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A Central Role for Cohesins in Sister Chromatid Cohesion, Formation of Axial Elements, and Recombination during Yeast Meiosis

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Summary

A multisubunit complex, called cohesin, containing Smc1p, Smc3p, Scc1p, and Scc3p, is required for sister chromatid cohesion in mitotic cells. We show here that Smc3p and a meiotic version of Scc1p called Rec8p are required for cohesion between sister chromatids, for formation of axial elements, for reciprocal recombination, and for preventing hyperresection of double-strand breaks during meiosis. Both Rec8p and Smc3p colocalize with chromosome cores independently of synapsis during prophase I and largely disappear from chromosome arms after pachytene but persist in the neighborhood of centromeres until the onset of anaphase II. The eukaryotic cell's cohesion apparatus is required both for the repair of recombinogenic lesions and for chromosome segregation and therefore appears to lie at the heart of the meiotic process.

Introduction

During meiosis, chromosome number is halved because two successive rounds of chromosome segregation occur without an intervening round of chromosome duplication. This enables the production of haploid gametes from diploid cells. Chromosome segregation during the second meiotic "equational" division resembles that during mitosis in that sister centromeres are segregated to opposite poles of the cell. During the first "reductional" division, sister centromeres remain attached to each other as they are separated from their homologs (reviewed in Miyazaki and Orr-Weaver, 1994).

During mitosis, sister chromatids segregate away from each other because their kinetochores attach to microtubules that extend to opposite poles (Rieder and Salmon, 1998). During the early phases of mitosis, from prophase until metaphase, the tendency of microtubules to move sisters apart is counteracted by cohesion holding sisters together. Cohesion not only generates the tension by which cells align sister chromatids on the metaphase plate but possibly also ensures that sister kinetochores attach to spindles emanating from opposite poles. The sudden loss of cohesion both at centromeres and along chromosome arms, rather than an increase in the exertion of microtubules, is thought to trigger sister separation during anaphase (Moore and Orr-Weaver, 1998; Uhlmann et al., 1999).

Cohesion between mitotic sister chromatids depends on a multisubunit complex called cohesin, which contains at least four proteins: Smc1p, Smc3p, Scc1p, and Scc3p (Losada et al., 1998; Toth et al., 1999). Smc1 and Smc3 are thought to form a heterodimer in which the proteins are joined by two long stretches of antiparallel coiled coil that are separated by a hinge region forming a V-shaped molecule (Melby et al., 1998; Hirano, 1999; Nasmyth, 1999). Cohesion between sister chromatids is established during S phase, possibly at replication forks where sisters are in close proximity (Uhlmann and Nasmyth, 1998). In yeast, cohesin remains associated with chromosomes from S phase until metaphase. Two of its subunits, Scc1p and Scc3p, dissociate from chromosomes as sister chromatids separate at the onset of anaphase (Michaelis et al., 1997). In animal cells, however, most cohesin dissociates from chromosomes during prophase (Losada et al., 1998) even though cohesion persists until anaphase. What holds sister centromeres together during metaphase in animal cells and whether it involves residual cohesin is not known.

Sister chromatid cohesion and its dissolution is expected to have key roles during both meiotic divisions. Premeiotic DNA replication produces sister chromatids that are held tightly together. Homolog chromosomes then pair along their entire length and during pachytene form double, synapsed chromosomes that are associated with a proteinaceous scaffold called the synaptonemal complex (SC) (Moses, 1958; Kleckner, 1996; Roeder, 1997). This structure consists of two axial elements running along the longitudinal axes of sister chromatid pairs and a central element composed of proteins like Zip1 or Scp1 (Sym et al., 1993; Schmekel et al., 1996), which connect axial elements. DNA double-strand breaks (DSBs) induced by the Spo11 endonuclease (Bergerat et al., 1997; Keeney et al., 1997) lead to recombination between chromatids from homologous chromosomes and the formation of chiasmata, which become visible in many organisms after the synaptonemal complex has been destroyed. Chiasmata connect homologous chromosomes, and it has been proposed that sister chromatid cohesion along chromosome arms holds the homologs together (Maguire, 1974; Carpenter, 1994).

During meiosis I, sister kinetochores attach to spindles emanating from the same pole, while homolog kinetochore pairs connect to the opposite pole. As a consequence, homologs and not sister chromatids come under tension from the meiosis I spindle apparatus. Chromosome segregation at the first meiotic division therefore requires destruction of sister chromatid cohesion along chromosome arms. Meanwhile, cohesion between sister centromeres persists throughout the first meiotic division and permits alignment of sister chromatids on the meiosis II mitotic spindle. Its subsequent destruction is thought to trigger sister separation at anaphase II.

Genetic and biochemical analyses have identified

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both axial element constituents and proteins needed for sister chromatid cohesion. In yeast, Red1p is necessary for axial element formation and for efficient sister chromatid cohesion (Smith and Roeder, 1997). Red1p and its protein kinase Mek1p (Bailis and Roeder, 1998) are distributed in a patchy manner along pachytene chromosomes. However, neither of these proteins nor Hop1p (Hollingsworth et al., 1990), which has a similar distribution, persists at centromeres after the first meiotic division. In mammals, Scp3p (Cor1), which is found along the entire length of axial elements (Dobson et al., 1994; Lammers et al., 1995), does persist in the neighborhood of centromeres until anaphase II. *Drosophila melanogaster* MeiS332p, which has been characterized both genetically and cytologically, associates with centromeric regions only during metaphase I and persists there until the onset of anaphase II (Moore et al., 1998). It prevents precocious loss of cohesion between sister centromeres (Kerrebrock et al., 1995).

We began this study by asking whether two cohesin subunits known to be required for sister chromatid cohesion during mitosis were also essential during meiosis. We investigated the roles of Scc1p (also called Mcd1p or Rhc21p) (Guacci et al., 1997; Michaelis et al., 1997; Heo et al., 1998) and Smc3p (Michaelis et al., 1997). We found that Scc1p is expressed at very low levels in meiotic cells and that the *scc1-73* allele, which is conditionally lethal in mitosis, has only a modest influence on spore formation and viability. Smc3p, on the other hand, is expressed at high levels and, like mammalian Scp3 (Schalk et al., 1998), is found continuously along chromosome cores. Smc3p is furthermore essential both for the formation of axial elements and for meiotic sister chromatid cohesion, showing that the processes of chromosome axis formation and sister chromatid cohesion are intimately related in meiosis.

The budding yeast genome contains a second Scc1-like protein that, because of its weak homology to the Rec8 protein in *Schizosaccharomyces pombe* (DeVeaux and Smith, 1994; Molnar et al., 1995; Michaelis et al., 1997), we call Rec8p. Rec8p is expressed exclusively during meiosis. Like Smc3p, Rec8p colocalizes with chromosome cores containing either axial elements or synaptonemal complexes and is necessary for their formation. Neither Rec8p nor Smc3p is required for the initiation of normal levels of Spo11p-induced DSBs, but both are necessary for recombination between homologs. Interestingly, both Rec8p and Smc3p largely disappear from chromosome arms during the first meiotic division but persist in the vicinity of centromeres until the onset of anaphase II. Our results show that a "meiotic" cohesin that holds sister chromatids together is also required for repair of meiotic DSBs and strand exchange between homologs.

Results

Smc3p Is Needed for Sister Chromatid Cohesion during Meiosis

To address whether Scc1p or Smc3p cohesin subunits are required for sister chromatid cohesion during meiosis, we backcrossed the temperature-sensitive alleles *scc1-73* and *smc3-42* six times with a strain (SK1) that sporulates with high efficiency and does so even at 35°C. Diploid *scc1-73* cells produced four spored asci with an efficiency comparable to wild type (at both 25°C and at 35°C), but only 50% of their spores were viable. Diploid *smc3-42* cells, in contrast, failed to sporulate at either temperature. Even at 30°C, *smc3-42* cells failed to undergo any meiotic divisions and arrested with a single nucleus, which often degenerated (not shown).

