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Cohesins: Chromosomal Proteins that Prevent Premature Separation of Sister Chromatids

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Summary

Cohesion between sister chromatids opposes the splitting force exerted by microtubules, and loss of this cohesion is responsible for the subsequent separation of sister chromatids during anaphase. We describe three chromosmal proteins that prevent premature separation of sister chromatids in yeast. Two, Smc1p and Smc3p, are members of the SMC family, which are putative ATPases with coiled-coil domains. A third protein, which we call Scc1p, binds to chromosomes during S phase, dissociates from them at the metaphase-to-anaphase transition, and is degraded by the anaphase promoting complex. Association of Scc1p with chromatin depends on Smc1p. Proteins homologous to Scc1p exist in a variety of eukaryotic organisms including humans. A common cohesion apparatus might be used by all eukaryotic cells during both mitosis and meiosis.

Introduction

Because most of the instructions for building a cell reside within chromosomes, their duplication and segregation during cell proliferation must occur with a fidelity that far exceeds that of other biosynthetic processes. Having duplicated its chromosomes, the cell's next task is to generate a bipolar mitotic spindle and to attach sister kinetochores to microtubules that associate with opposite poles of this spindle. By means of tension exerted on sister kinetochores, these microtubules align each pair of sister chromatids on the metaphase plate (Skibbens et al., 1993). Chromosome alignment during metaphase depends not only on "splitting" forces exerted by microtubules on kinetochores but also on an opposing "cohesive" force exerted by tethers that hold sister chromatids together. The subsequent separation of sister chromatids to opposite poles, known as anaphase, is thought to be due to a sudden loss of cohesion between sister chromatids. Reduplication of chromosomes normally never occurs until sisters from the previous round have been separated. Failure to link these two processes or to align sister chromatid pairs on the metaphase plate could be responsible for the abnormal karyotypes of many human tumor cells and could therefore have an important role in oncogenesis (Lengauer et al., 1997).

Little or nothing is known about the nature of sister chromatid cohesion in mitotic cells. DNA replication gives rise to intercatenated sister chromatid molecules whose resolution at anaphase requires Topoisomerase II (DiNardo et al., 1984). It is, however, unlikely that this intertwining of DNA molecules alone holds sister chromatids together during G2 and M phases, because cohesion between circular sister minichromosomes in yeast is maintained in the absence of any catenation in cell cycle mutants arrested in metaphase (Koshland and Hartwell, 1987).

New insight into the process of sister chromatid separation has recently stemmed from analysis of B-type cyclin proteolysis, which occurs at the same stage of the cell cycle. Proteolysis of B-type cyclins by the proteasome is preceded by their multiubiquitination. Cyclin ubiquitination depends on a large multisubunit complex (King et al., 1995; Sudakin et al., 1995; Zachariae et al., 1996), which is also essential for the separation of sister chromatids during anaphase (Irniger et al., 1995) and has therefore been called the anaphase promoting complex (APC). The cyclin destruction machinery is tightly cell cycle regulated; it lies dormant during S, G2, and early M phases but springs into action during anaphase and remains highly active during the subsequent G1 (Amon et al., 1994).

The failure of APC mutants to separate sister chromatids cannot be explained by their failure to destroy B-type cyclins. Cells that overproduce nondegradable B-type cyclins continue to separate sister chromatids (Holloway et al., 1993; Surana et al., 1993). Anaphase onset presumably depends on the destruction of other proteins by the APC. One candidate is Pds1p, a nonessential protein necessary for arresting the cell cycle in the presence of DNA damage (Yamamoto et al., 1996). Its destruction shortly before the metaphase-to-anaphase transition is necessary for sister chromatid separation (Cohen-Fix et al., 1996). In fission yeast, proteolysis of Cut2p might have a similar function to that of Pds1p (Funabiki et al., 1996). If sister chromatid cohesion during G2 and M phases depends on proteinaceous bridges and not merely on DNA intertwining, then one of the APC's additional functions might be to mediate the destruction of proteins that are components of such bridges. Such structures have not yet been identified, nor indeed has any individual protein ever been directly implicated in sister chromatid cohesion in mitotic cells. It is not known, for example, whether Pds1p is bound to chromosomes, nor indeed whether it is needed to hold them together during G2 or M phase.

To identify proteins needed for sister chromatid cohesion that might be substrates of the APC, we set out to isolate mutants that lose chromosomes at a high frequency and are capable of separating sister chromatids in the absence of APC function. By this means, we identified four genes (*SCC1*, *SCC2*, *SMC1*, and *SMC3*) involved in sister chromatid cohesion. Scc1p (sister chromatid cohesion) binds to chromosomes during S phase, dissociates from them at the metaphase-to-anaphase transition, is at this stage degraded by the APC, and is essential for preventing premature sister chromatid separation. Smc1p and Smc3p are also chromosomal

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proteins. They belong to a family of proteins, members of which are important for chromosome condensation and form part of the longitudinal axis of mitotic chromosomes in vertebrates (Hirano et al., 1995; Koshland and Strunnikov, 1996). The association of Scc1p with chromatin depends on Smc1p. Proteins similar to Scc1p exist in fission yeast (Birkenbihl and Subramani, 1992), C. elegans, Drosophila, and in humans (McKay et al., 1996). Rec8, a related protein in fission yeast, is required for sister chromatid cohesion during meiosis (Molnar et al., 1995). A common cohesion apparatus might therefore be used during mitosis and meiosis in all eukaryotic cells.

Results

A Screen for Genes Needed for Sister Chromatid Cohesion in Metaphase Cells

If proteinaceous bridges holding sister chromatids together are destroyed by the APC, then it should be possible to identify components of these bridges by isolating mutants capable of separating sister chromatids in the absence of APC function. A modest defect in sister chromatid cohesion should be compatible with proliferation but might cause cells to lose chromosomes. We therefore sought mutants that lose chromosomes at high frequency during proliferation at 25°C and are capable of separating sister chromatids in the absence of APC function when shifted to 37°C. To inactivate the APC conditionally, we used a strain carrying a temperature-sensitive allele of *CDC16* (*cdc16–123*). The parental strain (*ade2–1*, *cdc16–123*, *CFIII*, *SUP11*) contained a "marker" chromosome and forms white colonies, whereas mutants that lose chromosomes with high frequency form colonies with multiple red sectors (Spencer et al., 1990).

We isolated 377 mutants that reproducibly formed colonies with multiple red sectors. These mutants were subsequently screened by microscopic analysis of their DNA and microtubule distribution. Upon incubation at 37°C, the parental strain fails to separate its chromosomes, which remain in a single mass at the bud neck. We found that eight mutants managed to separate their chromosomes into two adjacent masses on either side of the bud neck at 37°C, which indicated that they might be capable of separating sister chromatids. Tetrad analysis of progeny derived from crosses between all eight double mutant strains and wild type (*CDC16*) showed that chromosome loss (at 25°C) and partial nuclear division (at 37°C) were tightly linked and due to a single mutation, which caused ts lethality in an otherwise wild-type background.

