

Spo13 regulates cohesin cleavage

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A key aspect of meiotic chromosome segregation is that cohesin, the protein complex that holds sister chromatids together, dissociates from chromosome arms during meiosis I and from centromeric regions during meiosis II. The budding yeast protein Spo13 plays a key role in preventing centromeric cohesin from being lost during meiosis I. We have determined the molecular basis for the metaphase arrest obtained when *SPO13* is overexpressed during the mitotic cell cycle. Overexpression of *SPO13* inhibits anaphase onset by at least two mechanisms. First, Spo13 causes a transient delay in degradation of the anaphase inhibitor Pds1. Second, Spo13 inhibits cleavage of the cohesin subunit Scc1/Mcd1 or its meiosis-specific homolog, Rec8, by the separase Esp1. The finding that Spo13 did not prevent cleavage of another Esp1 substrate, Slk19, suggests that overexpression of *SPO13* is sufficient to prevent cohesin cleavage by protecting specific substrates from separase activity.

[Key Words: Spo13; separin; cohesin; sister-chromatid; segregation; meiosis; *S. cerevisiae*]

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To generate two daughter cells with the exact same complement of chromosomes, it is critical that sister chromatids remain tightly associated prior to chromosome segregation and promptly dissociate as nuclear division commences during anaphase. Essential to this process is the multisubunit protein complex known as cohesin, which provides the physical linkage between sister chromatids. In budding yeast, the cohesin complex consists of at least four subunits, Smc1, Smc3, Scc1/Mcd1, and Scc3 (for review, see Uhlmann 2001).

Cohesion between sister chromatids is established during DNA replication (Uhlmann and Nasmyth 1998) and is maintained until the onset of anaphase, when chromosome segregation is initiated by the loss of cohesion. At the onset of anaphase, the cohesin subunit Scc1/Mcd1 is cleaved by a protease known as separase (Esp1 in budding yeast), allowing sister chromatids to separate (for review, see Nasmyth et al. 2000). The activity of Esp1 is controlled by the anaphase inhibitor securin (Pds1 in budding yeast), which binds to and inhibits Esp1. A ubiquitin ligase called the Anaphase Promoting Complex or Cyclosome (APC/C), in association with the WD40 repeat protein Cdc20, targets Pds1 for degradation at the metaphase–anaphase transition (for review, see Peters 1998; Cohen-Fix 2001), thereby freeing Esp1 to initiate chromosome segregation and mitotic exit.

In contrast to mitosis, where cohesion is lost along the

entire length of the chromosome by the onset of anaphase, sister-chromatid cohesion is lost in a stepwise manner during meiosis (for review, see Miyazaki and Orr-Weaver 1994; Lee and Orr-Weaver 2001). Sister-chromatid cohesion is lost along chromosome arms at the onset of anaphase I, but centromeric cohesion is retained until the onset of anaphase II. Loss of cohesion on chromosome arms is required for homologs to segregate from each other (Buonomo et al. 2000), whereas retention of centromeric cohesion between sister chromatids beyond anaphase I and its subsequent loss at the onset of anaphase II are critical for proper segregation of sister chromatids during meiosis II (Tang et al. 1998). Budding yeast cells accomplish this stepwise loss of cohesion during meiosis in part by replacing the mitotic cohesin subunit Scc1/Mcd1 with a meiosis-specific homolog, Rec8. When *REC8* is replaced by *SCC1/MCD1*, sister-chromatid cohesion is lost along the entire length of the chromosome during the first meiotic division, suggesting that retention of cohesion around centromeres is a specific feature of Rec8-containing cohesin complexes (Toth et al. 2000). As cleavage of Scc1/Mcd1 is required for chromosome segregation in mitosis, Rec8 cleavage by Esp1 is required for chromosome segregation during meiosis (Buonomo et al. 2000). Cleavage of Rec8 at chromosome arms is necessary for anaphase I to occur, and cleavage of Rec8 at centromeric regions is also likely to be required for sister chromatids to separate during meiosis II. The mechanisms that bring about this differential control of Rec8 cleavage are unknown.

SPO13 is required to inhibit sister-chromatid separation during meiosis I. Cells lacking *SPO13* undergo a single meiotic division with a mixed chromosome segregation pattern; some chromosomes segregate preferen-

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tially in an equational manner (sister chromatids are separated), whereas others preferentially segregate reductionally (homologs are separated; Klapholz and Esposito 1980a,b; Hugerat and Simchen 1993). Furthermore, in *spo13Δ* mutants, Rec8 is lost at the chromosome arms and centromeres simultaneously (Klein et al. 1999).

SPO13 overexpression during the mitotic and meiotic cell cycle leads to a metaphase arrest and metaphase I delay, respectively (McCarroll and Esposito 1994). To examine how Spo13 functions to regulate chromosome segregation, we analyzed the consequences of *SPO13* overexpression on mitotic cell cycle progression. We find that when overexpressed during mitosis, *SPO13* inhibits anaphase onset by at least two mechanisms. High levels of Spo13 cause a transient delay in the degradation of the anaphase inhibitor Pds1. Importantly, Spo13 also inhibits the cleavage of the cohesin subunits *Sccl1/Mcd1* and Rec8 in a *PDS1*-independent manner but does not prevent cleavage of another Esp1 substrate, Slk19. Our results indicate that overexpression of *SPO13* is sufficient to specifically inhibit cleavage of the cohesin subunits *Sccl1/Mcd1* and Rec8 by Esp1.

Results

Overexpression of SPO13 arrests cells in metaphase

To determine the molecular basis whereby high levels of Spo13 prevent cell cycle progression, we analyzed the consequences of expressing *SPO13* from the galactose-inducible *GAL1-10* promoter (*GAL-SPO13*) or the tetracycline/doxycycline-repressible *TET* promoter system (*TET-SPO13*). Cells were arrested in the G₁ stage of the cell cycle with α -factor pheromone and released into the cell cycle under conditions where *SPO13* was expressed to high levels. Progression through S phase was not affected as judged by FACS analysis (data not shown), but progression through mitosis was inhibited (Fig. 1A,C). Cells overexpressing *SPO13* arrested as large budded cells with metaphase spindles and an undivided nucleus (Fig. 1A,C), indicating that high levels of Spo13 block entry into anaphase.