To observe chromosome cohesion and segregation, we integrated multiple tandem operators for the bacterial Tet repressor in the vicinity of the chromosome V centromere. These Tet operators were visualized by expressing a Tet repressor protein fused to green fluorescent protein (GFP) (Michaelis et al., 1997). Upon transfer of wild-type cells to sporulation medium, the centromere V (cenV)-GFP dots from each homolog were paired in about 85% of cells (due presumably to centromere clustering), but they temporarily separated before reassociating prior to segregation at the first meiotic division (Figure 1). In mutants that cannot undergo recombination and fail to synapse homologs, the fraction of uninucleate cells with paired cenV-GFP dots steadily declined (P. M. et al., unpublished), indicating that recombination-mediated pairing of homologs is responsible for the later associations. In wild type, binucleate cells usually contained two very closely juxtaposed cenV-GFP dots per nucleus (Table 1 and Figure 1), which then split into two at the onset of anaphase II. Tetranucleate cells always contained one GFP dot per nucleus (Table 1B and Figure 1).

The presence of three or four GFP dots in a uninucleate cell indicates separation of sister chromatids before the first meiotic division. Such cells were never seen in wild type or in *spo11* mutants (not shown) and only rarely in *scc1-73* mutants. In contrast, the fraction of uninucleate cells with three or four GFP dots rose to 50% in *smc3-42* mutants (Figure 1). This suggests that Smc3p but possibly not Scc1p is essential for sister chromatid cohesion prior to the first meiotic division. Analysis of the pattern of GFP dots in binucleate cells confirmed this conclusion (Table 1A). Wild-type (WT) and *scc1-73* binucleate cells usually had a pair of closely juxtaposed dots in each nucleus and produced four spored asci with a single GFP dot in each spore (Table 1B). In contrast, *spo11* mutants showed a pattern consistent with random segregation of unseparated homologs, that is, binucleates with two "twin" dots distributed at random in the two nuclei. Those few *smc3-42* mutant cells that managed to complete a first nuclear division (when sporulated at 25°C) predominantly had separated at least one sister pair, that is, at least one of the two nuclei contained two or more distinct signals (Table 1A).

The *SCC1*-like *REC8* Gene Is Required for Sister Chromatid Cohesion during Meiosis

The largely normal cenV-GFP segregation in *scc1-73* mutants might be due to another protein with similar properties to Scc1p that functions only during meiosis. The budding yeast genome encodes a 77 kDa protein (YRP007C) whose amino acid sequence shares 16% identity and 34% similarity to Scc1p (Michaelis et al., 1997). This Scc1 homolog also has limited homology to Rad21p of *S. pombe* (Birkenbihl and Subramani, 1995)

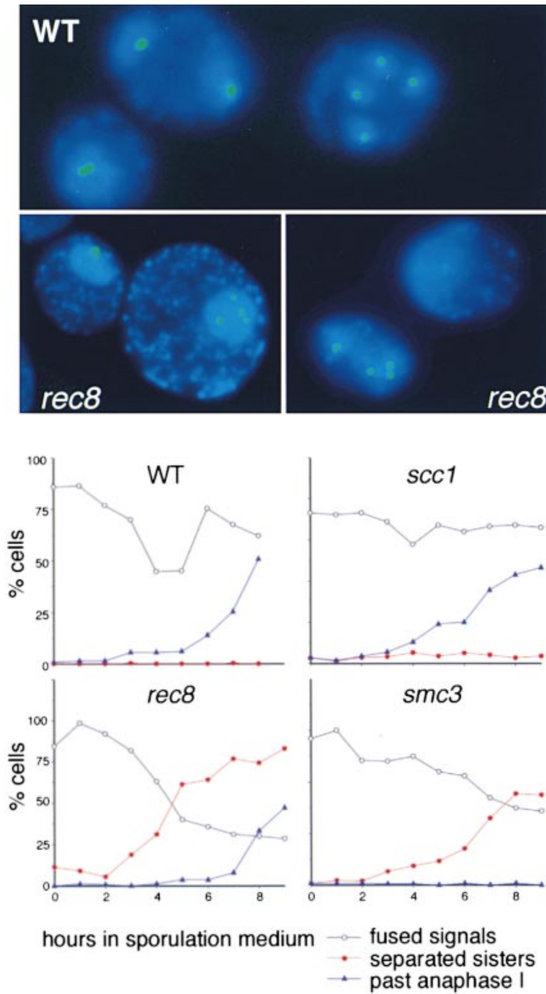

Figure 1. Meiotic Sister Chromatid Cohesion and Homolog Pairing Defects in *rec8*Δ and *smc3-42* Mutants

The top panel shows green cenV-GFP signals in mono-, bi-, and tetranucleate wild-type cells (FKY1012) (DAPI-stained nuclei are blue). Below, four cenV-GFP signals in a mononucleate cell and three signals in one nucleus of a binucleate cell indicate precocious sister separation in the *rec8*Δ mutant (FKY1022). Blue triangles show the percentage of cells with more than one nucleus as determined by DAPI staining, that is, cells that have completed early anaphase I. Open circles show the percentage of uninucleate cells with a single GFP signal corresponding to four homozygously tagged chromatids of chromosome V. Chromatids were tagged close to the centromere. Closed red circles show the percentage of uninucleate cells with at least three GFP signals separated, indicating loss of sister chromatid cohesion. Both *rec8*Δ and—even more so—*smc3-42* cells were delayed in their progression through anaphase I. All cells started out with a high level of fused GFP signals (t = 0) due to centromere clustering (Jin et al., 1998), some vegetative interactions between homologs (Weiner and Kleckner, 1994), and the Rabl orientation (J. Loidl, personal communication). Meiotic pairing replaced vegetative associations in WT (FKY1012 *MATa/α*, cenV-GFP/cenV-GFP) and *scc1-73* (FKY1043 *MATa/α*, cenV-GFP/cenV-GFP, *scc1-73/scc1-73*) cells but not in *smc3-42* (FKY792 *MATa/α*, cenV-GFP/cenV-GFP, *smc3-42/smc3-42*) or *rec8*Δ (FKY1022 *MATa/α*, cenV-GFP/cenV-GFP, *rec8*Δ*::KanMX4/rec8*Δ*::KanMX4*) mutants.

Table 1. *smc3-42* and *rec8* Mutations Cause Chaotic Chromosome Segregation

n = 200	Wild Type (1012)	*scc1* (1043)	*rec8* (1022)	*smc3* (792)	*spo11* (800)
(A) Meiosis I Cells					
	87	72	32	11	50.25
	3	7	7	32	0
	8	17	27	29.5	0
	0	0	21	21.5	0
	0	3	11	6	49.75
Others	2	1	2	0	0
(B) Meiosis II Cells					
	98.75	98.5	6	nd	50.5
	0.25	0	22	nd	0.25
	0.5	1	28	nd	0
	0	0	13	nd	3.75
	0	0.5	10	nd	45.5
	0	0	13	nd	0
	0	0	1	nd	0
	0	0	3	nd	0
	0	0	3	nd	0
Others	0.5	0	1	nd	0

Segregation of chromosome V in Meiosis I (MI) cells (A) and Meiosis II (MII) cells (B) was followed by virtue of its GFP tag close to the centromere. The first column illustrates the main classes of GFP segregation observed. WT and *scc1* mutants had close to 100% normal segregation, that is, two signals per nucleus after the first and one per nucleus after the second division. A fraction of MI cells show separated GFP dots, probably indicating onset of anaphase II. In *rec8* and *smc3-42* mutants, other prominent classes were discovered, especially 3:1 segregation in MI cells and 2:1:1:0, 3:1:0:0, 2:2:0:0, and 4:0:0:0 in *rec8* MII cells (*smc3-42* cells very rarely underwent a second division). These classes can best be explained by a high rate of loss of sister chromatid cohesion, followed by random segregation. The absence of recombination in *spo11* mutant cells causes a very different spectrum of missegregation (50% 4:0 in MI and 50% 2:2:0:0 in MII). The strain identity is indicated below the mutant allele. FKY800 *MATa/α*; cenV-GFP/cenV-GFP, *spo11::URA3/spo11::URA3*. nd, not determined.

and slightly more homology to a related *S. pombe* protein called Rec8p (DeVeaux and Smith, 1994) (Figure 3B). Because of its similar sequence and functions (see below), we called the second *Saccharomyces cerevisiae* *SCC1*-like gene *REC8*. The similarities between Scc1- and Rec8-like proteins are largely confined to their N- and C-terminal domains. *rec8* mutants in *S. pombe* are

