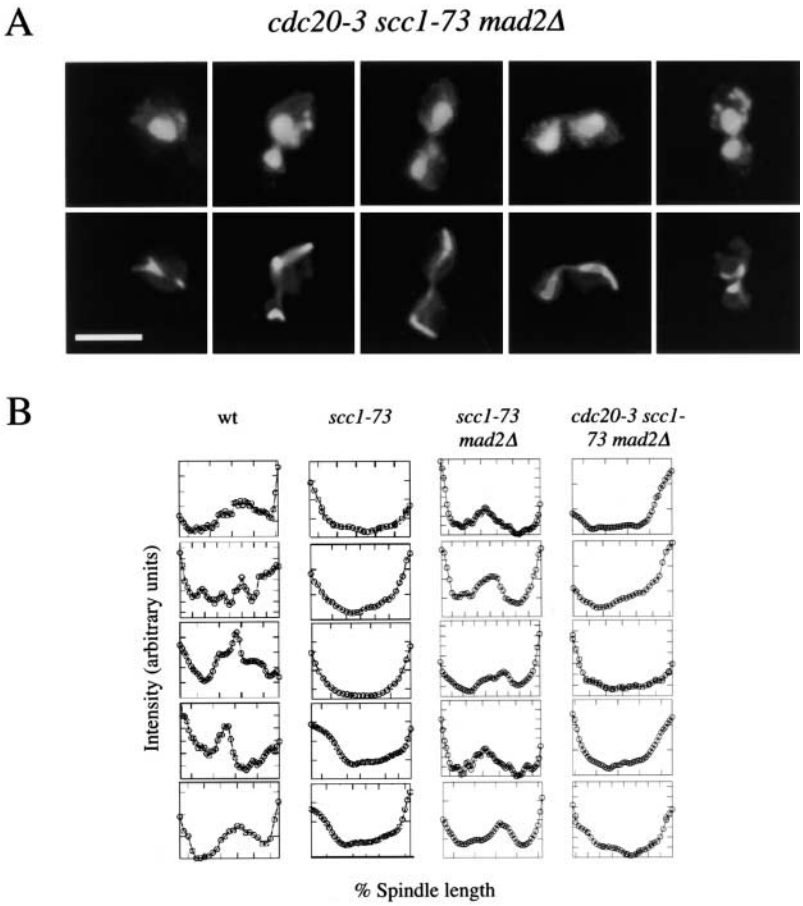

Figure 2. **The rescue of spindle fragility in *scc1* mutants by *MAD2* deletion requires functional APC.** G1 cells of a *cdc20-3 scc1-73 mad2Δ* strain (TH885) were collected by centrifugal elutriation and released in YEPD at 37°C. At the indicated time points, cells were collected for FACS® analysis of the DNA contents (unpublished data) and spindle immunostaining (A and B). Spindles were photographed at 120 min after the release. Fluorescence intensity along spindle length (B) was measured using Metamorph software (Universal Imaging) and compared with that of wild-type (wt; TH560), *scc1-73* (TH572), and *scc1-73 mad2Δ* (SP1250) cells treated in the same conditions. Bar, 5 μm.

chromatid separation before APC activation, allowing spindles to attempt elongation in the presence of inactive APC. To test this idea, we inactivated the spindle checkpoint in an *scc1-73* mutant. A synchronous culture of G1 *scc1-73 mad2Δ* double mutant cells, obtained by elutriation, was released into the cell cycle at 37°C. As shown in Fig. 1 A, lack of Mad2 caused *scc1-73* cells to elongate spindles and undergo cytokinesis (unpublished data) with wild-type kinetics compared with the onset of budding. We confirmed these results by measuring spindle lengths through the cell cycle (Fig. 1 C). Thus, as suggested previously (Skibbens et al., 1999) the presence of monopolarly attached kinetochores triggers activation of the spindle assembly checkpoint in yeast like in higher eukaryotic cells. Strikingly, lack of Mad2 also rescued the defect in spindle stability of *scc1-73* cells (Fig. 1 B). This result suggests that both the spindle stability defect and the cell cycle delay observed in *scc1-73* cells are due to activation of the spindle checkpoint.