Crosses between single mutant strains showed that their ts lethalities were recessive to wild type and that they fell into four different complementation groups. Wild-type genes isolated by rescuing the mutants' ts lethality also suppressed their high frequency of chromosome loss. The first corresponded to *SMC1*, which encodes a protein related to a family of proteins associated with mitotic chromosomes in vertebrates (Hirano and Mitchison, 1994). Previously characterized *smc1* mutants lose chromosomes at high frequency and fail to complete mitosis (Strunnikov et al., 1993). The second corresponded to a previously uncharacterized open reading frame that encodes another member of the SMC family, which we called *SMC3*. The third encodes a protein related to *Rad21* from the fission yeast S. pombe (Birkenbihl and Subramani, 1992). Because of its vital role in holding together sister chromatids, we called this gene *SCC1* (sister chromatid cohesion). Proteins related to Scc1p also exist in C. elegans and in humans (McKay et al., 1996). The last gene, which we provisionally called *SCC2*, encodes a protein with homology to an uncharacterized open reading frame in S. pombe. This genetic analysis showed that we had isolated one allele each of *smc1* and *smc3*, two alleles of *scc1*, and four different alleles of *scc2*. Tetrad analysis of spores derived from diploids heterozygous for *scc1*, *scc2*, and *smc3* deletions showed that these three genes are essential for proliferation at 25°C as well as 37°C.

SCC1 Is Needed for Sister Chromatid Cohesion

Because individual chromosomes cannot be visualized in S. cerevisiae, we characterized sister chromatid cohesion in *scc1* mutants initially using fluorescent in situ hybridization (FISH). Wild-type and *scc1–73* mutant cells growing at 25°C were incubated in the presence of nocodazole for 3 hr and then shifted to 37°C for 1.5 hr. In wild-type cells, fluorescent probes from the left arm of chromosome XVI produced a single fluorescent dot in 83% and two fluorescent dots in 17% of all nuclei. In *scc1–73* mutant cells, on the other hand, the same probe produced two dots in 69% of all nuclei (Figure 1A). Similar results were obtained using a probe close to the centromere (data not shown). Damage to the mitotic spindle caused by nocodazole is detected by a surveillance mechanism that blocks sister chromatid separation in wild-type cells but fails to do so in *scc1–73*. *scc1* mutants are still capable of detecting and responding to spindle damage (see below), which suggests that they might have a specific defect in holding sister chromatids together.

Sister Chromatids Separate Prematurely in *scc1* mutants

An alternative way of detecting the location of chromosomal loci is to mark them with binding sites for sequence-specific DNA binding proteins. Lac Repressor fused to green fluorescent protein (GFP) binds to tandem Lac operators integrated at unique sites within yeast chromosomes and thereby enables them to be visualized as small green fluorescent dots within the yeast nucleus (Straight et al., 1996). We developed a similar technique using the Tetracycline Operator/Repressor system. We integrated 336 Tet operators adjacent to the centromere of chromosome V in a cell that expresses a Tet repressor fused to GFP. The Tet operators were visible as green fluorescent dots within the nuclei of either live or fixed cells. In asynchronous cultures of haploid wild-type cells, we observed single dots in unbudded cells, in cells with small buds, and in some cells with large buds, but two dots only in cells with large buds (Figure 1B).

We followed sister chromatid cohesion by sampling

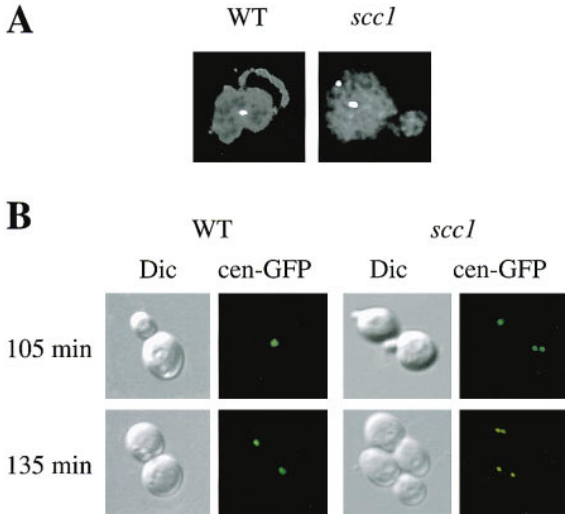

Figure 1. Scc1p Is Required for Sister Chromatid Cohesion

(A) Wild-type (K699) strain and the *scc1–73* mutant (K5832) were grown in YEPD to early log phase, arrested with nocodazole for 3 hr, and then shifted to the nonpermissive temperature of 37°C for additional 90 min. Samples were processed for FISH analysis and hybridized with a DNA fragment distal of the centromere of chromosome XVI.

(B) Wild-type (K6745) and *scc1–73* (K6752) cells containing the Tet-GFP fusion and the tandem repeats of the tet operator were collected by centrifugal elutriation and released into YEPD at 37°C. Cells of similar bud size were analyzed for the existence of one or two dots, corresponding to paired or separated sister chromatids, respectively. In large budded wild-type cells, sister chromatids separated into the mother and daughter cell, while small budded cells contained only one dot. In *scc1* mutant cells, separated sister chromatids could be detected shortly after DNA replication in small budded cells. Large budded cells often showed both dots in one bud (at 135 min, 40% showed two dots in one cell bud and 60% had separated dots like wild-type cells).

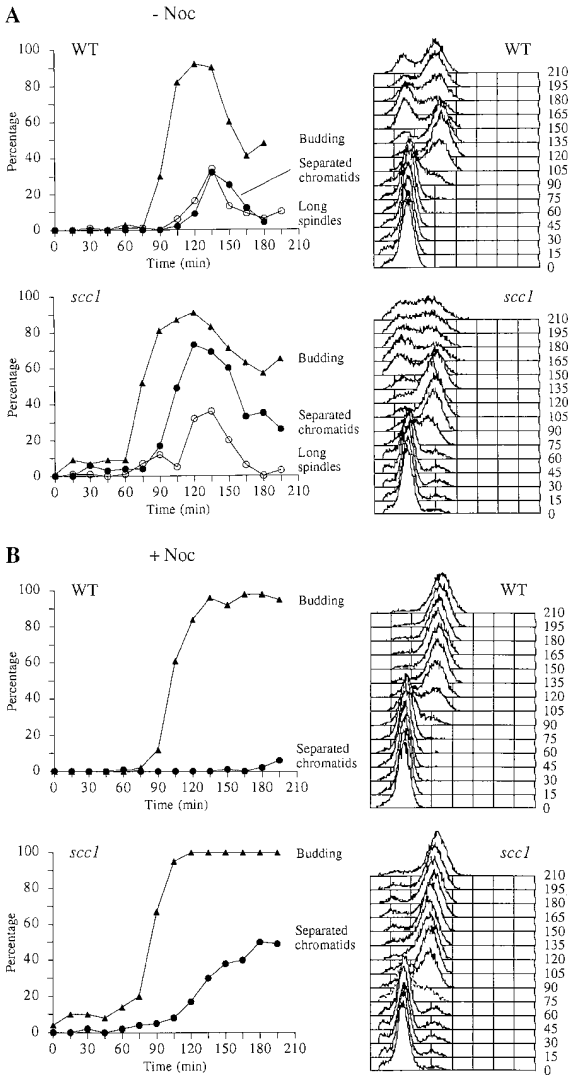

Figure 2. Sister Chromatids Separate Prematurely in the *scc1* Mutant

(A and B) Small unbudded wild-type (K6745) and *scc1–73* (K6752) cells were isolated by elutriation and incubated in YEPD at 37°C in the absence (A) or in the presence of nocodazole (B). Samples were taken every 15 min and analyzed for their budding index, the existence of paired or separated sister chromatids, the presence of fully elongated spindles by indirect immunofluorescence using an antitubulin antibody, and DNA content by FACS.