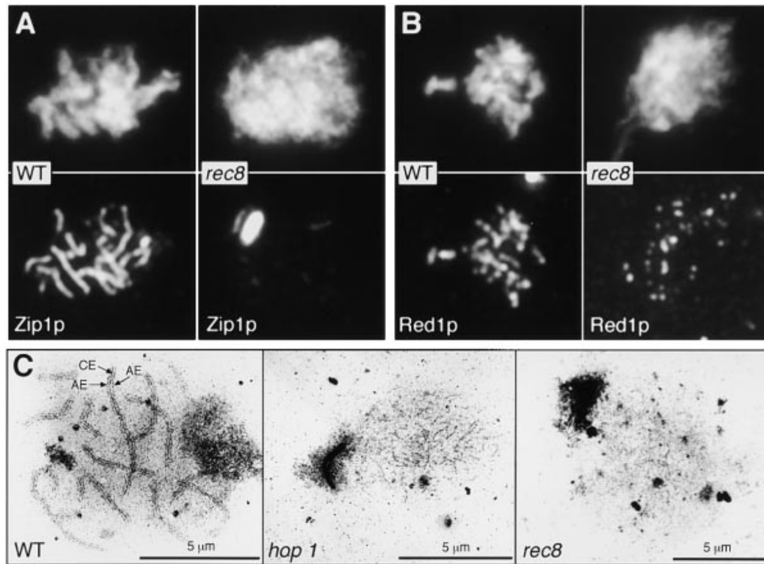

Figure 2. Synapsis and Formation of Axial Elements Are Defective in *rec8*Δ Mutants

(A) Synapsed regions, assayed using synapsis-specific anti-Zip1 antibody, were detected in WT (FKY1) but not in *rec8*Δ(FKY1067 *MATa/α, rec8Δ::KanMX4/rec8Δ::KanMX4*) or *smc3-42* (FKY454 *MATa/α, smc3-42/smc3-42*) mutants (not shown). In *rec8*Δ mutants, chromatin failed to condense properly, and Zip1 aggregated in a polycomplex that is typically observed in asynaptic mutants.

(B) Red1p-labeled nuclei from WT and a *rec8*Δ mutant. Red1p was never found in linear structures in the *rec8*Δ mutant. This suggests that axial element formation is defective in *rec8*Δ mutants, because Red1p normally resides also on unsynapsed axial element fragments.

(C) Electron micrographs from WT (FKY1), *hop1* (FKY21 *MATa/α, hop1::LEU2/hop1::LEU2*), and *rec8* (FKY1067) mutants. In pachytene cells from WT, the tripartite structure of the synaptonemal complex (axial elements, AEs; central element, CE) can be visualized in the electron microscope after silver staining. In a *hop1* null mutant, which does not undergo synapsis (F. K. and B. Byers, unpublished), unsynapsed axial element fragments can still be detected. The *rec8* nuclei possess nucleoli and duplicated spindle pole bodies but no axial elements.

defective in recombination (DeVeaux and Smith, 1994), in linear element formation, and in sister chromatid cohesion (Molnar et al., 1995).

To investigate the function of *S. cerevisiae REC8*, we deleted one copy from an SK1 diploid. Upon tetrad dissection of asci produced from this strain, all four spores produced similar sized colonies, which confirms that *REC8* (which has also been called *SPO69*) (Chu et al., 1998) is not required for mitosis. Diploid cells homozygous for the *rec8* deletion underwent two meiotic divisions but produced very few and abnormal spores at 30°C (not shown). Chromosome segregation was highly abnormal, largely because sister chromatids separated before the first meiotic division. Up to 70% of the cells contained three or four cenV-GFP dots prior to the first division (Figure 1). Cohesion between the distal tips of sister chromatid arms was investigated using a strain in which Tet operators were integrated near the right-hand telomere of chromosome V (FKY1024 *MATa/α*, TelV-GFP/TelV-GFP, FKY1039 *MATa/α*, TelV-GFP/TelV-GFP, *rec8Δ::KanMX4/rec8Δ::KanMX4*). By 9 hr, the GFP signals had split into three or four in 50% of uninucleate *rec8*Δ cells (not shown). *REC8* is therefore required for sister chromatid cohesion both at centromeres and at the arms near telomeres. Chromosome V segregation in *rec8*Δ binucleates resembled that seen in *smc3-42* mutants. Furthermore, we observed a prominent class of tetranucleate cells in which two nuclei contained one dot, one nucleus contained two dots, and one nucleus lacked any dot (Table 1), which can be attributed to precocious sister separation.

When we analyzed a *rec8*Δ strain in which only one chromosome V was marked by Tet operators, 31% of the binucleates had a pair of closely juxtaposed dots in only one nucleus, 35% had a single cenV-GFP dot in each nucleus, and 34% had two separate cenV-GFP dots in one of the two nuclei. This is the pattern one would expect if sister chromatids had separated precociously in 70% of cells but then segregated at random at the first division. A very similar pattern was seen in *spo11 rec8*Δ double mutants, suggesting that recombination has little effect on the precocious sister chromatid separation in *rec8*Δ mutants. In contrast, in wild-type cells sister centromeres almost always remained paired and segregated to the same pole.

REC8 and *SMC3* Are Required for the Formation of Synaptonemal Complexes and Axial Elements

To investigate chromosome structure in *rec8* and *smc3* mutants, we prepared chromosome spreads at two hourly intervals after transfer of wild-type, *rec8*Δ, and *smc3-42* mutant cells to sporulation medium (at 30°C). We used fluorescence microscopy and immunofluorescence to analyze chromatin condensation and the distribution of proteins diagnostic for synapsis (Zip1p; Sym et al., 1993) and chromosomal axis formation (Red1p; Smith and Roeder, 1997). Nuclei containing synaptonemal complexes with a centrally located Zip1 protein along the entire length of chromosomes and a more patchy distribution of Red1p along the axial elements peak in abundance around 6 hr in wild type (Figure 2). Neither Red1p nor Zip1p was found in such structures in *rec8*Δ (Figures 2A and 2B) or *smc3-42* mutants (not shown) at any time point. In both mutants, most Zip1 protein aggregated as a large complex (as found in other asynaptic mutants), whereas residual Red1p was found in small dots on chromatin that had failed to condense into chromosomal structures (Figure 2B). These data suggest that *rec8*Δ and *smc3-42* mutants are defective in the formation of both synaptonemal complexes and axial elements. This conclusion was confirmed by electron microscopy. Tripartite synaptonemal complexes were seen in wild type, whereas axial element fragments were seen in *hop1* mutants, which are defective in homolog pairing (Hollingsworth et al., 1990) (Figure 2C). Neither synaptonemal complex nor axial element fragments were found in *rec8*Δ (Figure 2C) or *smc3-42* mutants (not shown).