Anaphase onset is triggered by the degradation of securin Pds1, which liberates Esp1 to initiate sister-chromatid separation (for review, see Peters 1998). To determine if the *SPO13*-induced metaphase arrest was caused by Pds1 stabilization, we analyzed Pds1 protein levels in *TET-SPO13* cells. Pds1 protein levels initially remained elevated during the arrest but then declined despite cells remaining arrested in metaphase (Fig. 1B,C). Levels of the mitotic cyclin Clb2 remain high during the *SPO13*-induced arrest, and the cyclin-dependent kinase (CDK) inhibitor Sic1 did not accumulate, indicating that exit from mitosis did not occur (Fig. 1B). These results suggest that elevated Pds1 levels are in part responsible for the metaphase arrest observed in cells overexpressing *SPO13*. The finding that Pds1 protein levels declined and cells were nevertheless arrested with metaphase-like spindles raised the possibility that high levels of Spo13 also prevent anaphase onset by means other than inhibiting Pds1 degradation.

Pds1 stabilization caused by high levels of Spo13 is largely independent of the DNA damage and mitotic spindle checkpoints

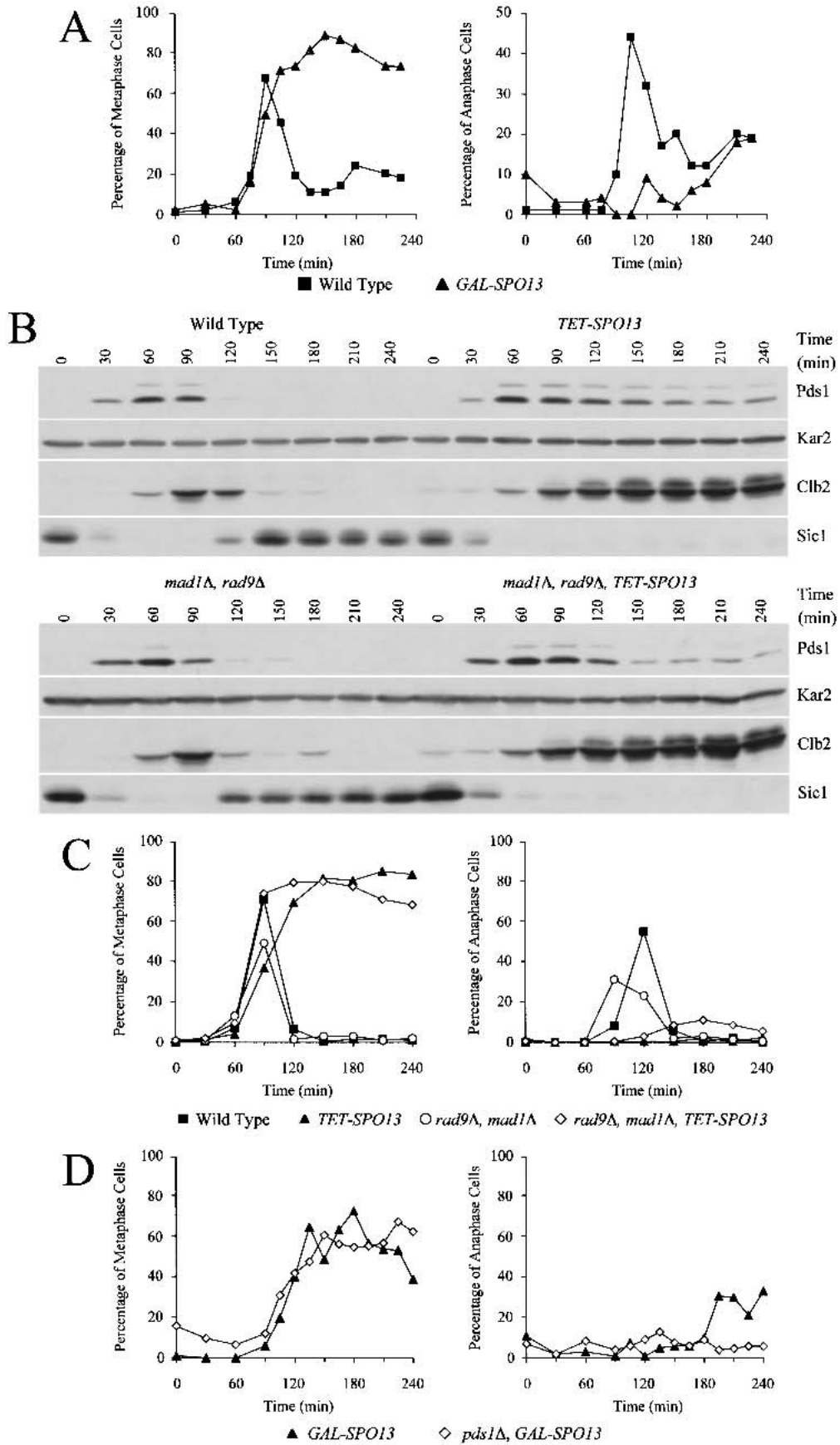
DNA or mitotic spindle damage lead to the activation of the DNA damage and spindle checkpoints, respectively, both of which bring about cell cycle arrest through inhibition of Pds1 degradation (Gardner and Burke 2000; for review, see Melo and Toczyski 2002). To determine whether the stabilization of Pds1 observed in cells overexpressing *SPO13* was caused by activation of either checkpoint, we analyzed Pds1 levels in *TET-SPO13* cells deleted for the DNA damage checkpoint component *RAD9* or the mitotic spindle checkpoint component *MAD1*. Pds1 levels remained elevated in cells lacking *RAD9* (data not shown). Inactivation of the mitotic spindle checkpoint, or both the DNA damage and spindle checkpoints, caused a minor decline in Pds1 protein levels in cells overexpressing *SPO13* (Fig. 1B; data not shown). We conclude that the stabilization of Pds1 brought about by high levels of Spo13 is not caused by activation of the DNA damage checkpoint and is only in part caused by activation of the mitotic spindle checkpoint. Inactivation of either checkpoint or both did not alleviate the metaphase arrest caused by overexpression of *SPO13* (Fig. 1C; data not shown) indicating that mechanisms other than activation of these checkpoints prevented entry into anaphase in cells expressing high levels of Spo13.