cells from synchronous cultures prepared by centrifugal elutriation. We isolated unbudded G1 cells from cultures growing at 25°C and incubated them at 37°C in the presence or absence of nocodazole. We measured cellular DNA content (by FACscan), budding, the extension of bipolar spindles (by tubulin staining), and the number of green fluorescent dots per cell. In wild-type cells, separation of the Tet operators at *URA3* and segregation of spindle poles took place at approximately the same time, 45 min after the completion of DNA replication and bud formation (Figure 2A). Nocodazole blocked sister separation, cytokinesis, and rereplication (Figure 2B). In *scc1–73* cells, on the other hand, sister separation commenced much earlier (Figure 2A) and took place in the presence of nocodazole, albeit more slowly than in its absence, even though both cytokinesis and rereplication were completely blocked (Figure 2B).

To compare the timing of sister separation in wild-type and mutant cells, we used the fraction of budded cells and cells that possessed two dots to estimate the cumulative fraction of cells that had separated their sister chromatids at different time points (see Experimental Procedures). This showed that, whereas wild type separated sister chromatids about 45 min after budding, *scc1–73* mutants did so after about 30 min. Sister chromatid separation never occurs in wild cells with small buds but does so in *scc1* mutants (Figure 1B). Sister chromatids rapidly move into mother and bud in wild-type cells upon their separation but are frequently found in the same half of the cell in *scc1* mutants at the equivalent stage of the cell cycle (Figure 1B).

The FACscan profiles of *scc1* mutant cells (Figure 2A) showed that they undergo cytokinesis around 25 min later than wild-type cells. However, we frequently detected two dots in *scc1* mutant cells that had just undergone cytokinesis. Despite this missegregation of chromosomes, many *scc1* mutant cells rereplicate their genomes.

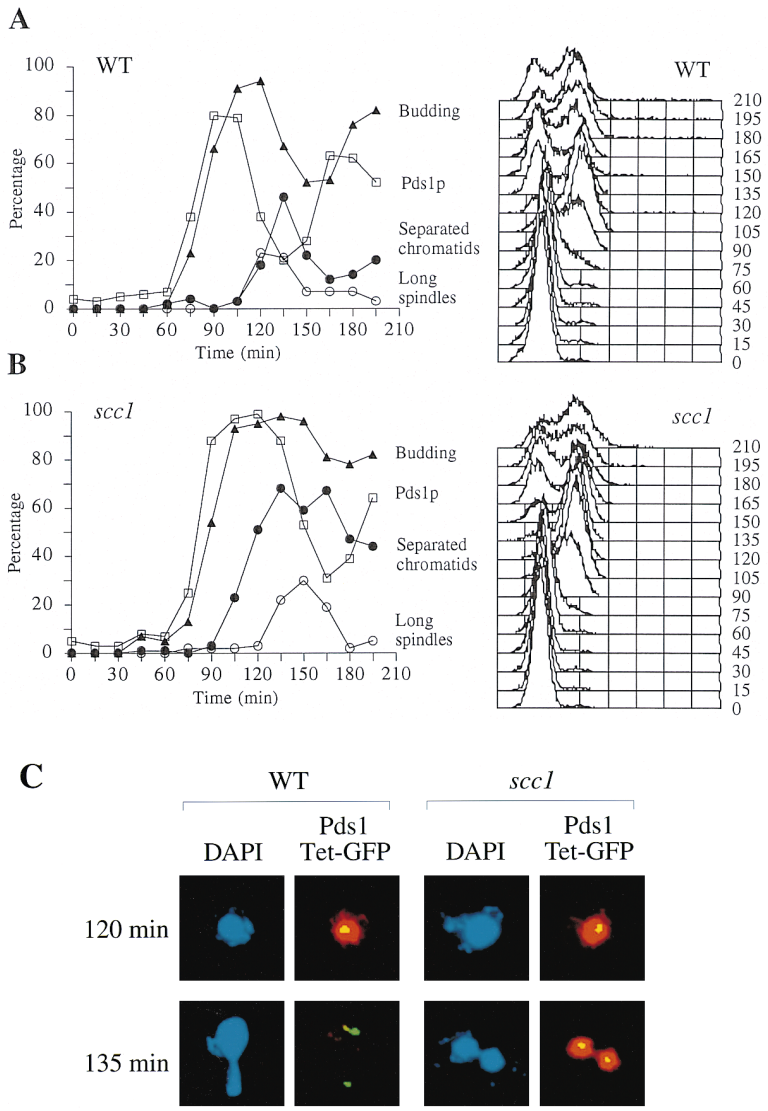

Figure 3. Sister Chromatids Separate prior to Pds1p Proteolysis in *scc1* Mutants

Small unbudded wild-type (K6803) and *scc1–73* (K6800) cells were isolated by centrifugal elutriation and incubated in YEPD at 37°C. The percentage of separated sister chromatids and the percentage of budded cells were determined from ethanol fixed cells. The level of Pds1p was determined using an anti-myc antibody, and the spindles were analyzed using an antitubulin antibody by indirect immunofluorescence. Pds1p appears in the nucleus at the time of budding and replication in both strains. In wild-type cells, Pds1p is degraded prior to sister chromatid separation (A), while in *scc1–73* Pds1p is degraded after sister chromatid separation (B). Pds1p destruction and spindle elongation seems to be delayed in *scc1–73*. (C) Chromosomal DNA is shown by staining with DAPI, Pds1p is detected using an Cy3 coupled secondary antibody, and the Tet-GFP fusion protein enabled the detection of separated sister chromatids using indirect immunofluorescence of formaldehyde-fixed cells. Green dots on a red (Cy3) background show up as yellow dots.

Sister Chromatids Separate prior to APC-Mediated Pds1p Proteolysis in *scc1* Mutant Cells

To address whether the premature separation of sister chromatids in *scc1–73* might be caused by premature destruction of Pds1p, we tagged the *PDS1* gene with 18 myc epitopes (inserted at the endogenous *Pds1* locus), which enabled us to measure its accumulation within nuclei by in situ immunofluorescence. We were thus able to repeat the elutriation experiments with strains that permitted the simultaneous measurement of sister chromatid separation and Pds1p levels.

In wild-type cells, Pds1p destruction occurred approximately 30 min after budding and slightly before the onset of anaphase (Figures 3A and 3C). In *scc1–73* mutants, on the other hand, the disappearance of Pds1p occurred about 60 min after budding; that is, it was not advanced by the mutation but actually delayed by 30 min (Figures 3B and 3C). As a consequence, it occurred 45 min after the separation of sister chromatids. These data show that *scc1–73* mutant cells separate sister chromatids long before they destroy Pds1p. Unlike wild-type cells whose sister separation depends on Pds1p destruction, *scc1* mutants clearly separate sister chromatids in the presence of high levels of Pds1p. The delayed destruction of Pds1p in *scc1* mutants might be due to a surveillance mechanism that is responsive to the tension of mitotic spindles, which might also cause the delay in cytokinesis.