In principle, the rescue of the spindle defects in cells with monopolarly attached chromosomes by a *MAD2* deletion could be due to a direct effect of Mad2 on spindle stability rather than to the restoration of the normal timing of APC activation with respect to chromosome segregation. To check whether the rescue of the spindle stability defect by deletion of *MAD2* was mediated by the APC, we introduced in the *scc1-73 mad2Δ* double mutant a loss-of-function mutation affecting the APC activator Cdc20. We then analyzed spindle structure in a synchronous culture of a *cdc20-3 scc1-73 mad2Δ* triple mutant obtained by elutriation and released at 37°C. The spindles in the triple mutant were as unstable as in *scc1-73* single mutant cells (Fig. 2 A), suggesting that rescue of the spindle instability in *scc1-73* cells by *MAD2* deletion is mediated by the APC. This result was confirmed by measuring the intensity of spindle staining along its length in *cdc20-3 scc1-73 mad2Δ* versus *scc1-73 mad2Δ* cells (Fig. 2 B). The figure shows that unlike wild-type and *scc1-73 mad2Δ*, *cdc20-3 scc1-73 mad2Δ* central spindles, like those of *scc1-73*, lack the increase in spindle staining due to overlapping microtubules (Fig. 2 B).

One possibility was that spindle instability in an *scc1-73* mutant was a direct consequence of the absence of functional Scc1/Mcd protein itself. Therefore, we analyzed the effect on spindle stability of monopolar attachment obtained by preventing duplication of chromosomes. Cells lacking the initiation replication protein Cdc6 do not replicate their DNA at S phase but nevertheless undergo a haploid mitosis with unduplicated chromosomes segregating randomly to the poles (Piatti et al., 1995). However, Scc1 can load normally onto these monooriented chromosomes (Uhlmann and Nasmyth, 1998). To deplete cells of Cdc6, we used strains in which Cdc6 was under the control of the galactose-inducible *GAL1-10* promoter (*GAL-ubiCDC6*) (Piatti et al., 1996). We generated a population of G1 cells lacking Cdc6 (see Materials and methods) and followed their progress through mitosis. Again, the presence of monopolarly attached chromosomes caused spindles to become very