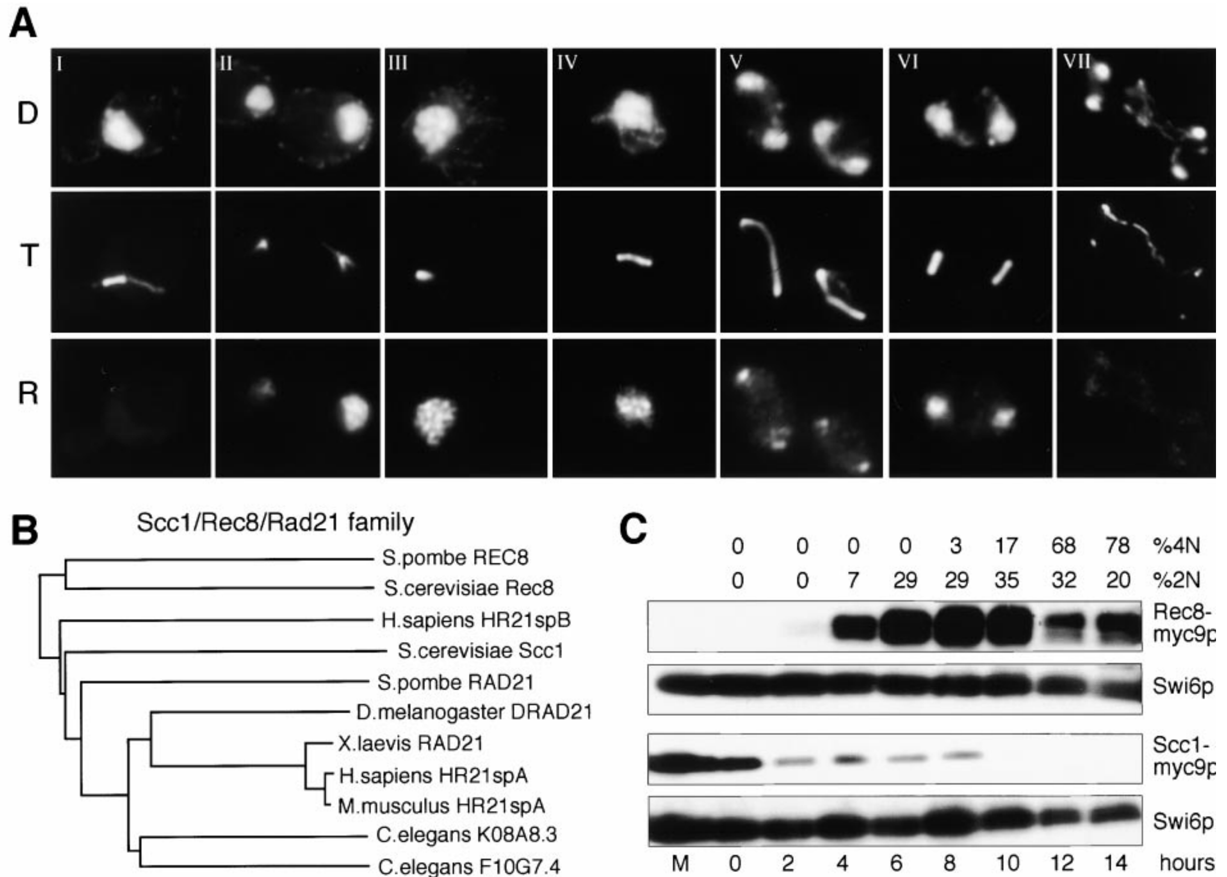

Figure 3. Rec8p Is Expressed Specifically in Meiosis, Decreases after Pachytene, and Disappears after Anaphase II

(A) The distribution of Rec8-HA3p (FKY1091 *MATa/α*, *REC8::HA3::URA3/REC8::HA3::URA3*) as detected by in situ immunofluorescence. We could distinguish different meiotic stages according to the morphology of DNA (D), tubulin (T), and Rec8p (R) consistent with those based on tubulin and DAPI alone (Hayashi et al., 1998). From left to right, (I), mitotic; (II), early prophase and zygotene; (III), pachytene; (IV), metaphase I; (V), anaphase I (two cells); (VI), metaphase II; (VII), telophase II. The intensity of Rec8 signals varied strongly between pachytene (very high) and anaphase I and metaphase II (very low), which is not apparent from these pictures due to optimized exposure times. Rec8p appears shortly before premeiotic DNA replication and accumulates to maximal levels during pachytene, when it becomes organized in thread-like structures. Rec8p's abundance declines by metaphase I, where it is concentrated in only part of the nucleus. It declines even further around the onset of anaphase I, at which point the remaining protein is clustered around the spindle poles. The amount of residual Rec8p, as well as the degree of focus around the spindle pole bodies, was variable from cell to cell between anaphase I and metaphase II, but it was invariably much less abundant than in pachytene. This variability was not found in immunostained spreads (Figures 5, 6, and 7), suggesting that it might derive from residual Rec8p (possibly cleaved) that is no longer tightly associated with DNA. Rec8p disappeared from cells undergoing anaphase II.

(B) A family tree linking members of the Scc1/Rec8/Rad21 family, which shows that Rec8 homologs belong to a separate branch, was constructed by the Phylo_Win Program using the Neighbor Joining method (Galtier et al., 1996).

(C) The abundance of Rec8-Myc9p (FKY1090 *MATa/α*, *REC8::MYC9::URA3/REC8::MYC9::URA3*) and Scc1-Myc18p (FKY443 *MATa/α*, *SCC1::MYC18::TRP1/SCC1::MYC18::TRP1*) during mitosis (M) and meiosis (0–14 hr) as detected by Western blotting. The numbers alongside %2N and %4N at the top indicate the percentage of bi- and tetranucleate cells, respectively. Swi6p was selected as a loading control because of its constant levels throughout meiosis. FACs analysis (not shown) suggests that Rec8p starts to be expressed shortly before premeiotic DNA replication.

Distribution and Abundance of Rec8p, Smc3p, and Scc1p during Meiosis

To detect Scc1p, Smc3p, and Rec8p, we replaced their endogenous genes with versions that encode multiple Myc or HA epitopes at their C termini. We used Western blotting to compare the levels of Scc1-Myc18p and Rec8-Myc9p during vegetative growth and sporulation. Scc1-Myc18p was expressed at high levels in vegetative cells, but Rec8-Myc9p was undetectable. This situation was reversed after cells were transferred to sporulation medium. The level of Scc1-Myc18p declined rapidly, while Rec8-Myc9p accumulated to high levels around the time of premeiotic DNA replication (Figure 3C and not shown).

We next analyzed the abundance and distribution of Myc-tagged proteins using in situ immunofluorescence (Figure 3A). Based on double labeling of tubulin and Rec8p and chromatin morphology, we identified several classes of cells whose frequency peaked in a defined order consistent with their being successive meiotic stages. Scc1-Myc18p was barely detectable in meiotic cells by this means (not shown), whereas Rec8-Myc9p

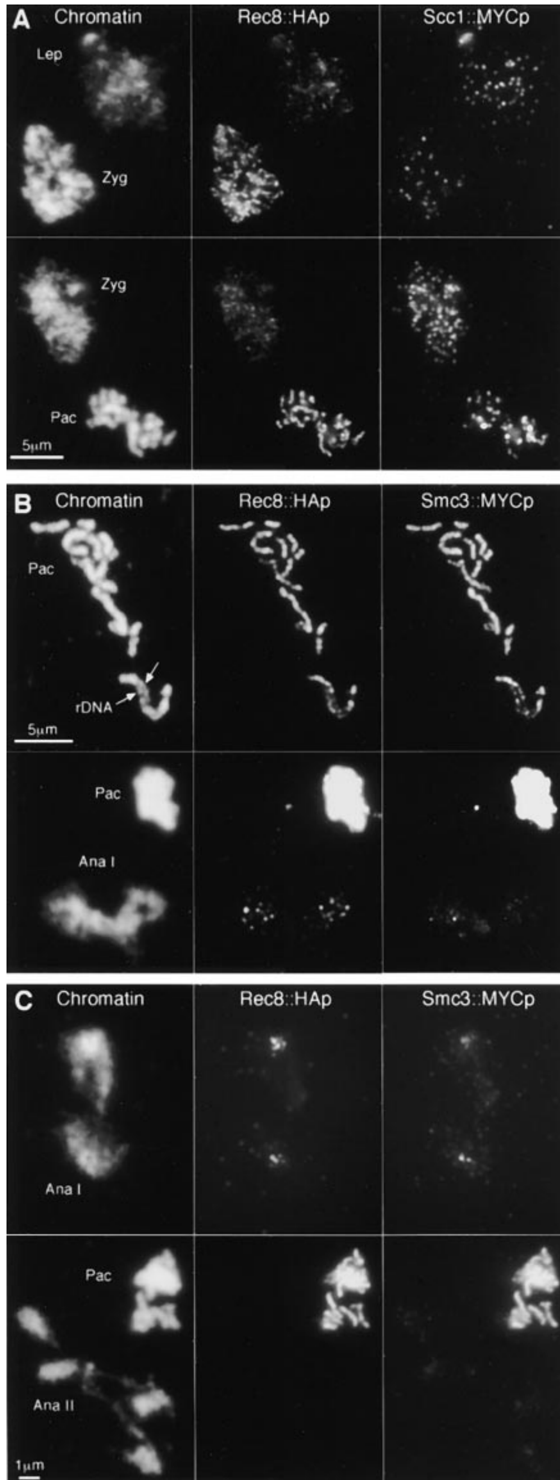

Figure 4. Rec8p and Smc3p but Not Scc1p Decorate the Axes of Pachytene Bivalents and Localize in Reduced Amounts Close to the Spindle Poles in Anaphase I Cells but Are Absent after Anaphase II

From left to right, chromatin (DAPI), Rec8-HA3p, and Scc1-Myc18p or Smc3-Myc6p. Different channels of the same image were acquired after double immunolabeling of meiotic spreads.