Scc1p Is Necessary for Sister Chromatid Cohesion in *apc* Mutants

The separation of sister chromatids prior to Pds1p destruction in *scc1* mutants suggests that loss of Scc1 function allows sister separation to occur in the absence of APC activity. To address this directly, we compared the kinetics of sister chromatid separation in *scc1* and *cdc16* single mutants with that in *scc1 cdc16* double mutants. In *cdc16–123* mutants, fewer than 20% of the cells managed to separate sisters within 2 hr of their having completed DNA replication (Figure 4). This confirms that *CDC16* is normally essential for sister chromatid separation. In *scc1 cdc16–123* double mutants, on the other hand, sister separation took place with kinetics that were similar, if not identical, to that in *scc1* single

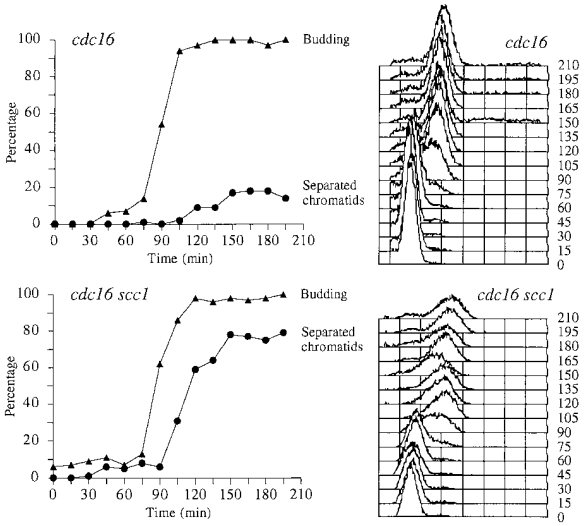

Figure 4. Premature Sister Separation in *scc1* Mutants Is Independent of APC

Small unbudded *cdc16–123* (K6798) and *scc1–73 cdc16–123* (K6755) cells were isolated by centrifugal elutriation and incubated in YEPD at 37°C. *cdc16–123* arrested in metaphase with unseparated sister chromatids. *scc1–73 cdc16–123* double mutants separated sister chromatids but still arrested as large budded cells with a 2C DNA content.

mutants (Figures 2A and 4). Thus, the premature separation of sister chromatids in *scc1* mutants occurs in the absence of Cdc16 function.

Scc1p Is an Unstable Protein Whose Abundance Fluctuates during the Cell Cycle

To detect the protein encoded by the *SCC1* gene, we tagged the endogenous gene with 18 myc epitopes at the C terminus. The myc-tagged protein (Scc1-myc18) was functional in yeast. It migrated in SDS polyacrylamide gels with an apparent molecular mass of 110 kDa, 20 kDa larger than the predicted size of 90 kDa. We measured Scc1-myc protein levels by Western blotting as cells progress through the cell cycle. The protein was absent in early G1 cells, accumulated during S, G2, and metaphase, and declined during anaphase (Figure 5A). Scc1-myc protein accumulated 30 min earlier than the mitotic cyclin Clb2 and declined 15 min earlier (Figure 5A). Indirect immunofluorescence showed that Scc1p is a nuclear protein which is absent in early G1 cells. It also showed that Scc1-myc's proteolysis during anaphase is not completed before full separation of the spindle poles. Unlike Pds1-myc, some Scc1-myc can still be detected in cells that have undergone both anaphase A and B (Figure 5C). These data suggest that Scc1p is degraded later than Pds1p but earlier than Clb2p. We found that *SCC1* mRNAs were absent in early G1 and accumulated to maximum levels in late G1/S phase (data not shown), which suggests that transcriptional control also contributes to the changes in Scc1p levels during the cell cycle.

To address whether Scc1p might be degraded as a consequence of its ubiquitination by the APC, we analyzed the stability of Scc1-myc protein expressed from the *GAL* promoter in wild-type and *cdc23–1* mutant cells arrested in G1. Cells growing in raffinose were arrested in G1 using mating pheromone, then treated with galactose for 30 min to induce synthesis of Scc1-myc, and finally transferred to glucose medium to repress any further synthesis. Western blotting showed that Scc1-myc protein made in pheromone-arrested cells is rapidly degraded in wild-type but less so in *cdc23-1* mutant cells (Figure 5D). *SCC1* mRNA was in contrast equally unstable (Figure 5D).

Scc1p Associates with Chromosomes during S Phase and Dissociates from Them at the Metaphase-to-Anaphase Transition

To establish whether Scc1p might be directly involved in holding sister chromatids together, we tested its association with chromatin in chromosome spreads (Klein et al., 1992). Diploid cells sampled every 15 min from a synchronous culture were lysed on microscope slides. Chromosomes but little else settle onto and stick to the slide, which enables the subsequent detection of proteins associated with them by indirect immunofluorescence. Soluble nuclear proteins like Cse1p are washed away, whereas known chromosomal proteins like Orc2p are tightly associated with chromosomes (Tanaka et al., 1997).

Colocalization of Scc1-myc and chromatin was detected in samples derived from S, G2, and metaphase cells (Figure 6A). As expected, Scc1-myc was not detected on chromatin in early G1 cells that lack the protein. However, Scc1-myc associates with chromatin as soon as it accumulates within nuclei in late G1/early S phase. In most cases, all the chromatin from each nucleus sticks together, but, in some cases, the chromatin is much more disperse. In such "super-spreads," we noticed that Scc1-myc is distributed in discrete spots (100 or more) throughout the genome (Figure 6A).

Colocalization between Scc1-myc and chromatin was lost abruptly at the onset of anaphase (Figures 6A and 6B). A comparison between nuclear and chromosomal staining showed that Scc1p dissociates from chromatin more rapidly during anaphase than it declines in abundance (Figure 6B). It is possible to detect chromosomes that are just in the process of sister chromatid separation because their DAPI staining forms a characteristic dumbbell (Figure 6A). The frequency of these "DAPI dumbbells" and that of early anaphase cells rose to a maximum at the same time, confirming their identity as chromosomes in early anaphase (data not shown). Remarkably, we never detected colocalization between Scc1-myc and chromatin in "dumbbell" chromosome spreads (Figure 6A). This suggests that Scc1p fully dissociates from chromosomes at the same time as sister chromatid separation occurs (or possibly even slightly before). This point coincides with the onset of Scc1p proteolysis but clearly precedes its completion. Scc1-myc can still be detected in all early anaphase nuclei of fixed cells (Figure 5C).