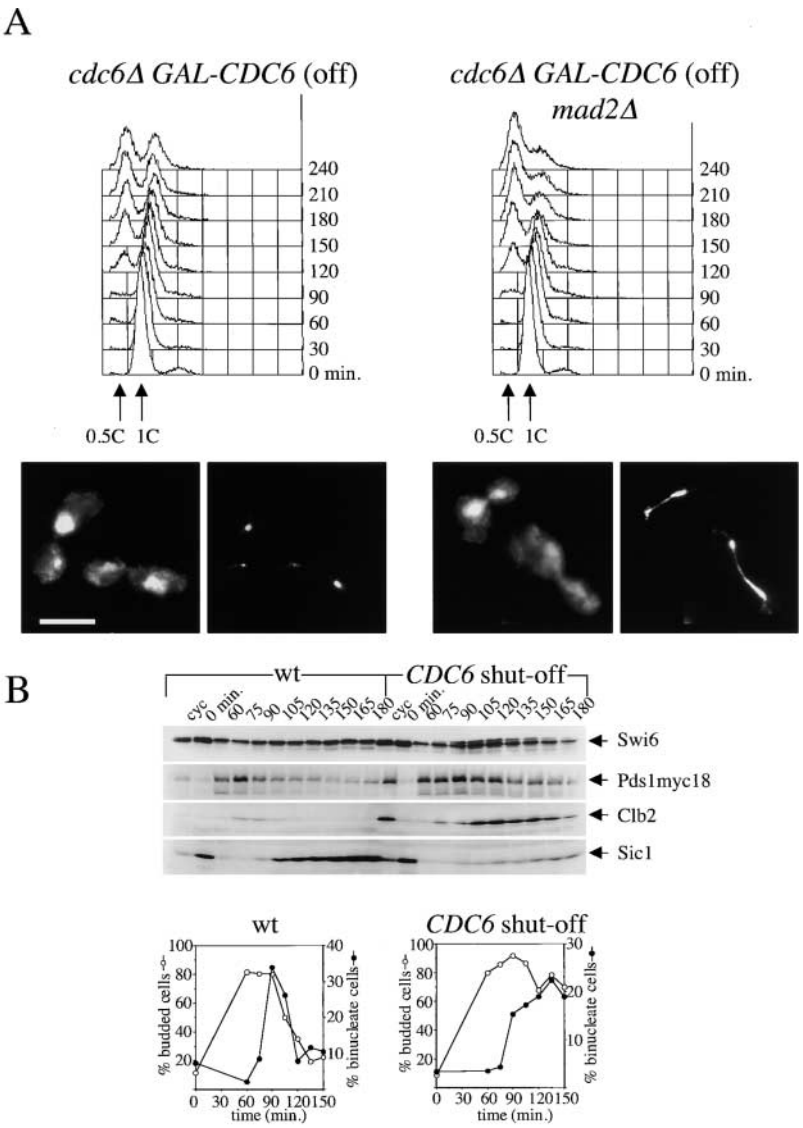

Figure 3. **Replication mutants show a Mad2-dependent spindle instability and are unable to timely activate the APC while undergoing haploid mitosis.** (A) *cdc6Δ GAL-ubiCDC6* (SP847) and *cdc6Δ GAL-ubiCDC6 mad2Δ* cells (SP911) were grown in YEPRG and arrested in S phase by hydroxyurea treatment. After 210 min, cells were released in YEPD medium to switch off *CDC6* transcription (off) in the presence of α-factor to arrest cells in the next G1 phase ($t = 0$). Subsequently, Cdc6-depleted cells were released into fresh YEPD medium at 37°C. At the indicated times, cells were collected for FACS® analysis of the DNA contents (histograms) and tubulin staining (photographs). (B) Wild-type (SP441) and *GAL-ubiCDC6* cells (SP1336) were grown in the presence of galactose (YEPRG) and arrested in G2 by nocodazole treatment. After 150 min, cells were released from the nocodazole arrest into fresh medium lacking galactose (YEPD) and containing α-factor to arrest them in the next G1 phase. Subsequently, both cultures were released from the G1 block in YEPD at 37°C ($t = 0$). After 90 min, when >90% of the cells had budded 10 μg/ml of α-factor were readded to prevent cells from entering a new cell cycle. During the release at 37°C in YEPD, cells were collected for Western blot analysis of Pds1myc18, Clb2, and Sic1 and to analyze the kinetics of budding and nuclear division (graphs). Swi6 has been used as a loading control. Bar, 5 μm.

unstable (Fig. 3 A). Furthermore, like in *scc1* mutants *MAD2* deletion allowed proper spindle elongation in cells depleted of Cdc6 (Fig. 3 A). To confirm that the spindle checkpoint was indeed activated upon Cdc6 depletion, we monitored markers of cell cycle progression such as degradation of securin (Pds1), cyclin B (Clb2), and accumulation of the cyclin B CDK inhibitor Sic1 by Western blot. These parameters reflect activation of the APC because Pds1 and Clb2 are targeted to degradation through APC-dependent ubiquitination, whereas accumulation of Sic1 depends on the APC-mediated activation of the phosphatase Cdc14 (for review see Zachariae and Nasmyth, 1999). Lack of Cdc6 caused a pronounced delay in Pds1 degradation (Fig. 3 B) and dramatically affected Clb2 proteolysis and reaccumulation of Sic1 (Fig. 3 B).