(A) Rec8p and Scc1p from strain FKY1119 (*MATa/α, REC8::HA3::URA3/REC8::HA3::URA3, SCC1::MYC18::TRP1/SCC1::MYC18::TRP1*) are associated with chromatin throughout meiotic prophase stages, such as leptotene, zygotene, and pachytene. For Rec8p, initially isolated foci coalesce with time to form continuous SC, while most but not all Scc1p foci disappear. A small number (30 to 50) of Scc1 foci remain on chromosomes.

(B) Rec8p and Smc3p from strain FKY1111 (*MATa/α, SMC3::MYC6::LEU2/SMC3::MYC6::LEU2, REC8::HA3::URA3/REC8::HA3::URA3*) are distributed continuously along the axes of pachytene bivalents, including the unsynapsed rDNA region. Both proteins produce narrower signals than chromatin, consistent with them being at the core of the bivalents. The lower three panels illustrate loss of both Rec8p and Smc3p from chromosomes between pachytene and anaphase I. A pachytene nucleus lies next to an anaphase I cell. To see the residual cohesins at the spindle pole cluster, the pachytene nuclei were strongly overexposed.

(C) Upper panels show Rec8p and Smc3p clustering around spindle poles after the onset of anaphase I. In the lower panels, a pachytene nucleus is shown alongside an anaphase II cell. Neither Rec8p nor Smc3p were detected on chromatin in anaphase II nuclei.

accumulated to high levels within nuclei 4–6 hr after transfer to sporulation medium and remained at this level until the formation of meiosis I mitotic spindles. Rec8-Myc9p colocalized with chromosomes during pachytene. Its abundance within nuclei declined rapidly after pachytene, but a variable fraction clearly persisted as clusters of protein within nuclei until the second meiotic division, whereupon it disappeared completely. An identical pattern was seen with Rec8-HA3p (not shown). The distribution during meiosis of an Smc3 protein tagged with Myc epitopes was similar to that of Rec8p (not shown), except that it was also present in mitotic cells.

Rec8p and Smc3p but Not Scc1p Colocalize with Chromosome Cores during Pachytene

The association of these proteins with chromosomes was analyzed by immunostaining of chromosome spreads (Klein et al., 1992). Rec8-HA3p and Scc1-Myc18p were associated with DNA from premeiotic DNA replication until the first meiotic division. Both proteins had a punctate distribution during leptotene and zygotene, but their foci did not colocalize (Figure 4A). By pachytene, Rec8p was found in a continuous line along the longitudinal axes of chromosomes (Figures 4A and 4B), while Scc1p was found in about 30–50 discrete foci. Smc3-Myc6p, on the other hand, colocalized with Rec8-HA3p on pachytene chromosomes (Figure 4B). The lines of Rec8- and Smc3-specific staining were compact and clearly narrower than that of the chromosomal DNA, as detected by DAPI. They were, however, broader than those of Zip1. The distribution of Rec8p and Smc3p is distinct from that of Red1p (Smith and Roeder, 1997) in being more continuous with each chromosome's axial element. Costaining with antibodies against the centromeric protein Ndc10p (Goh and Kilmartin, 1993) showed that Rec8p was also present in the vicinity of centromeres on pachytene chromosomes (Figure 5B) and that it was preferentially associated with centromeric DNA as early as zygotene (Figure 5A).

Zip1p and Hop1p are absent from ribosomal DNA, but like Red1p (Smith and Roeder, 1997), both Rec8p and Smc3p staining runs along the axes of both homologs in this region of chromosome XII (Figure 4B). Likewise,

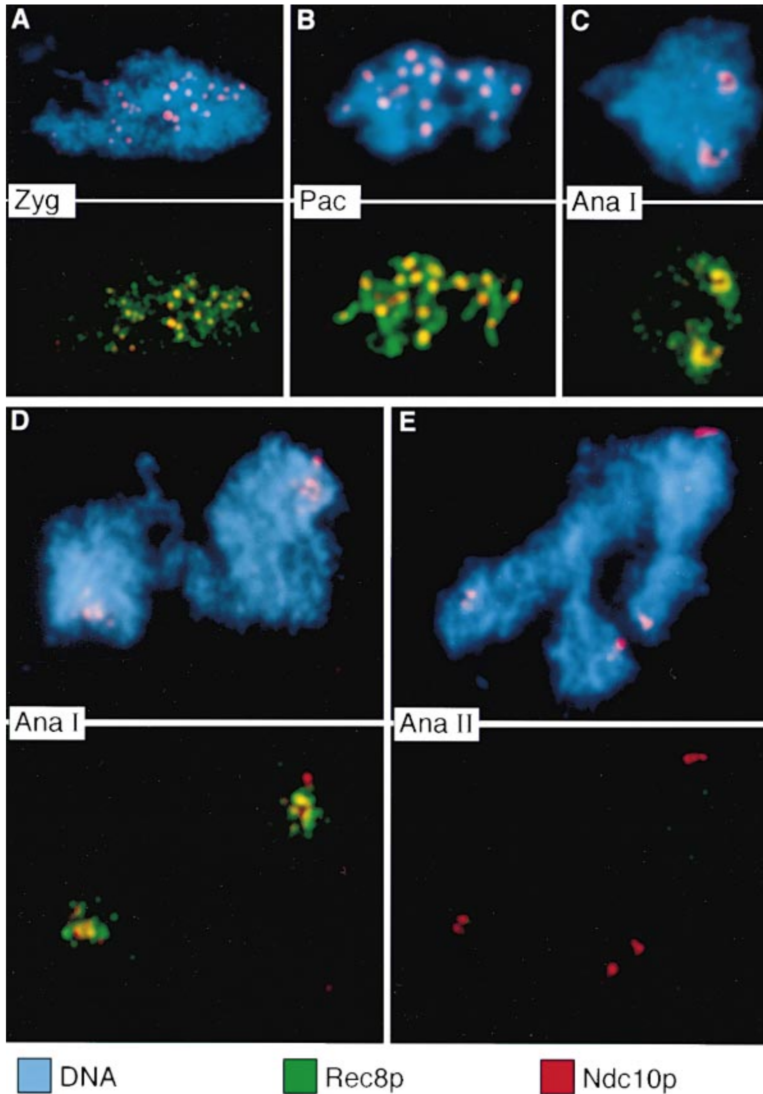

Figure 5. Centromeric Protein Ndc10p Colocalizes with Rec8p during Most of Meiosis

The blue and corresponding green images below show Ndc10p (red) on chromatin (blue) or Rec8p (green) from strain FKY1091. Ndc10p on chromatin accordingly appears pink, whereas colocalized Rec8p and Ndc10p appear yellow or orange.

(A) Early prophase, where little or no synapsis has taken place (leptotene or early zygotene). Note that almost all of the Ndc10p signals overlap with Rec8p foci at this stage and that Ndc10p foci coincide preferentially with the strongest Rec8p foci, suggesting preferential binding of Rec8p to regions around the centromeres.

(B) Pachytene, during which each of the 16 Ndc10p foci (synapsed centromeres) resides on a different Rec8p backbone.

(C) Early anaphase I, where centromeres but not the mass of chromatin have segregated to opposite spindle poles and much, but not all, Rec8p has disappeared from chromosome arms. The centromeres are tightly clustered around two DAPI-negative sites (spindle pole bodies), and the strongest Rec8p signals coincide with this cluster, suggesting specific protection of the protein at these clusters.

(D) Late anaphase I, by which stage Rec8p is almost exclusively present very close to the polar clusters. Note, however, that there is not a perfect overlap between Ndc10p and Rec8p. Rec8p could be associated with chromatin adjacent to kinetochores.

(E) Anaphase II, where Ndc10p marks four clusters of centromeres, whose position indicates the direction of these two nuclear divisions. Rec8p has completely disappeared from chromatin by this stage.

both proteins decorated the chromosome cores of univalents found in asynaptic *spo11* mutants (Figure 6A).