Association of Scc1p with Chromosomes Depends on Smc1p

Scc1p is associated with sister chromatids during metaphase and is essential for their cohesion. Thus, Scc1p's

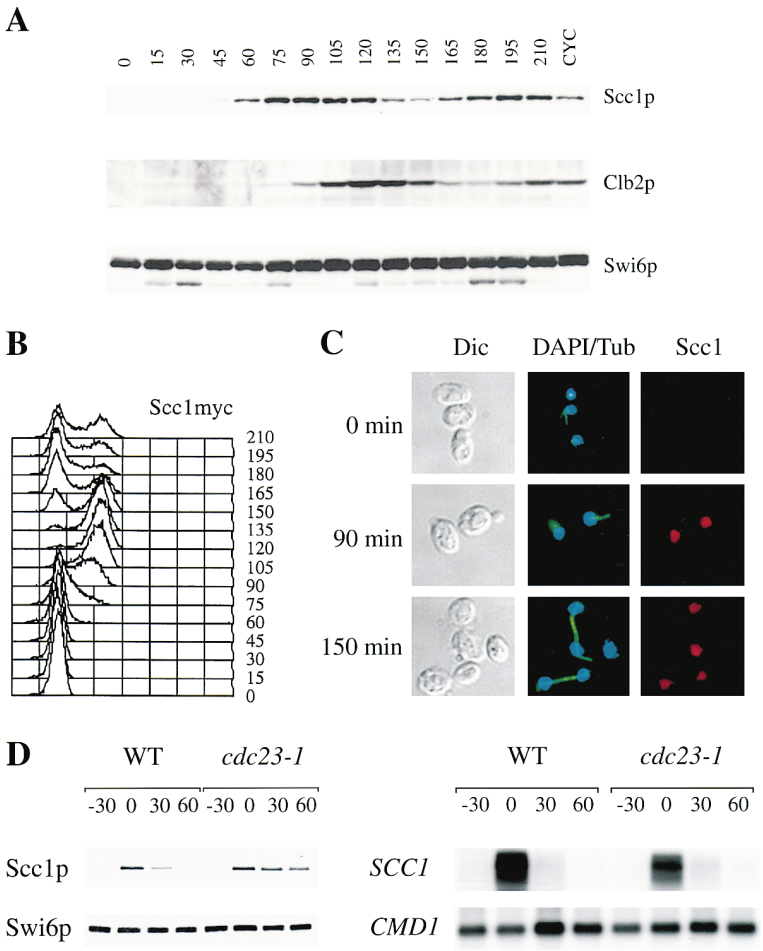

Figure 5. Scc1p Is an Unstable Nuclear Protein

Diploid wild-type cells carrying the *SCC1* gene fused to 18 copies of the myc epitope were synchronized by centrifugal elutriation, and the small unbudded cells were released into YEPRaf at 25°C. Samples were collected every 15 min and (A) Scc1p, Clb2p, and Swi6p levels were determined by Western blot analysis of protein extracts, (B) DNA replication was measured by FACScan, and (C) localization of Scc1p within fixed cells detected by indirect immunofluorescence. (D) The myc-tagged *SCC1* gene was placed under the *GAL1–10* promoter and integrated into the *URA3* locus of wild-type cells (K6843) and *cdc23–1* (K6844). Cells were grown at 25°C in YEPRaf, arrested for 150 min in early log phase by the addition of α factor in G1, and then shifted to 33°C to inactivate the APC. After incubation for 30 min (−30) at 33°C, expression of *SCC1* was induced by the addition of 2% galactose. The GAL1–10 promoter was repressed by collecting cells by filtration and incubation in YEPD and α factor 30 minutes later (0). Levels of Scc1-myc protein were measured by Western blotting and RNA levels measured by Northern blotting.

dissociation from chromosomes could be a causal factor in the separation of sister chromatids. How does Scc1p bind to chromosomes and how might this process be regulated? To investigate whether Scc1p's association with chromosomes depends on Scc2p or the two members of the SMC family, Smc1p and Smc3p, we used chromosome spreading. We analyzed Scc1-myc's association with chromatin as wild-type, *smc1*, *smc3,* and, *scc2* mutant cells progress through the cell cycle at 37°C following release from a G1 arrest induced by mating pheromone. In wild-type cells, Scc1-myc appeared within nuclei between 15 and 30 min after pheromone release; that is, around the time cells underwent DNA replication. The kinetics of its nuclear accumulation were similar in all three mutants (data not shown). Neither the timing nor the extent of Scc1-myc's association with chromatin was affected by *smc3* or *scc2* mutations. However, Scc1-myc's chromosomal association was greatly reduced in the *smc1* mutant at all time points following the release (Figure 6C). This suggests that Scc1p does not associate with chromosomes by binding to DNA alone. Its association depends critically on Smc1p or at least on an aspect of chromosome architecture dependent on this protein.

If Scc1p's association with chromosomes were important for sister chromatid cohesion and Scc1p fails to associate with chromatin in *smc1* mutants, then *smc1* mutants should also be defective in holding sister chromatids together. This is indeed the case. Using the Tet-GFP system, we found that a large fraction of chromosome V kinetochores separate in *smc1–259 cdc16–123* mutant cells (Figure 7A). Furthermore, this separation occurs earlier than it does in wild type. Similar data were obtained with *smc3–42* and *scc2–4* mutants (Figures 7B and 7C). Thus Smc1p, Smc3p, and Scc2p are required to prevent premature separation of sister chromatids.

Discussion

It is nearly 120 years since Walter Flemming concluded that "the impetus causing nuclear threads to split longitudinally acts simultaneously on all of them." It has of course been recognized for some time that the force that splits chromosomes at mitosis is exerted by microtubules attached to kinetochores on each sister chromatid. The splitting force exerted by these microtubules has been measured and found to arise long before splitting actually occurs. During alignment of sister chromatids on the metaphase plate, the splitting force mediated by microtubules is opposed by an equal and opposite cohesive force that holds sister chromatids together (Nicklas, 1988). It is suspected that the actual impetus for chromosome splitting does not arise from any major change in the forces exerted by microtubules but rather

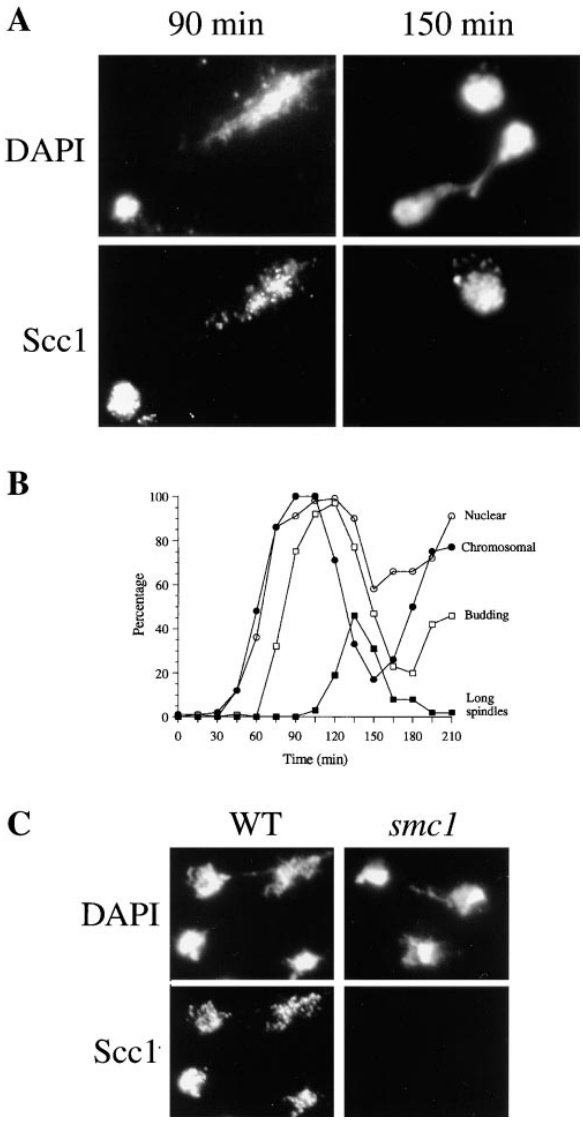

Figure 6. Cell Cycle–Regulated Association of Scc1p with Chromosomes

(A) Chromosome spreads were examined by indirect immunofluorescence for the localization of Scc1p.