Therefore, it appears that both *cdc6* and *scc1* mutants engage the spindle checkpoint. In principle, one would not expect the spindle checkpoint to be activated in a simple model in which occupancy of kinetochores by microtubule attachment was detected. Our results suggest that bipolarity plays an important role as well, perhaps by generating the "tension" at kinetochores that arises when they are bipolarly attached (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). However, in none of our conditions is there a full cell cycle arrest as is seen after depolymerization of microtubules by nocodazole. More likely, the cell monitors both attachment and tension, thus ensuring the highest fidelity of chromosome segregation possible under different circumstances.

Taken together, the analysis of *cdc6* and *scc1* mutants shows that the simple mechanical link hypothesis is not sufficient to explain how correct spindle elongation is triggered. Our experiments suggest rather that spindle elongation must be coupled to APC activation for correct formation of the spindle midzone. The most likely role for APC activation in spindle stabilization is via destruction of the securin *PDS1*, which is both degraded at the metaphase/anaphase transition in a Cdc20/APC-dependent manner (Yanagida et al., 1999; Zachariae and Nasmyth, 1999) and localized on spindles in *S. cerevisiae* (Jensen et al., 2001) and *S. pombe* (Funabiki et al., 1996). Therefore, we deleted *PDS1* in *scc1-73* cells to check whether removal of Pds1 would bypass the requirement of APC activation in spindle stability. We found that *PDS1* deletion was sufficient to stabilize spindles in *scc1-73* cells (Fig. 4). Furthermore, *scc1-73 pds1Δ* cells un-

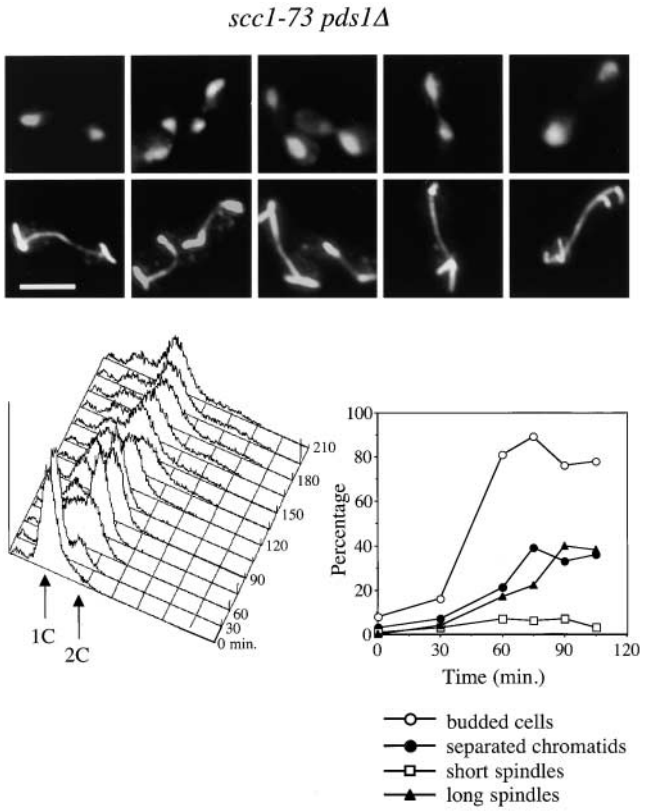

Figure 4. **Deletion of *PDS1* is sufficient to bypass the cytokinesis delay and the spindle defects of *scc1*.** G1 cells of a *scc1-73 pds1Δ* strain (SP1749) were obtained by elutriation and released in YEPD at 37°C. At the indicated time points, cells were collected for FACS® analysis (histograms), spindle morphology (pictures and graph), and to monitor the kinetics of budding and sister chromatid separation (graph). Spindles were photographed at 90 min after the release. Bar, 5 μm.

derwent cytokinesis and spindle elongation with wild-type kinetics (Fig. 4). This result suggests that the spindle checkpoint-dependent accumulation of Pds1 in *scc1* cells is responsible for destabilizing anaphase spindles and for delaying mitotic exit and cytokinesis.