High levels of SPO13 inhibit anaphase entry in the absence of PDS1

The observation that cells overexpressing *SPO13* arrest in metaphase despite declining levels of Pds1 raised the possibility that *SPO13* could arrest cells in this stage of the cell cycle without stabilizing Pds1. To test this possibility directly, we examined the consequences of overexpressing *SPO13* in cells lacking *PDS1*. *GAL-SPO13* or *TET-SPO13* cells lacking *PDS1* arrested in metaphase as judged by mitotic spindle morphology (Fig. 1D; see Figs. 3C, 4A, below), showing that high levels of *SPO13* can cause a metaphase arrest in the absence of *PDS1*.

Overexpression of SPO13 prevents sister-chromatid separation by inhibiting Sccl1/Mcd1 cleavage

High levels of Spo13 could inhibit entry into anaphase by either preventing sister-chromatid separation or interfering with mitotic spindle elongation. To distinguish between these possibilities, we analyzed whether overexpression of *SPO13* could arrest cells in metaphase in the absence of sister-chromatid cohesion. To inactivate cohesion between sister chromatids we used a temperature-sensitive allele of the *SCC1/MCD1* cohesin subunit, *mcd1-1* (Guacci et al. 1997). Loss of cohesion brought about by the *mcd1-1* allele causes activation of the mitotic spindle checkpoint (Severin et al. 2001). Therefore, we analyzed the effects of the *mcd1-1* mutation on the *GAL-SPO13*-induced metaphase arrest in



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cells deleted for the mitotic spindle checkpoint component *MAD1*. Loss of sister-chromatid cohesion through heat inactivation of the *mcd1-1* allele allowed *GAL-SPO13* cells to enter anaphase (Fig. 2A). Cells were then delayed in exit from mitosis, as judged by the persistence of cells with anaphase spindles (Fig. 2A), which is consistent with the finding that cells expressing high levels of *SPO13* accumulate high levels of the mitotic cyclin Clb2 and fail to accumulate the CDK inhibitor Sic1. Our results indicate that overexpression of *SPO13* does not inhibit mitotic spindle elongation, but rather interferes with the dissolution of sister-chromatid cohesion and mitotic CDK inactivation.

At the onset of anaphase, the cohesin subunit Scc1/Mcd1 is cleaved, and the cleavage products transiently accumulate during anaphase (Uhlmann et al. 1999). To determine whether overexpression of *SPO13* inhibited the onset of anaphase by preventing Scc1/Mcd1 cleavage, we analyzed whether Scc1/Mcd1 was cleaved in *GAL-SPO13* and *TET-SPO13* cells. Upon release from a pheromone-induced G_1 arrest, the Scc1/Mcd1 cleavage product was transiently observed in wild-type cells at the onset of anaphase, and simultaneously, full-length Scc1/Mcd1 levels declined (Fig. 2B; data not shown). In contrast, no Scc1/Mcd1 cleavage product was observed in cells overexpressing *SPO13*, and full-length Scc1/Mcd1 persisted (Fig. 2B; data not shown). The bulk of Scc1/Mcd1 was also not cleaved in *TET-SPO13* cells lacking *PDS1* (see Fig. 4B below), showing that overexpression of *SPO13* prevents Scc1/Mcd1 cleavage and that it does so in part by means other than Pds1 stabilization.

To test directly whether the metaphase arrest caused by high levels of Spo13 was caused by inhibition of Scc1/Mcd1 cleavage, we analyzed whether cleavage of Scc1/Mcd1 was sufficient to induce anaphase entry in cells overexpressing *SPO13*. We used a *SCC1/MCD1* allele in which one of the Esp1 cleavage sites is replaced by a cleavage site for the tobacco etch virus (TEV) protease (*scc1-TEV268*; Uhlmann et al. 2000). Cells overexpressing *SPO13* and carrying a *scc1-TEV268* construct as the sole source of *SCC1/MCD1* were released from a G_1 block, and production of the TEV protease (under the control of the *GAL1-10* promoter) was induced when cells entered the *TET-SPO13*-induced metaphase arrest (110 min after release from α -factor). Scc1/Mcd1 cleavage occurred efficiently soon after induction of the TEV protease, as judged by the appearance of the Scc1/Mcd1 cleavage product, chromosome segregation, and mitotic