(B) The percentage of nuclei and chromosomal masses containing Scc1p staining of budded cells and of cells containing long spindles were analyzed. Scc1p is localized on chromosomes at the same time as it appears in the nucleus, at replication or shortly before. With the onset of anaphase, it is not found on chromosomes of dividing nuclei (200 dumbbells were examined).

(C) Wild-type (K6565) and *smc1–259* (K 6577) cells carrying the *SCC1myc18* gene were arrested in G1 with α factor for 90 min. Cells were shifted to 37°C for 60 min and released into YEPD without α factor at 37°C. Samples were taken every 15 min, and chromosome spreads were analyzed for the presence of Scc1p. Scc1p bound to chromosomes of wild-type cells, but it was not localized on chromosomes in *smc1–259*.

from a sudden loss of cohesion between sisters. There exist in several organisms mutants that are defective in sister chromatid cohesion, but there is only one case where the protein involved has been shown to be localized to chromosomes. MeiS332 in Drosophila is a protein that binds to centromeric regions, is required for sister kinetochore cohesion during meiosis, and dissociates from chromosomes as sister centromeres separate at anaphase II (Kerrebrock et al., 1995). It is not, however, required for sister chromatid cohesion in mitotic cells.

Sister Chromatid Cohesion Proteins

By identifying mutations that allow yeast cells lacking APC function to undergo some semblance of anaphase, we have discovered four proteins necessary for sister chromatid cohesion. Two of these, Smc1p and Smc3p, are members of the SMC family of putative ATPases with coiled-coil domains (Hirano et al., 1995). The third, Scc1p, is an unstable protein that binds to chromosomes during late G1 or S phase (following its synthesis during late G1) and remains tightly associated with sister chromatids until metaphase. Proteins similar to Scc1 exist in a wide variety of eukaryotes including humans. Proteins of this family contain conserved domains at their N and C termini. Sequences related to the conserved C-terminal domain exist also in the Cdb4 protein from fission yeast, which is thought to bind bent DNA (Yamada et al., 1994). The fourth protein, Scc2p, has a homolog in S. pombe but remains otherwise uncharacterized.

In wild-type cells, sister chromatids remain associated for an appreciable period after formation of mitotic spindles. In *scc1*, *scc2*, *smc1*, and *smc3* mutants, sister chromatids separate prematurely, soon after the formation of bipolar spindles. Smc1p, Smc3p, and Scc1p are associated with chromosomes and are essential for the cohesive force that opposes microtubule-induced chromosome splitting. They might therefore be suitably called "cohesins." The premature separation of sister chromatids in *scc* and *smc* mutants is consistent with the currently accepted view that sister chromatids are under tension during metaphase and that this is an intrinsic aspect of chromosome alignment.

Loss of Cohesion at Anaphase

It is remarkable that Scc1p dissociates from chromatin at the onset of anaphase. This property alone could explain the loss of cohesion between sisters that is thought to be the trigger for their separation. Our data suggest that Scc1p is needed for sister cohesion near centromeres and within chromosome arms. In chromosome spreads where chromatin has been highly dispersed, we detected 100 or more distinct foci of Scc1p staining throughout the genome. These observations suggest that Scc1p might exert cohesion via numerous, but distinct, sites within each chromosome.

Decatenation of sister chromatids by Topoisomerase II after replication is clearly also needed for the "resolution" of sister chromatids during prophase and for their full separation during anaphase. However, there is no evidence that a lack of Topo II activity is responsible for holding sisters together during metaphase. It could of course be argued that Scc1p opposes sister separation by inhibiting Topo II. This is unlikely because Scc1p presumably does not oppose the Topo II activity needed to "resolve" sister chromatids from their postreplicative tangle during prophase.

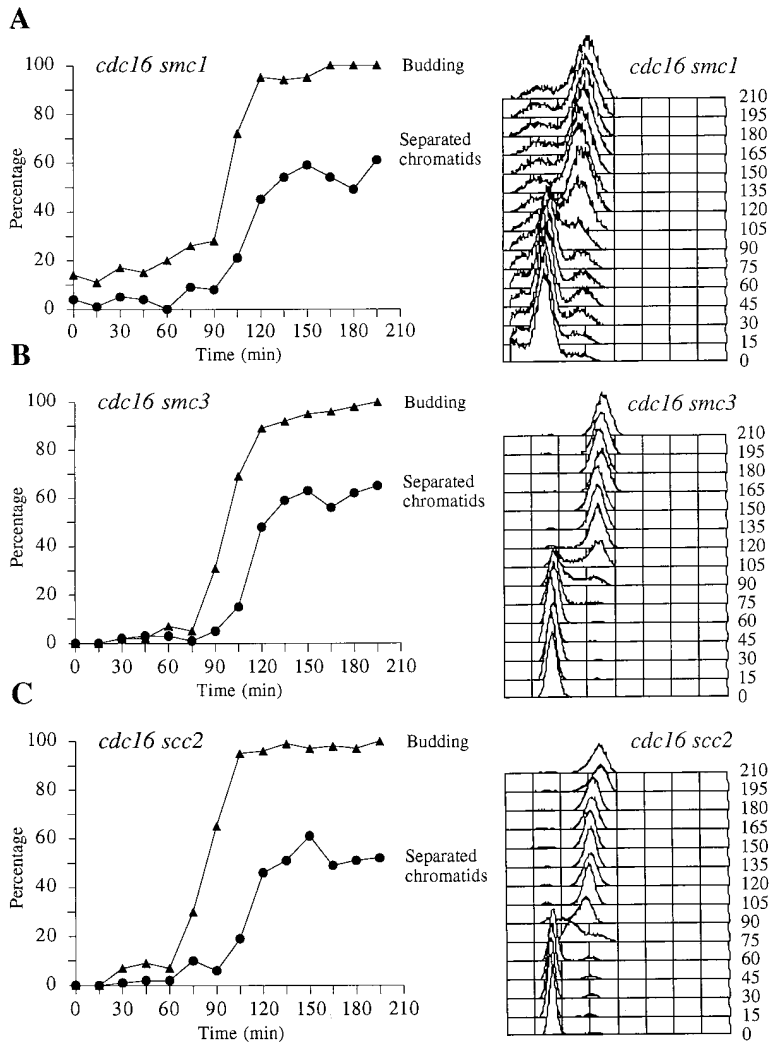

Figure 7. *SMC1*, *SMC3*, and *SCC2* Are Required for Sister Chromosome Cohesion

(A) *smc1–259 cdc16–123* (K6759), (B) *smc3–42 cdc16–123* (K7058), and (C) *scc2–4 cdc16–123* (K7059) cells containing the Tet-GFP fusion were collected by centrifugal elutriation and released into YEPD at 37°C. Cells were analyzed for the existence of one or two dots, corresponding to paired or separated sister chromatids, respectively, and for the percentage of budded cells. All double mutants arrested at the nonpermissive temperature as large budded cells with a 2C DNA content. A large fraction of cells of all three double mutants arrested with separated sister chromatids.