The APC-dependent destruction of Pds1 leads to activation of the separase Esp1, which cleaves Scc1, thus causing dissolution of the bonds between sister chromatids (Uhlmann et al., 2000). Recent experiments have suggested that Esp1 could be required for proper spindle elongation (Jensen et al., 2001) and stabilization (Uhlmann et al., 2000). Therefore, we checked whether in our experiments spindle stabilization caused by *MAD2* deletion was mediated by Esp1. Cells carrying the *scc1-73*, *esp1-N5*, and *mad2Δ* mutations were arrested with α-factor and released at a nonpermissive temperature. The kinetics of spindle elongation and spindle morphology of the triple mutant during anaphase B was the same as for the *scc1 mad2Δ* double mutant and wild-type (Fig. 5). We obtained similar results with *scc1-73 mad2Δ* mutants containing another allele of *ESP1*, *esp1-1* (data not shown; Fig. S1 available at http://www.jcb.org/cgi/content/full/jcb.200104096/DC1). We also analyzed spindle elongation in a double *scc1-73 esp1-N5* mutant and found that its kinetics of spindle elongation were comparable to those of

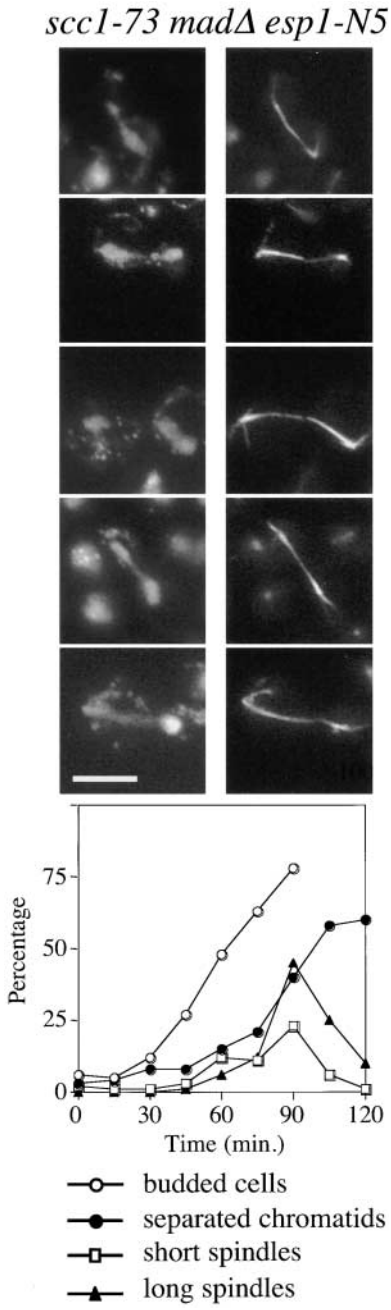

Figure 5. **Esp1 is not required for spindle stability of *scc1-73 mad2Δ* cells.** A cycling culture of a *scc1-73 mad2Δ esp1-N5* mutant (SP2126) was arrested in G1 with α-factor ($t = 0$) and released at 37°C. At different time points, cells were collected for analysis of the spindle structure by in situ immunofluorescence (photographs and graph) and to monitor the kinetics of budding and sister chromatid separation. Spindles were photographed at 90 min after the release. Bar, 5 μm.

scc1-73 single mutants (data not shown; Fig. S2 available at http://www.jcb.org/cgi/content/full/jcb.200104096/DC1). Therefore, we conclude that in contrast to previously published experiments (Jensen et al., 2001) Esp1 activation is not required for spindle elongation during anaphase. Rather, Esp1 might be required as suggested previously (Uhlmann et al., 2000; Sullivan et al., 2001) for proper spindle stability at telophase after elongation.