Rec8p and Smc3p Persist at Centromeres until Anaphase II

It is difficult to distinguish unambiguously diplotene chromosomes, whose preexisting synaptonemal complex has just dissolved and is poised to undergo the first meiotic division, from zygotene chromosomes, which have not yet formed SC. However, cells that have just initiated anaphase can be identified unambiguously. Figure 4B (lower panels) shows that the amount of both Rec8p and Smc3p associated with anaphase I chromosomes is much less than that associated with pachytene chromosomes. Rec8p was present in a number of small foci (Figure 5B) that were usually clustered around DAPI-negative sites corresponding to spindle pole bodies (Figures 3A_v, 4B, 5C, and 5D). The distribution of Smc3p was more diffuse at this stage, but it was also clustered around spindle poles along with Rec8p (Figure 4C). Rec8-HA3p colocalized closely with Ndc10p in both dense and loose clusters in cells that had just completed anaphase I (Figures 5C and 5D), demonstrating that Rec8 persists in the vicinity of centromeres after the separation of homologs. In contrast, Rec8-HA3p (and Smc3-Myc6p) was undetectable in tetranucleate spreads (Figure 4C) that contained four sets of Ndc10 foci (Figure 5E). These data suggest that Rec8p and Smc3p disappear from centromeric regions shortly after the onset of anaphase II.

Spo13p Controls Rec8p's Disappearance from Centromeres

Our data suggest that Rec8p associated with centromeric chromatin has an important role in holding sister centromeres together until the onset of anaphase II. As predicted by this hypothesis, Rec8p persisted at centromeres during the first meiotic division in *spo11* mutants even though homologs segregated at random (Figure 6B). Rec8p only disappeared from centromeric foci in *spo11* mutants when sisters separated at the second meiotic division (not shown). To test further the connection between the disappearance of Rec8p from centromeres and loss of cohesion between them, we

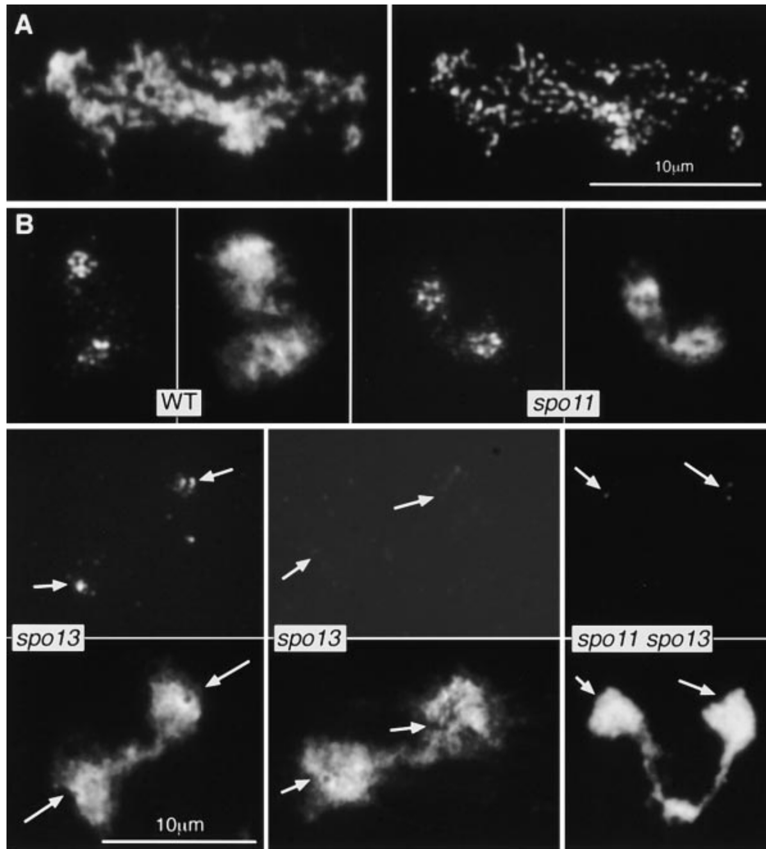

Figure 6. Rec8p in Chromosome Spreads from *spo11* or *spo13* Mutants

(A) Chromatin (left) and Rec8p (right) in a *spo11* mutant (FKY1145 *MATa/α, spo13::hisG/spo13::hisG, spo11::URA3/spo11::URA3, REC8::HA3::URA3/REC8::HA3::URA3*). *spo11* mutants fail to synapse but form axial element fragments and undergo chromatin condensation. Rec8p localizes to the cores of the unsynapsed univalents. The same was true for Smc3p in *spo11* cells (FKY458 *MATa/α, spo11::URA3, SMC3::MYC6::LEU2, spo11::URA3, SMC3::MYC6::LEU2*, not shown).

(B) The upper two panel pairs show Rec8p clusters and the corresponding chromatin during anaphase I from WT and a *spo11* mutant (FKY1108 *MATa/α, spo11Δ::URA3/spo11Δ::URA3, REC8::MYC9::URA3/REC8::MYC9::URA3*). Rec8p clusters in these two strains were indistinguishable. The three lower panel pairs show Rec8p and chromatin during anaphase in *spo13* (FKY1115 *MATa/α, spo13::hisG/spo13::hisG, REC8::MYC9::URA3/REC8::MYC9::URA3*) or *spo13 spo11* (FKY1145) cells. Either much less (left) or almost no Rec8p (right) was found in *spo13* mutants. In *spo13 spo11* double mutants, Rec8p was barely detectable at centromere clusters.

analyzed the distribution of Rec8p in chromosome spreads from *spo13* mutants that separate a subset of sister chromatids instead of segregating homologs at the first meiotic division and then fail to undergo a second meiotic division (Klapholz and Esposito, 1980a, 1980b; Hugerat and Simchen, 1993). In *spo13* mutants, spreads from cells that had just undergone anaphase I either contained faint clusters of Rec8p at the spindle poles or none at all (Figure 6B). Association of Rec8p with chromosome axes earlier during meiosis, for example during pachytene, was unaffected by the *spo13* mutation (not shown). Very little—but still detectable—amounts of Rec8p were found associated with binucleate spreads from *spo11 spo13* double mutants in which 100% of cells undergo a single equational division (Figure 6B). We conclude that *SPO13* is required to delay Rec8p's removal from centromeres until the second meiotic division.

REC8 and *SMC3* Are Needed for Recombination but Not for Formation of Double-Strand Breaks

During mitotic divisions, reciprocal exchanges occur more frequently between sister than nonsister chromatids. This situation is reversed during meiosis, where exchanges between homologs are vital for their stable association and correct segregation. Because this bias could be mediated by meiotic cohesion, we tested whether *REC8* and *SMC3* had any influence on recombination between homologs. To obtain viable progeny, we measured the frequency of recombination between *arg4* and *his4* heteroalleles at different times following the transfer of sporulating cells to vegetative conditions, upon which they resume a mitotic division. *Rec8Δ* and *smc3-42* mutants rapidly lost viability 4 hr after transfer to sporulation medium (at 25°C). Nevertheless, among the survivors, there were 20- to 40-fold fewer *ARG4* or *HIS4* recombinants in both mutants than in wild type (Figure 7A). Both the loss of viability and the reduction of recombinants in *rec8* and *smc3-42* mutants were comparable to *com1/sae2* mutants, which are defective in the processing of Spo11-induced DSBs (Prinz et al., 1997). These data suggest that *SMC3* and *REC8*, like their *S. pombe* counterparts, are necessary for efficient recombination between homologs.

The lack of recombination in *rec8* and *smc3-42* mutants could be due either to their failure to produce DSBs or to use such breaks to promote recombination between homologs. Fragments generated by DSBs at the *HIS4:LEU2* hot spot (Cao et al., 1990) appeared in both mutants with normal kinetics but were resected to a greater extent than wild type and persisted at stages when they were repaired by recombination in wild type (Figure 7B). This result, along with the observation that accumulation of DSBs in *rad50S rec8Δ* (FKY1076 *MATa/α, rec8Δ::KanMX4/rec8Δ::KanMX4, rad50S::URA3/rad50S::URA3*) and *rad50S smc3-42* (FKY1137 *MATa/α, rad50S::URA3/rad50S::URA3, smc3-42/smc3*-42) double mutants was identical to that in *rad50S* single mutants (FKY329 *MATa/α, his4B::LEU2-MluI/his4X::LEU2-MluI-BamHI::URA3, rad50S::URA3/rad50S::URA3*) at a natural hot spot close to *THR4* (not shown), implies that DSBs are produced normally in the mutants but are not