The Trigger for Scc1p's Dissociation from Chromatin

Given that dissociation of Scc1p from chromosomes might be instrumental in triggering sister chromatid separation, it is important to establish the proximate cause of this event. Dissociation of Scc1p from chromosomes depends on the APC, suggesting that it might be triggered by the destruction of proteins that inhibit Scc1p dissociation. Expression of a nondegradable version of Pds1p blocks Scc1p's dissociation from chromatin, whereas the expression of a nondegradable mitotic cyclin Clb2 has no effect (data not shown). This raises the possibility that Pds1p blocks anaphase by stabilizing the cohesion between sister chromatids mediated by Scc1p. Pds1p cannot, however, be the sole regulator of such cohesion because *pds1* mutants, unlike *scc1* mutants, do not separate sister chromatids prematurely at 37°C (R. Ciosk, personal communication). Furthermore, there is no evidence that Pds1p is a chromosomal protein. This suggests that Pds1p might interfere with the dissociation of Scc1p from chromosomes and with sister chromatid separation, either by blocking APC's access to other key substrates or by blocking access to chromosomes of yet unknown factors that promote dissociation of Scc1p.

One candidate for such an APC substrate is Scc1p itself. It too is degraded at the metaphase-to-anaphase transition and continues to be degraded in an APC-dependent manner during the subsequent G1 period. It is unclear, however, whether Scc1p's proteolysis is essential for sister separation. Though destruction of Scc1p commences at the metaphase-to-anaphase transition, it is clearly not complete until cells undergo cytokinesis. Crucially, we detect appreciable amounts of Scc1p protein within nuclei after sister chromatids have fully separated and after Scc1p has fully dissociated from chromosomes. Thus, Scc1p's proteolysis might be a consequence and not a cause of its dissociation from chromosomes.

Chromosome Architecture, Condensation, and Cohesion

Smc1p and Smc3p are members of a large protein family. The yeast genome encodes two other proteins of this type, called Smc2p and Smc4p (Koshland and Strunnikov, 1996), whose vertebrate homologs, called

XCAP-C and XCAP-E in Xenopus, are distributed along the central axis of mitotic chromosomes and are components of a 13S particle, called condensin, that is necessary for chromosome condensation (Hirano et al., 1997). Smc2p and Smc4p have also been implicated in chromosome condensation in yeast (Strunnikov et al., 1993; Saka et al., 1994).

Vertebrates also possess Smc proteins whose sequence resembles that of Smc1 and Smc3. The bovine equivalent of Smc3 is a component of a complex called RC-1, which is capable of DNA strand exchange (Jessberger et al., 1996). The functions of these members of the SMC family in vertebrates remains, however, uncertain. We tagged Smc1p and Smc3p with myc epitopes and found that both proteins are associated with chromatin in mitotic cells (data not shown). This raises the possibility that, in addition to their role in sister cohesion, Smc1 and Smc3 might be part of structures that form the longitudinal axis of yeast chromosomes. Scc1p fails to associate with chromatin in *smc1- 259* mutant cells. This suggests that Scc1's function depends on that of Smc1. It also suggests that Scc1p does not simply bind to DNA but does so only via Smc1p or via structures involving it (and possibly Smc3p). We currently do not know, however, whether Scc1p actually associates with Smc1p on chromosomes and, if so, whether it does so at only some or at all of Smc1p's chromosomal locations.

The connection between Scc1p and Smc1p may be a direct one. Guacci et al. (1994) have shown that overexpression of *SCC1* (which they call *MCD1*) can suppress the ts lethality of *smc1* mutants and that the two proteins can be coprecipitated (Guacci et al., [this issue of *Cell* 1997]).

It is axiomatic that the definition of a chromatid's longitudinal axis and its subsequent condensation must involve mechanical systems capable of distinguishing whether a given stretch of DNA belongs to the same chromatid or to its sister. We currently have no clue how this is achieved, but it would not be surprising if cohesion proteins were involved. It should likewise not be surprising to find that proteins like Smc1p and Smc3p, which may be involved in defining chromatid architecture, are also involved in cohesion. Our observations raise the possibility that these two aspects of mitotic chromosome architecture are intimately connected. Yeast chromosomes remain fully transcriptionally active throughout mitosis. Their structure during G2 and early M phase might therefore resemble that of lampbrush chromosomes in frogs, in which sister chromatids are paired at the base of transcriptionally active radial loops or bights (Callan, 1986). Our data raise the possibility that Smc1p, Smc3p, and Scc1p might exist at the bases of such loops. If so, these proteins might serve not only to hold sister chromatids together but also to generate the loops themselves and/or to bring adjacent loops on each chromatid together.

Cohesion during DNA Repair and Meiosis

Budding yeast possesses a second protein with unknown function (SC31900_7) that contains N- and C-terminal domains similar to those of Scc1p. The fission yeast S. pombe also possesses two such proteins: Rad21p, most similar to Scc1p, and Rec8p, which is more similar to SC31900_7. Mutants in *rad21* are hypersensitive to double strand breaks induced by gamma rays (Birkenbihl and Subramani, 1992). This phenotype could in principle be explained by defective sister chromatid cohesion. Like Scc1p, Rad21p is essential for mitotic divisions.

The sensitivity of *rad21* mutants to γ irradiation raises the possibility that Scc1p and Rad21p might be directly involved in the process of DNA repair in addition to sister chromatid cohesion. It is remarkable in this regard that the bovine homolog of Smc3p, which like Scc1p is needed for sister chromatid cohesion in yeast, has been found in a particle with DNA strand exchange activity (Jessberger et al., 1996). It is therefore possible that the mechanisms by which sister chromatids are held together are also employed during DNA repair.

Rec8p, which is only expressed during meiosis I, has not previously been recognized as a member of the Scc1/Rad21 family because of a potential DNA sequencing error that eliminated the N-terminal half of its amino acid sequence (V. Morozov, personal communication). *rec8* mutants are defective in the pairing and recombination of homologous chromosomes. Remarkably, *rec8* mutants are also defective in sister chromatid cohesion (Molnar et al., 1995). Thus, Rec8's primary function might be to maintain sister chromatid cohesion following premeiotic S phase, which might be essential for the subsequent pairing of homologs. If so, similar mechanisms might be utilized for sister chromatid cohesion during meiosis and mitosis.

The establishment of cohesion during DNA replication, its condensation along the longitudinal axis of chromosomes during prophase, and its destruction at the metaphase-to-anaphase transition presumably involves a sophisticated mechanical system, the workings of which remain shrouded in mystery. A difference in the timing of the loss of cohesion within chromosome arms and centromeres is a key aspect of meiosis. Our results suggest that similar mechanisms might promote chromatid cohesion during mitosis and meiosis in all eukaryotic cells.