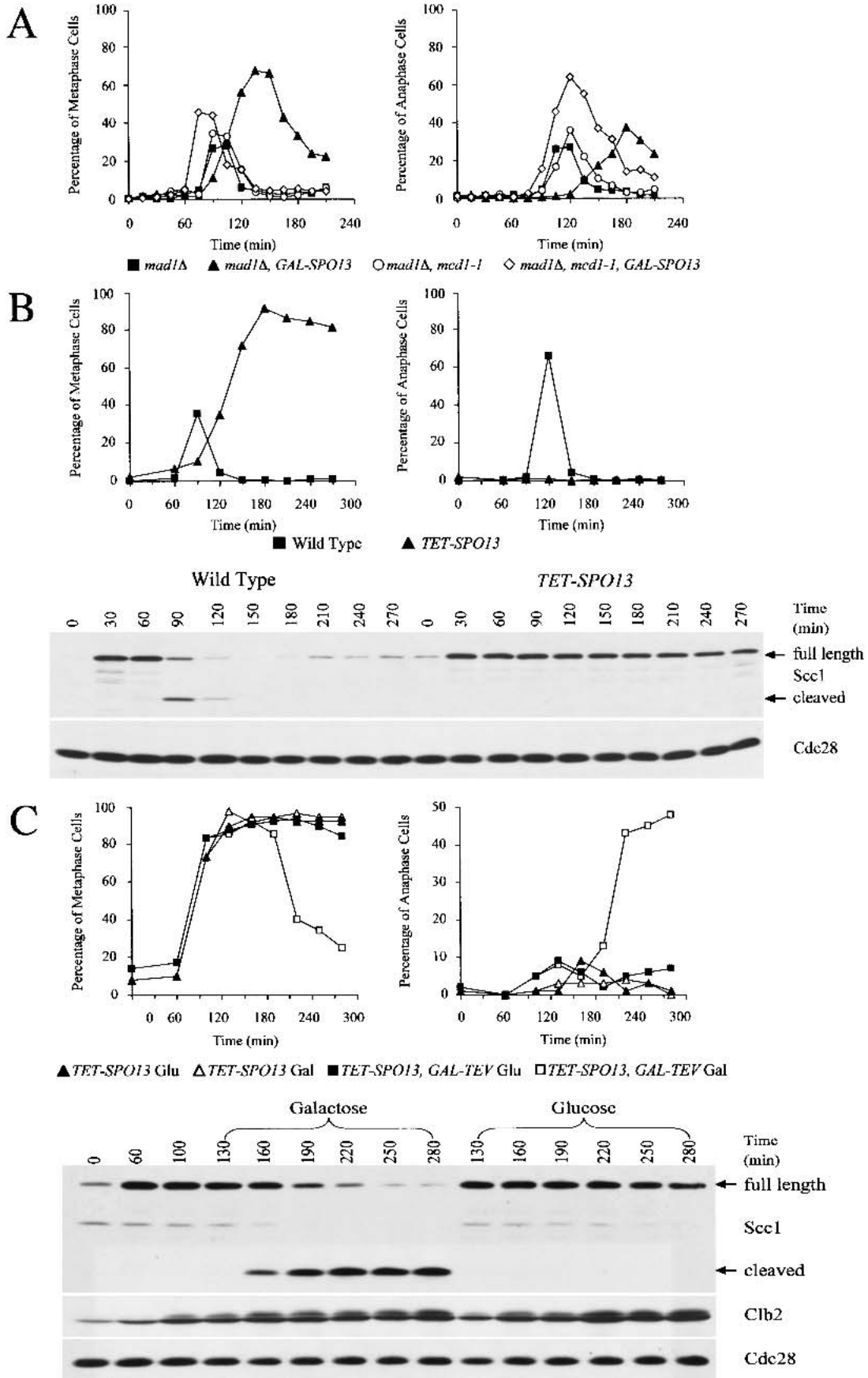
spindle elongation (Fig. 2C, +Galactose). Exit from mitosis, however, did not occur, as Clb2 protein levels remained elevated and mitotic spindles did not disassemble (Fig. 2C), indicating that Spo13 overexpression also prevented mitotic CDK inactivation. Under conditions wherein expression of the TEV protease was inhibited (+Glucose), Scc1/Mcd1 cleavage did not occur and cells remained arrested in metaphase (Fig. 2C). Our results show that cleavage of Scc1/Mcd1 was sufficient to induce anaphase in *TET-SPO13*-arrested cells. We conclude that Spo13 inhibits anaphase by inhibiting Scc1/Mcd1 cleavage. This inhibition is in part caused by a transient stabilization of Pds1 but is also caused by a *PDS1*-independent mechanism that affects cleavage of Scc1/Mcd1 directly.

Overexpression of *SPO13* inhibits *Rec8* cleavage

During meiosis, Pds1 is degraded at the metaphase I–anaphase I transition, which liberates Esp1 to cleave the meiotic cohesin subunit Rec8 on chromosome arms (Buonomo et al. 2000; Salah and Nasmyth 2000). Thus, Pds1 is not likely to be involved in preventing Esp1 from cleaving Rec8 at centromeric regions during the first meiotic division. Spo13, on the other hand, is required for retention of centromeric cohesion during anaphase I (Klein et al. 1999). Our analysis of the effects of *SPO13* overexpression on mitotic cell cycle progression showed that Spo13 inhibits cleavage of Scc1/Mcd1 in part through a *PDS1*-independent mechanism. The *PDS1*-independent mechanism whereby Spo13 inhibits Scc1/Mcd1 cleavage during mitosis may be similar to the mechanism whereby Spo13 protects centromeric Rec8 during anaphase I. We were, unfortunately, not able to test the possibility that overexpression of *SPO13* prevented Rec8 cleavage during the meiotic cell cycle directly. For reasons that are unclear, overexpression of *SPO13* during meiosis resulted in a failure of cells to enter metaphase I, with the majority failing to initiate premeiotic S phase (B. Lee, unpubl.). This early meiotic cell cycle arrest made it impossible to test whether high levels of Spo13 interfered with Rec8 cleavage and anaphase I onset. Furthermore, deletion of *PDS1* in meiosis caused cells to arrest in prophase I (data not shown).