Table I. **Table of strains**

Name	Relevant genotype
TH560	*MATa, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*
TH572	*MATa, scc1-73, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*
TH885	*MATa, scc1-73, cdc20-3, mad2::HIS3, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*
SP441	*MATa, pds1::PDS1myc18::LEU2*
SP847	*MATa, cdc6::hisG, ura3::URA3 GAL-ubiCDC6, scc1::SCC1myc18::TRP1*
SP911	*MATa, cdc6::hisG, ura3::URA3 GAL-ubiCDC6, scc1::SCC1myc18::TRP1, mad2::TRP1*
SP1250	*MATa, scc1-73, mad2::TRP1, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*
SP1336	*MATa, pds1::PDS1myc18::LEU2, cdc6::hisG, ura3::URA3 GAL-ubiCDC6*
SP1749	*MATa, scc1-73, pds1::URA3, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*
SP2126	*MATa, scc1-73, mad2::TRP1, esp1::kanMX4, CEN::TRP1::esp1-N5, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO*

In conclusion, our work has shown that in addition to controlling sister chromatid separation Pds1 destruction is required to allow successful spindle elongation at anaphase. How then would Pds1 destruction stabilize spindle elongation? One possibility is that an as yet unidentified Pds1 target influences spindle stability during elongation. Alternatively, since degradation of Pds1 has been implicated in full activation of the APC (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999), Pds1 might inhibit the APC-dependent degradation of hypothetical inhibitor(s) of microtubule growth during spindle elongation. Further work on different Pds1 targets or analysis of APC substrates will be required to address these issues.

Materials and methods

Strains and media

All yeast strains were derivatives of or were backcrossed at least three times to W303 (*ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, ssd1*). Strains used for this work are listed in Table I. Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with either 2% glucose (YEPD) or 2% raffinose (YEPR) or 2% raffinose plus 1% galactose (YEPRG). α-Factor was used at 2 μg/ml, nocodazole at 5 μg/ml, and hydroxyurea at 150 mM.

Cdc6 depletion

Yeast strains carrying a *CDC6* deletion were kept alive by one copy of the galactose-inducible *GAL-ubiCDC6* fusion (Piatti et al., 1996). Cells were grown in YEPRG and then arrested either in G2 by nocodazole or in S phase by hydroxyurea. Cultures were released from the block in YEPD to repress *GAL-ubiCDC6* transcription containing α-factor. In these conditions, Cdc6-depleted cells arrest in G1 and upon release from α-factor cannot replicate their DNA but nevertheless undergo a "reductional" anaphase where unduplicated chromosomes segregate randomly (Piatti et al., 1995).

Western blot analysis

Western blot analysis was performed as described (Piatti et al., 1996). 50 μg of protein were transferred to nitrocellulose membranes and detected using chemiluminescence detection (ECL; Amersham Pharmacia Biotech). Myc-tagged Pds1 was detected with 9E10 Mab; polyclonal antibodies were used to detect Clb2 (Amon et al., 1994), Sic1 (Skowyra et al., 1997), and Swi6 (Moll et al., 1992).

Other techniques

Centrifugal elutriations were performed as described (Piatti et al., 1995). Flow cytometric DNA quantitation was determined according to Epstein and Cross (1992). Visualization of Tet operators integrated at the *URA3* locus of chromosome V (35 Kb from the centromere) using the GFP-tetR fusion was performed as described (Michaelis et al., 1997). Immunofluorescence was performed according to Nasmyth et al. (1990). Images were captured with either a Coolsnap CCD camera (Photometrics) mounted on an Eclipse E600 microscope (Nikon).

Online supplemental material

In Fig. S1, the *esp1-1* mutation does not affect spindle stability of *scc1-73 mad2Δ* cells. In Fig. S2, a double *scc1-73 esp1-N5* mutant elongates spindles with similar kinetics to *scc1-73* single mutant cells.

We are grateful to Kim Nasmyth and Wolfgang Zachariae for helpful discussions and strains, M. Tyers for anti-Sic1 antibodies, Sanne Jensen for *esp1-N5*, and Arshad Desai, Suzanne Eaton, and Giovanna Lucchini for critical reading of the article. This work was supported by Training and Mobility of Researchers contract ERBFMRXCT98-0212 and a grant from Associazione Italiana Ricerca sul Cancro to S. Piatti.

Submitted: 23 April 2001
Revised: 10 October 2001
Accepted: 10 October 2001

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