Experimental Procedures

Yeast Strains and Media

Complete media was used as previously described (Rose et al., 1990). All strains were derived from W303 (K699, *MATa ade2–1 trp1–1 can1–100 leu2–3,12 his3–11,15 ura3 GAL psi+*). Strain YPH278 containing *CFIII URA3*, *SUP11* (Spencer et al., 1990) was backcrossed four times with wild type (K5043) and with *cdc16–123* (K5555).

To tag the *SCC1* gene, a C-terminal ClaI-XbaI fragment was cloned into pBluescript II Sk$^-$ (Stratagene). An SpeI site was introduced in front of the stop codon by PCR mutagenesis, where two SpeI cassettes containing 9 myc epitopes each were inserted. The fragment was cloned into an integrative vector, linearized with BsmI, and introduced into the diploid wild-type strain K842 at the endogenous *SCC1* locus. This strain was dissected, and the haploid strains K6565 (*MATa*) and K6566 (*MAT*α) were generated. The *SCC1myc18* gene was found to be fully functional.

To generate an inducible gene, *SCC1myc18* was amplified by PCR from the strain K6565 and cloned under the control of the *GAL1–10* promoter into an integrative vector. The construct was linearized with NsiI and integrated at the *URA3* locus.

Genetics

K5555 (*MATa cdc16–123 CFIII* (CEN3. L. YPH278) *URA3SUP11*), was mutagenized with Ethyl methanesulfonate to 40%–60% survival and approximately 8×10^5 colonies were plated as previously described (Spencer et al., 1990). Cells were grown at 23°C for 5–7 days. Clones showing the sectoring phenotype (2300) were rescreened. Selected mutants (377) were freshly grown on YEPD plates, replica plated, and shifted to 37°C for 3 hr. Eight mutants, which showed two DNA masses and a more extended spindle than *cdc16–123* at 37°C, were isolated. The mutants were backcrossed to W303, and the sectoring phenotype was found to be linked to temperature-sensitive lethality independent of the *cdc16–123* mutation. All eight mutations were recessive. Crosses between single mutants identified four complementation groups. For the described experiments, the alleles *scc1–73*, *scc2–4*, *smc1–259*, and *smc3–42* were used.

The *SMC1*, *SMC3*, *SCC1*, and *SCC2* genes were isolated by complementing the temperature-sensitive phenotype of the corresponding mutant. A CEN vector–based genomic library was transformed into *smc1–259* (K6013), *smc3–42* (K5824), *scc1–73* (K5832), and *scc2–4* (K5828) and transformants selected at 37°C. The *smc1* and *smc3* mutants were each rescued by one plasmid, the *scc1* mutant by three independent plasmids, and the *scc2* mutant by two independent plasmids carrying the corresponding full-length genes.

Deletion alleles were constructed by transforming heterozygous diploid strains carrying the temperature-sensitive alleles of either *smc3–42*, *scc1–73*, or *scc2–4* and the wild-type copy of either gene, with the deletion constructs. For disruption of *SMC3* an internal BamHI-HpaI and for disruption of *SCC1* and *SCC2* internal NsiI-XhoI fragments were replaced by *URA3*. The disruption constructs were released from the vectors and transformed into the diploid strains. Resulting temperature-sensitive diploids were analyzed by tetrad analysis. All three genes were found to be essential because in a tetrad two spores were inviable and the other two were temperature sensitive but never carried the *URA3* gene. Therefore, the deletion was found to be linked to the temperature-sensitive lethality. *smc1–259* was crossed to *smc1–2* (Strunnikov et al., 1993) and found to be allelic.

Chromosome Spreading

Mitotic chromosomes were spread as described for meiotic chromosomes by Klein et al. (1992) with the following modifications. Cells were grown in YEPD or YEPRaf to early log phase, 10 ml of the culture was harvested and washed in sheroplasting solution (1.2 M sorbitol, 0.1 M potassiumphosphate [pH 7.4], 0.5 M $MgCl_2$) and digested in 300 μl of the same solution containing 10 mM DTT and 150 μg/ml Zymolase 20 T (Seikagaku Kyogo Co.) at 37°C for 20 min. The digest was stopped and the cells spread as described by Klein et al. (1992).

Visualization of Tet Operators Using GFP

A 350 bp fragment containing seven *tetO2* operators (Gossen and Bujard, 1992) was amplified by PCR from plasmid pUHD10–3. This fragment was gradually head-to-tail multimerized, producing a tandem repeat of 112 *tetO* operators, which was cloned into the plasmid pRS306 (Sikorski and Hieter, 1989). p306*tetO112* was triple integrated into the *URA3* locus, 35 kbp away from the centromere on the left arm of chromosome V. The visualization of the *ura3*::3x*URA3tetO 112* locus was achieved by the binding of the tet repressor-GFP (tetR-GFP) fusion protein to the tet operators. TetR was amplified by PCR from the plasmid pWH520 (Berens et al., 1992). A sequence encoding the SV-40 large T-antigen nuclear localization signal (NLS) was introduced after the ATG and the GFP (amplified by PCR from pSF313) was introduced at the C terminus. For optimal expression, we used the *URA3* promoter. Efficient termination of the transcript was ensured by cloning the alcohol dehydrogenase I terminator derived from pAAH5 after the stop codon (Ammerer, 1983). The whole construct was integrated into the *LEU2* locus.

Freshly grown cells not exceeding an OD of 2 were used for analysis. For scoring dots, an aliquot of the ethanol-fixed cells was mixed with H_2O, sonicated, concentrated by short centrifugation, and immobilized on a slide covered with a thin layer of 2% agarose. For every sample we analyzed 100 cells.

Estimating Cumulative Sister Separation Using the Budding Index

The percentage of cells with one or two green dots never reaches 100% because cells rapidly undergo cytokinesis after sister chromatid separation. To estimate the timing of sister separation relative to DNA replication and budding, it is best to analyze the cumulative fraction of the starting population that has undergone sister separation at each time point (S). Before cells reinitiate the second cell cycle, S will be given by the formula $(U/(2 - U)) \times (TD + 1) + TD$, where U is the fraction of unbudded cells and TD the fraction of cells with two dots. Using the above formula, it is possible to use the GFP dot data to derive percent figures for cumulative sister separation. Plots of this parameter with time were roughly parallel to the onset of budding. The delay between these two curves gives the interval between budding and sister separation in WT and mutant cells.

Other Techniques

Cells were arrested with α factor and the microtuble depolymerization drug nocodazole as previously described (Irniger et al., 1995). Elutriation was performed as described by Schwob and Nasmyth (1993). Small G1 cells were grown in YEPRaf and released into YEPD at 37°C or YEPRaf at 25°C. RNA isolation, Northern blot analysis, and flow cytometric DNA quantitation were performed as previously described (Schwob and Nasmyth, 1993). Protein extracts, Western blot analysis, in situ immunofluorescence, photomicroscopy, and antibody dilutions were performed as described by Piatti et al. (1996). Fluorescent in situ hybridization (FISH) was performed as previously described (Guacci et al., 1994).

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EMBL Accession Numbers

The cloned sequences are available in the EMBL nucleotide sequence databank under the accession numbers: Y14278 (*SMC3*), Y14279 (*SCC2*) and Y14280 (*SCC1*). *SCC1* is identical to the *MCD1* gene identified by Guacci et al. (1997).