To determine whether Spo13 could at least in principle inhibit Rec8 cleavage in a *PDS1*-independent manner, we expressed *REC8* in vegetatively growing *pds1* Δ cells from the *SCC1/MCD1* promoter and analyzed the

Figure 1. Overexpression of *SPO13* causes cells to arrest in metaphase. (A) Wild-type (■, A940) and *GAL-SPO13* (▲, A1813) cells were arrested in G_1 with α -factor (5 μ g/mL) in YEPR (YEP + 2% raffinose). After 1 h, galactose was added to 2% to induce *SPO13* expression. Cells were then released into medium containing galactose but lacking pheromone. The percentage of cells with metaphase (left graph) or anaphase (right graph) spindles was determined at the indicated times. (B,C) Wild-type (■, A1951); *TET-SPO13* (▲, A4318); *rad9* Δ , *mad1* Δ (○, A5309); and *rad9* Δ , *mad1* Δ , *TET-SPO13* (◇, A5310) cells containing a *PDS1-HA* fusion were grown in YEPR containing doxycycline (5 μ g/mL) to inhibit *SPO13* expression. Cells were then washed to remove the doxycycline and arrested in G_1 with α -factor (5 μ g/mL) for 4 h, followed by release into YEPR lacking pheromone and doxycycline. After 100 min, when cells had budded, 5 μ g/mL α -factor was added to prevent entry into the second cell cycle. The total amount of Pds1, Clb2, and Sic1 (B), as well as the percentage of cells with metaphase (C, left graph) or anaphase (C, right graph) spindles were determined at the indicated times. Kar2 was used as a loading control in Western blot analyses. (D) *GAL-SPO13* (▲, A1767) and *GAL-SPO13 pds1* Δ (◇, A1801) cells were grown as described in A, and the percentage of cells with metaphase (left graph) or anaphase (right graph) spindles was determined.



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effects of *SPO13* overexpression on Rec8 cleavage. We first determined whether Rec8 is indeed cleaved by separase when expressed during mitosis. Upon release from a pheromone block, about half of the total amount of Rec8 protein was degraded during S phase in a separase independent-manner (Fig. 3B, +Nocodazole). The remaining Rec8 was degraded at the onset of anaphase in a separase-dependent manner. Full-length Rec8 levels declined, and the Rec8 C-terminal cleavage product, which is the same as the C-terminal cleavage observed in cells undergoing meiosis (data not shown), appeared during anaphase onset. In contrast, in cells treated with nocodazole, which inhibits Esp1 activity (Uhlmann et al. 1999), or in cells carrying a temperature-sensitive *esp1-1* mutant, full-length Rec8 persisted, and the accumulation of the Rec8 C-terminal cleavage product was prevented (Fig. 3B; data not shown). In cells overexpressing *SPO13*, Rec8 behaved in a manner similar to that of cells treated with nocodazole (Fig. 3D). About half of the total amount of Rec8 was degraded during S phase, but the remaining pool of Rec8 was stable (Fig. 3D). Furthermore, the C-terminal cleavage product of Rec8 was not observed, suggesting that high levels of Spo13 can prevent cleavage of the meiosis-specific cohesin subunit Rec8. This observation is also in agreement with the finding that overexpression of *SPO13* prevented Rec8 from dissociating from chromosomes during mitosis (M. Shonn and A. Murray, pers. comm.; see accompanying paper by Shonn et al. in this issue).

SPO13 overexpression does not prevent cleavage of *Slk19*

The recent identification of another substrate of Esp1, the kinetochore protein *Slk19* (Sullivan et al. 2001), enabled us to test whether Spo13 specifically inhibited cleavage of the cohesin subunits *Scc1/Mcd1* and *Rec8* or whether it inhibited Esp1 activity toward all its known substrates. We analyzed Spo13's effect on cleavage of *Slk19* in cells lacking *PDS1* to characterize specifically the consequences of *SPO13* overexpression that occur independently of *Pds1* stabilization. In contrast to *Scc1/Mcd1*, *Slk19* cleavage occurred in *TET-SPO13* cells (Fig. 4B). As observed in earlier experiments, Spo13 also inhibited mitotic CDK inactivation, as *Clb2*-associated ki-

nase activity remained elevated throughout the course of the experiment (Fig. 4B). We conclude that Spo13 specifically protects *Rec8* and *Scc1/Mcd1* from separase activity.

Discussion

Our results show that overexpression of *SPO13* during mitosis inhibits sister-chromatid separation. *SPO13* causes this metaphase arrest by two means. First, high levels of Spo13 cause a transient delay in *Pds1* degradation. How Spo13 brings about the transient stabilization of *Pds1* is not known. It is only to some extent through activation of the mitotic spindle checkpoint, as *Pds1* protein levels persist for a slightly shorter time in *TET-SPO13* cells lacking the mitotic spindle checkpoint component *MAD1*. Second, *SPO13* also prevented sister-chromatid separation in the absence of *PDS1*. We could exclude the possibility that this inability to enter anaphase was caused by defects in mitotic spindle elongation and showed that it was caused by a failure to cleave the cohesin subunit *Scc1/Mcd1*.

A key question is whether the mechanism in which high levels of Spo13 inhibit mitotic cell cycle progression provides insight into the function of Spo13 during meiosis. The finding that overexpression of *SPO13* is sufficient to prevent cohesin cleavage suggests that Spo13 is a regulator of *Rec8* cleavage during meiosis. However, overexpression of *SPO13* alone does not recapitulate all aspects of cohesion regulation that occur during meiosis, as high levels of Spo13 prevent *Scc1/Mcd1* cleavage during mitosis, but wild-type levels of Spo13 cannot protect *Scc1/Mcd1* from being cleaved when *SCC1/MCD1* is expressed instead of *REC8* during meiosis (Toth et al. 2000). Furthermore, high levels of Spo13 appeared to prevent a large fraction of *Scc1/Mcd1* and *Rec8* from being cleaved rather than only the presumably small pool that localizes to centromeres (Figs. 3, 4). Clearly, therefore, overexpression of *SPO13* during mitosis alone cannot bring about a meiosis-specific chromosome segregation pattern. However, our results nevertheless show that overexpression of *SPO13* is sufficient to prevent Esp1 from cleaving *Scc1/Mcd1* or *Rec8*. Importantly, the inhibition of cohesin cleavage brought about by Spo13 is, as in meiosis, independent of *PDS1*