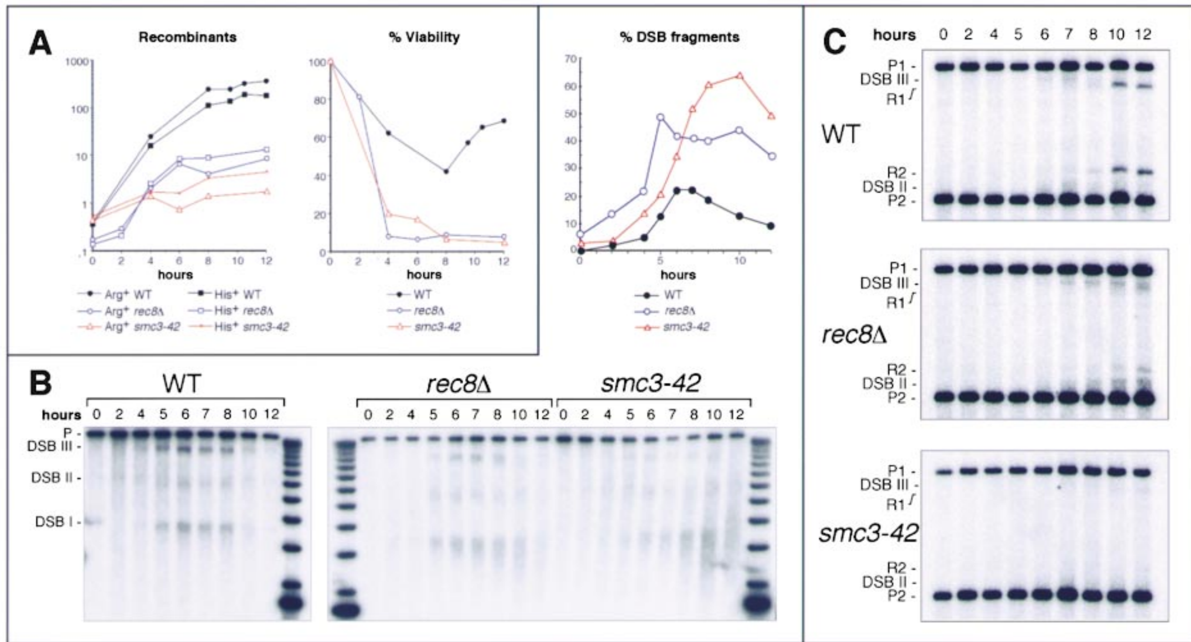

Figure 7. Meiotic Recombination Is Initiated but Not Completed in *rec8* and *smc3-42* Mutants

(A) *rec8* (FKY1122 *MATa/α*, *his4X::LEU2-MluI-BamHI::URA3/his4BLEU2-MluI*, *arg4-Nsp/arg4-BgI*, *rec8Δ::KanMX4/rec8Δ::KanMX4*) and *smc3-42* (FKY1132 *MATa/α*, *his4X::LEU2-MluI-BamHI::URA3/his4BLEU2-MluI*, *arg4-Nsp/arg4-BgI*, *smc3-42/smc3-42*) mutants lose viability upon entry into meiosis. The production of Arg^+ or His^+ cells from heteroalleles (parental constructs provided by D. Bishop, L. Xu, and N. Kleckner) by gene conversion is reduced in survivors following a return to growth experiment (WT is FKY361 *MATa/α his4X::LEU2-MluI-BamHI::URA3/his4BLEU2-MluI*, *arg4-Nsp/arg4-BgI*). Cells were pulled out of meiosis every 2 hr and plated on YPD. The left graph plots Arg^+ or His^+ cells per 10^4 colony-forming cells. As their colony-forming ability decreased, the ratio of Arg^+ (His^+) cells per viables increased, while the absolute yield of recombinants stayed close to constant. This result is similar to that obtained for other mutants, proficient in recombination initiation but defective in its repair (e.g., *com1/sae2*, Prinz et al., 1997).

(B) DSBs are formed with normal kinetics at the artificial *LEU2HIS4* recombination hot spot (Cao et al., 1990) in *rec8* (FKY1122) and *smc3-42* (FKY1142) mutants, but failure to repair them leads to hyperresection of DNA ends. Genomic DNA was isolated at different time points in meiosis, digested with PstI, blotted, and probed with probe 291 (Xu et al., 1995), highlighting a 3.9 kb, a 6.2 kb, and a 10 kb transient band corresponding to DSBI, DSBII, and DSBIII break sites. (The DSBIII site maps right in the promoter of the his4× allele and was not described for the locus by Xu and Kleckner [1995] and Storlazzi et al. [1995].) In WT (FKY361), these bands peak at 6 hr and are almost invisible by 10 hr. In contrast, in *rec8* and *smc3-42* mutants, these bands spread out over time, as the fragments are abnormally degraded. The graph illustrates the results from quantitating the sum of the DSB signals with a phosphoimager.

(C) Recombination products detected by a physical assay are reduced in *rec8* (FKY1122) and *smc3-42* (FKY1132) mutants. We measured reciprocal recombination at the *LEU2HIS4* hot spot using the BamHI/MluI system (Xu and Kleckner, 1995). Whereas in WT normal levels of recombinant fragments were detected, strongly reduced recombination levels were found in *rec8Δ* and no significant increase over background in *smc3-42* mutants (the reduction must be at least 10-fold). Bands corresponding to DSBII and DSBIII were found in the mutants. Parental fragments P1, 19.7 kb and P2, 12.3 kb; recombinant fragments R1, 18.2 kb and R2, 13.8 kb; DSBIII, 18.5 kb; and DSBII, 13.1 kb.

properly repaired. We therefore analyzed the formation of recombinant molecules in the region of the *HIS4LEU2* hot spot using a system in which parental chromosomes carry an appropriately designed restriction fragment polymorphism (Storlazzi et al., 1995; Xu and Kleckner, 1995). Physical analysis showed that crossing over was reduced to very low levels in both mutants (Figure 7C). Thus, homolog recombination is strongly reduced in *rec8Δ* and *smc3-42* mutants, and DSBs are rarely if at all repaired using sequences from homologs.

Discussion

It has long been recognized that cohesion between sister chromatids along chromosome arms is destroyed at the first meiotic division whereas that within centromeres is destroyed only at the second meiotic division and that this difference is a crucial aspect of meiosis. Apart from MeiS332p in *Drosophila* (Kerrebrock et al., 1995), which associates with centromeres during metaphase I and is required to hold them together until anaphase II, very little is known about the proteins that mediate meiotic sister chromatid cohesion. Scc1p and Smc3p are subunits of a cohesin complex necessary for holding sister chromatids together during mitotic divisions. We show here that Smc3p and a meiotic variant of Scc1p called Rec8p are essential for sister chromatid cohesion in budding yeast. Both proteins are found along the entire length of meiotic chromosomes during pachytene and largely disappear from chromosomes around the first meiotic division but persist at centromeres until metaphase II. Their eventual disappearance correlates with the separation of sister centromeres at anaphase II. Our data raise the possibility that differences in the distribution along chromosomes of a meiotic version of cohesin are responsible for the separation of chromatid arms during meiosis I and centromeres during meiosis II.

Rec8p: A Meiosis-Specific Cohesin Subunit?

Scc1p and Smc3p are two of four subunits of a cohesin complex essential for sister chromatid cohesion during mitotic divisions. Smc3p but probably not Scc1p is essential for sister chromatid cohesion during meiosis. Our results suggest that Scc1p's role in holding sister chromatids together is carried out during meiosis by a related protein only expressed during this stage of the yeast life cycle, which we call Rec8p. We have not yet investigated the roles during meiosis of the other cohesin subunits, Smc1p and Scc3p.

S. pombe not only possesses two Scc1-like proteins, Rad21p (needed for mitosis) and Rec8p (needed during meiosis), but also two Scc3-like proteins, one of which (Rec11p) has a role similar to Rec8p (Li et al., 1997). The *S. cerevisiae* genome, in contrast, encodes only one Scc3-like protein. To explain these observations, we propose that both *S. pombe* and *S. cerevisiae* use meiotic variants of cohesin to generate sister chromatid cohesion during premeiotic DNA replication. In *S. cerevisiae*, Scc1p is replaced by Rec8p, whereas in *S. pombe*, Rad21p (Scc1p) and its mitotic equivalent of Scc3p are replaced by Rec8p and Rec11p, respectively. As predicted by this hypothesis, Rec8p and Smc3p can be efficiently coimmunoprecipitated from meiotic cells (S. B. C. B., unpublished). Similar meiotic versions of cohesin might also be used in mammals, including humans, that possess a Rec8-like gene expressed largely only in meiotic cells (Parisi et al., 1999).