Figure 2. *SPO13* inhibits *Scc1/Mcd1* cleavage. (A) *mad1Δ* (■, A928); *mad1Δ, GAL-SPO13* (▲, A1796); *mad1Δ, mcd1-1* (○, A4264); and *mad1Δ, GAL-SPO13, mcd1-1* (◇, A5413) cells were grown as described in Figure 1A except cells were released into YEPR medium containing galactose at 37°C. The percentage of cells with metaphase (left graph) or anaphase (right graph) spindles was determined at the indicated times. (B) Wild-type (■, A3160) and *TET-SPO13* (▲, A4003) cells containing an *SCC1-18MYC* fusion were grown as described in Figure 1B. The percentage of cells with metaphase (left graph) or anaphase (right graph) spindles was determined at the indicated times. The total amount of *Scc1-Myc* was determined by Western blot analysis. *Cdc28* was used as a loading control. (C) *TET-SPO13, GAL-TEV, SCC1-TEV268-HA* (■, A3475) and *TET-SPO13* (▲, A3382) cells were grown in YEPR containing doxycycline (5 μg/mL). Cells were then washed and grown for 2 h in YEPR lacking doxycycline to induce *SPO13* expression. Then 5 μg/mL α-factor was added to arrest cells in G₁, followed by release into YEPR lacking pheromone and doxycycline. After 110 min, when cells were arrested in metaphase, the cultures were split and galactose was added to 2% (open symbols) or glucose to 2% (closed symbols) to induce or inhibit the expression of the TEV protease, respectively. The percentage of cells with metaphase (left graph) and anaphase spindles (right graph) was determined at the indicated times. The total amount of *Scc1-HA* and *Clb2* for strain A3475 was determined by Western blot analysis. *Cdc28* was used as a loading control.

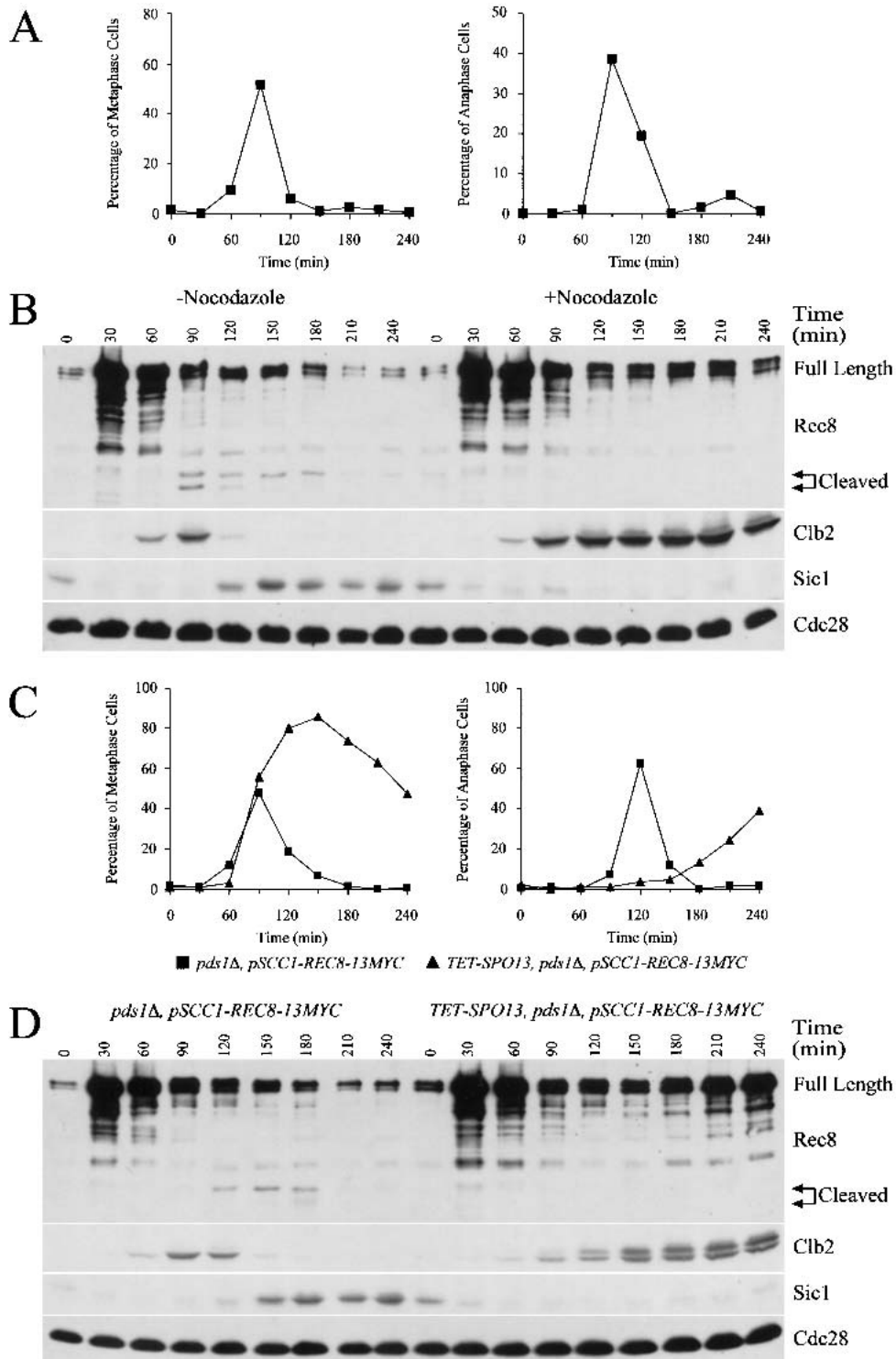


Figure 3. Spc13 inhibits cleavage of Rec8. (A,B) *pSCC1-REC8-13MYC* (■, A5468) cells were grown as described in Figure 1B except that after release from α -factor arrest, the culture was split and 15 μ M nocodazole was added to one-half of the cells. After 110 min, when cells had budded, 5 μ M α -factor was added to prevent entry into the second cell cycle. The percentage of cells with metaphase (A, left graph) or anaphase (A, right graph) spindles and the total amount of Rec8-Myc, Clb2, and Sic1 were determined at the indicated times. (B) Cdc28 was used as a loading control in Western blot analyses. (C,D) *pds1Δ, pSCC1-REC8-13MYC* (■, A5635) and *pds1Δ, pSCC1-REC8-13MYC, TET-SPO13* (▲, A5645) cells were grown as described in Figure 1B, and the percentage of cells with metaphase (C, left graph) or anaphase (C, right graph) spindles was determined at the indicated times. (D) The total amount of Rec8-Myc, Clb2, and Sic1 was determined by Western blot analysis. Cdc28 was used as a loading control.

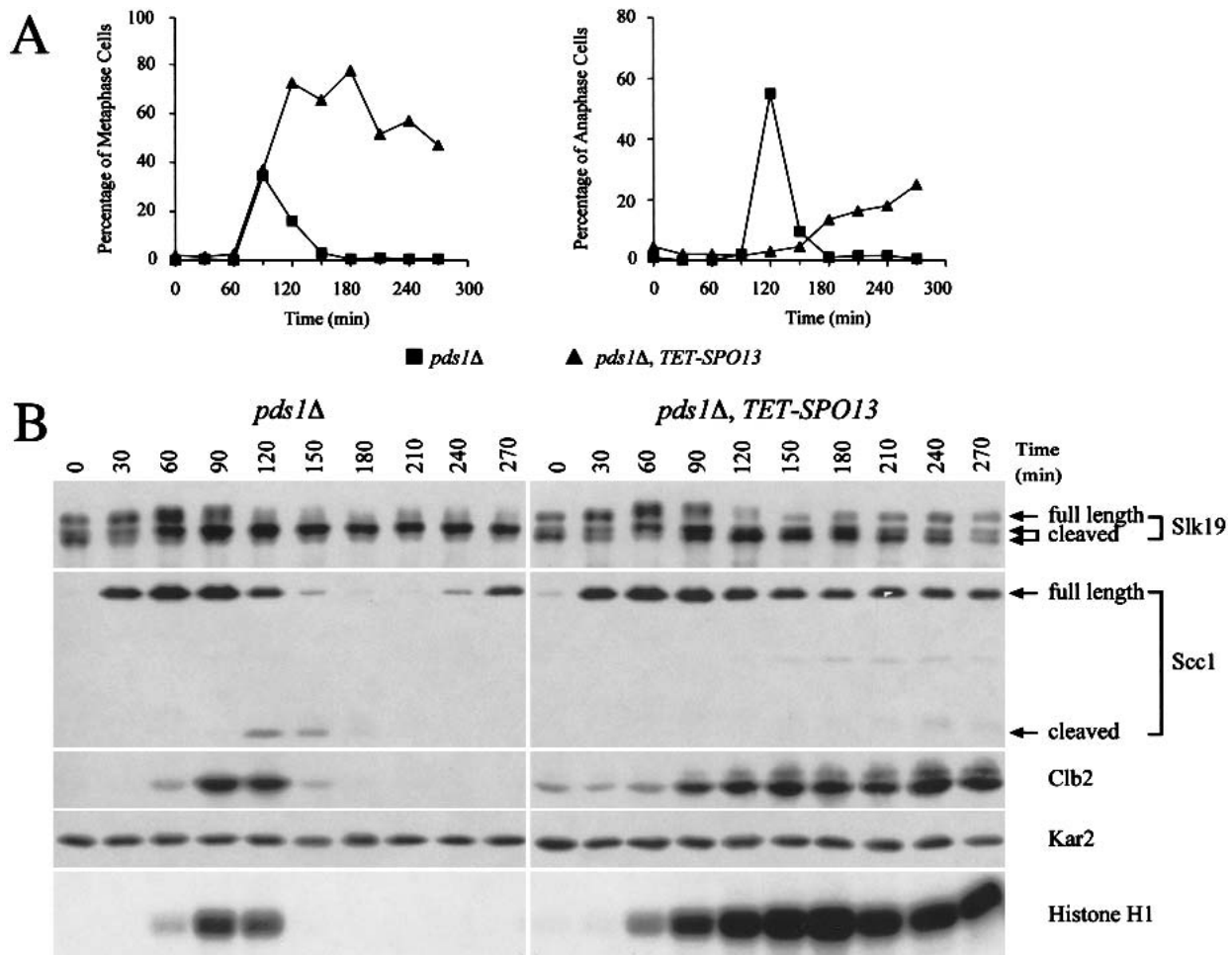


Figure 4. Spo13 does not inhibit cleavage of Slk19. *pds1Δ* (■, A4188) and *pds1Δ, TET-SPO13* (▲, A4187) cells containing *SCC1-3HA* and *SLK19-13MYC* fusions were grown as described in Figure 1B to determine the percentage of cells with metaphase (A, left graph) or anaphase (A, right graph) spindles. (B) The total amount of Scc1-3HA, Slk19-13MYC, and Clb2 was determined by Western blot analysis. Clb2-associated kinase activity was determined using Histone H1 as a substrate. At the 0 time point, three forms of Slk19-13MYC are visible. The upper band is full-length Slk19, which becomes phosphorylated as cells progress through the cell cycle. The bottom band corresponds to the C-terminal cleavage product of Slk19; and the middle band is the phosphorylated form of the cleavage product.

stabilization, suggesting that the effects of *SPO13* overexpression during mitosis to some extent recapitulate Spo13's function during meiosis. This observation, together with the finding that Rec8 is lost at centromeres during meiosis I in the absence of *SPO13* (Klein et al. 1999), suggests that Spo13 is an important factor in the control of cohesin cleavage in meiosis.

SPO13 specifically inhibits Scc1/Mcd1 and Rec8 cleavage when overexpressed during mitosis

Overexpression of Spo13 inhibited Scc1/Mcd1 and Rec8 but not Slk19 cleavage. The finding that Spo13 can prevent cleavage of a subset of separase substrates raises the interesting possibility that mechanisms exist that determine whether or not a substrate is cleaved by separase. Modifications such as phosphorylation on Esp1 itself

(Stemmann et al. 2001) could determine whether or not Esp1 could recognize a certain substrate. Alternatively, substrate modifications and/or accessibility could be the source of specificity. Phosphorylation of Scc1/Mcd1 by the polo-like kinase Cdc5 has been shown to greatly enhance Esp1's ability to cleave Scc1/Mcd1 (Alexandru et al. 2001). Perhaps Cdc5-dependent phosphorylation or other modifications are important to induce cleavage of some substrates but not others.

How does SPO13 prevent Scc1/Mcd1 or Rec8 cleavage?

As high levels of *SPO13* inhibit Scc1/Mcd1 and Rec8 cleavage but not that of another Esp1 substrate, Slk19, it is clear that Spo13 does not generally inhibit Esp1 activity, but rather specifically prevents Scc1/Mcd1 or Rec8 from being cleaved. The mechanism whereby Spo13 pro-

tecs *Sccl/Mcd1* or *Rec8* from being cleaved, however, remains obscure. One possibility we considered was that *Spo13* affects the function of *Cdc5*, a protein kinase that aids *Esp1* in cleaving *Sccl/Mcd1* (Alexandru et al. 2001). The finding that overexpression of *CDC5* did not lead to *Sccl/Mcd1* cleavage in cells with high levels of *Spo13* whereas overexpression of *ESP1* did (B. Lee, unpubl.) argues against this possibility. Another possibility is that *Spo13* binds directly to the cohesin complex and occludes access of *Esp1* to its substrate. This hypothesis would predict that *Spo13* and components of the cohesin complex physically interact. We have not observed such a physical interaction (B. Lee, unpubl.). Additionally, *Spo13* does not prevent *Sccl-Tev268* from being cleaved by the TEV protease, suggesting that the TEV cleavage site and presumably the *Esp1* site that the TEV cleavage site replaces are not physically obscured by *Spo13*. Therefore, we believe that *Spo13* does not itself prevent *Esp1* from cleaving *Sccl/Mcd1* or *Rec8* but plays a regulatory role in this process.

Further support for the idea that *Spo13* functions in a regulatory manner comes from the multiple phenotypes associated with *SPO13* overexpression during the mitotic cell cycle. *Spo13* not only inhibited cohesin cleavage but also prevented mitotic CDK inactivation. This inability to inactivate mitotic CDKs is not an indirect consequence of *GAL-SPO13* or *TET-SPO13* cells failing to separate sister chromatids. When *Spo13*'s inhibitory effect on sister-chromatid separation was alleviated, either by using a temperature-sensitive *mcd1-1* allele or by inducing *Sccl/Mcd1* cleavage by the TEV protease, mitotic CDK inactivation was still delayed or prevented. The inhibition of mitotic CDK inactivation brought about by high levels of *Spo13* was also not an indirect consequence of a delay in *Pds1* degradation, as *Spo13* also prevented mitotic CDK inactivation in cells lacking *PDS1*. How *Spo13* regulates sister-chromatid cohesion and mitotic CDK activity is still unclear. It is, however, interesting to note that the protein phosphatase *Cdc14* is required for mitotic CDK inactivation and for accurate meiotic chromosome segregation (Sharon and Simchen 1990; Visintin et al. 1998; Jaspersen et al. 1999). Perhaps *Spo13* exerts its multiple functions through the regulation of *Cdc14*.

Materials and methods

All strains were derivatives of the yeast strain W303 (A2587). The *TET-SPO13* construct was created by ligating a PCR fragment containing the *SPO13* open reading frame (ORF) into pCM190 (Gari et al. 1997). Subsequently, the 2 μ origin in pCM190 was deleted creating an integration plasmid that was then 3x-integrated at the *URA3* locus. The *GAL-SPO13-TRP1* fusion was constructed by ligating the *SPO13* ORF under the control of the *GAL1-10* promoter fragment. The fusion was subsequently cloned into YIplac204 (Gietz and Sugino 1988) and integrated at the *TRP1* locus. The *SCC1-3HA*, *SLK19-13MYC*, and *REC8-13MYC* fusions were constructed by a one-step PCR method (Longtine et al. 1998). To clone *REC8* under the control of the *SCC1* promoter (*pSCC1-REC8-13MYC*), the *GAL1-10* promoter fragment in *pFA6a-pGAL* (Longtine et al. 1998) was

replaced by a 1-kb *SCC1*-promoter PCR fragment. The resulting plasmid was used as a template in a one-step PCR-based method (Longtine et al. 1998) to replace the endogenous *REC8* promoter in a *REC8-13MYC* strain with the *SCC1* promoter. *SCC1-18MYC* and *PDS1-HA* constructs were described in Michaelis et al. (1997) and Cohen-Fix et al. (1996), respectively.

Growth conditions for individual experiments are described in the figure legends. Unless otherwise noted, cells were grown at 25°C. Immunoblot analysis was performed as described in Cohen-Fix et al. (1996). To visualize *Rec8* and its cleavage products, extracts were prepared by precipitating cells with trichloroacetic acid (TCA) and lysed as described in Moll et al. (1991). *Clb2*-associated kinase activity was assayed as described in Surana et al. (1993). Indirect in situ immunofluorescence methods and antibody concentrations are described in Visintin et al. (1999). Metaphase spindles were defined as mitotic spindles spanning an undivided nucleus. Anaphase spindles were defined as mitotic spindles spanning a divided nucleus.

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