The abundance of Scc1p greatly declines as cells proceed with the meiotic program. Scc1p does not disappear until shortly before the first meiotic division, but it neither colocalizes with Smc3p nor Rec8p and is instead found at distinct foci scattered evenly across the nucleus. The frequency and distribution of these foci on pachytene chromosomes resemble that of Msh4p, which is believed to be at the sites of late recombination nodules. Because of its weak but consistently regular focal pattern in meiotic prophase, we think it quite unlikely that the observed late Scc1p foci are merely dispensable remnants of earlier associations. Furthermore, spore viability is reduced to ~50% in *scc1-73* mutants, which is comparable to other mutations, such as *msh4* and *zip1*, known to be involved in meiotic recombination (Sym et al., 1993; Ross-Macdonald and Roeder, 1994). As is believed for these genes, *SCC1* might act in a partially redundant pathway to achieve correct chromosome segregation.

The cohesion between sister chromatids generated by Scc1p during mitotic cell cycle and Rec8p during meiosis must differ in important aspects. Whereas Scc1p is removed in a single step from mitotic chromosomes due to cleavage induced by the separin Esp1p, Rec8p disappears from meiotic chromosomes in at least two stages. The bulk of Rec8p and Smc3p is detached from chromosome arms by anaphase I, but a subpopulation of Rec8p and Smc3p persists in the vicinity of centromeres until the onset of anaphase II. The *S. pombe* Rec8 protein behaves in a similar fashion (P. Nurse, personal communication). This suggests that "centromeric" cohesin is responsible for maintaining cohesion between sister centromeres between the first and second meiotic division. In *Drosophila*, the MeiS332 protein binds to centromeric heterochromatin during metaphase of meiosis I, is required to prevent precocious sister centromere separation, and disappears from chromosomes at the onset of anaphase II. This raises the possibility that proteins like MeiS332 might protect cohesin at centromeres from attack by separins until shortly before the second meiotic division. If so, one must postulate that cells produce factors that neutralize MeiS332-like cohesin protector proteins only at the onset of anaphase II. The premature production of such factors might be responsible for the precocious removal of Rec8p from centromeres in *spo13* mutants.

Cohesins and Axial Elements

The search for proteins that form the core of pachytene chromosomes has often been conducted on the premise that because axial elements and synaptonemal complexes are unique to meiotic cells, they might consist of meiosis-specific proteins. Many constituents have indeed been identified on this basis. Hop1p, Red1p, Zip1p, and others are meiosis-specific proteins necessary for the formation of synaptonemal complex. By taking the alternative route of investigating mitotic proteins, we have identified two important novel players. Unlike Red1p and its kinase Mek1p, which have a patchy distribution along chromosome cores, both Rec8p and Smc3p are present continuously along the longitudinal axes of pachytene chromosomes independent of synapsis. This raises the possibility that meiotic cohesin complexes are in fact part of axial elements seen by electron microscopy. However, cohesion both precedes and survives the period during which axial elements are present. Therefore, it is equally possible that Rec8 and Smc3 instead form an underlying chromosomal core structure to which the real constituents of axial elements must attach. The cohesin core presumably shares several proteins and properties with equivalent structures built during vegetative divisions but must be modified—for instance, by replacing Scc1p by Rec8p—if it is to serve as a scaffold for the assembly of axial elements whose constituents are possibly exclusive to meiotic cells.

Cohesins and Recombination

Rad21p in *S. pombe* (Birkenbihl and Subramani, 1992) and Scc1p in *S. cerevisiae* (not shown) are necessary for repairing double-strand breaks caused by ionizing irradiation during vegetative growth. Rec8p might therefore function during meiotic recombination. We found that Spo11-induced DSBs were generated with normal kinetics in *rec8Δ* and *smc3-42* mutants but that they were resected to a much greater extent than in wild type and not processed efficiently into recombinant DNA molecules. Although sister chromatid repair would go undetected in this assay, our observation that the mutants accumulate broken chromosomes and are greatly delayed in undergoing meiotic divisions suggests that many if not most lesions escape repair in *rec8* and *smc3* mutants. We suggest that both meiotic and mitotic cohesins are required to ensure that DSBs find their targets, which are either sister chromatids (during mitosis) or homologs (during meiosis). Whether cohesin's participation in recombinational repair depends on the establishment of sister chromatid cohesion is unclear.

Because of the strong impact of the mutations on recombination, we speculate that cohesins might have a role in repair that goes beyond establishing cohesion between sister chromatids.

Meiosis: Spinning Out Cohesion Loss over Two Divisions

In vegetative cells, sister chromatid cohesion established during DNA replication is essential for the alignment of chromosomes on the metaphase spindle, whereas its subsequent destruction is required for the segregation of sister chromatids to daughter cells during anaphase. How then can a single round of sister chromatid cohesion generated during premeiotic DNA replication be sufficient for two successive divisions during meiosis? The answer may lie in the eukaryotic cell's ability to destroy cohesion within chromosome arms with very different kinetics to that surrounding centromeres. Cohesion and its destruction might be "spun out" over two divisions. Having recombined homolog chromatids, cells utilize arm cohesion to maintain the link between homologs needed for the first reductional division, as has been suggested by Maguire (1974), and they possibly trigger this first division by destroying arm cohesion. At the same time, cohesion around centromeres is conserved for the second meiotic division. Why, in the face of this complication, do organisms (with rare exceptions like male *Drosophila* [McKee and Karpen, 1990; McKim et al., 1998]) rely on chiasmata (and by implication on sister chromatid cohesion) to hold homologs together during metaphase of meiosis I? Many eukaryotic cells have mechanisms for pairing homologs without recombination (Dernburg et al., 1998), and this pairing mechanism would be equally good for segregating homologs as it is in male *Drosophila*. One answer may be that by coupling lack of meiotic recombination with infertility (i.e., a failure to segregate chromosomes), most eukaryotic organisms protect their sexually-reproducing offspring from competition by recombination-deficient siblings that might well otherwise have short-term advantages, such as maintaining combinations of alleles optimized for a certain environment.

Experimental Procedures

Yeast Strains and Media

All strains were derivatives of SK1 (or at least 5× crossed to SK1 strains, if originally W303). *scc1-73* and *smc3-42* mutations were isolated in W303 strain background (Michaelis et al., 1997). The *rec8*Δ mutation was introduced into SK1 by PCR-mediated gene replacement (Wach et al., 1994), replacing the complete sequence of ORF YRP007C (positions +4 to +2043, ATG = +1) with the heterologous *KANMX4* marker. C-terminal tagging of *SCC1* and *SMC3* genes resulted in *TRP1*-marked *SCC1::MYC18* and *LEU2*-marked *SMC3::MYC6* (Michaelis et al., 1997). The C-terminal tag of *REC8* was obtained by amplifying a fragment (positions +1531 to +2040) by PCR and introducing an XbaI site just before the stop codon. After cloning this PCR product into Yiplac211 (Gietz and Sugino, 1988), an XbaI-HA3-XbaI or SpeI-Myc9-SpeI cassette (Michaelis et al., 1997) was inserted into the XbaI site. The resulting plasmids were correctly integrated at the chromosomal *REC8* locus as confirmed by Southern blot analysis. In general, homozygous strains containing the tagged genes showed wild-type sporulation and viability. Rec8::Myc9, however, caused an ~50% decrease in spore viability, but its chromosomal distribution was indistinguishable from that of the fully functional Rec8::HA3. Chromosome V was visualized by a tet repressor–GFP fusion protein (tetR-GFP) binding to an array of tet operators (tetOs) integrated at either the *URA3* locus near the centromere (cenV-GFP) or at the *BMH1* locus 30 kb from the right telomere (TelV-GFP) (Ciosk et al., 1998).

Unless otherwise stated, strains were sporulated as described previously (Nairz and Klein, 1997). Briefly, cells were grown on a YPD plate for 24 hr, transferred to liquid YPA, and incubated until they reached a density of approximately 2×10^7 cells/ml, upon which the medium was replaced by half the volume 2% potassium acetate (t = 0).

Pairing and Sister Chromatid Cohesion Assay

Aliquots of sporulating cultures were fixed by addition of 1/10 volume 34% formaldehyde for 10 min, washed briefly in 96% ethanol, and resuspended in 0,5 μg/ml DAPI solution. Cells were inspected for chromatin and GFP in the same cells in a Zeiss fluorescence microscope. *smc3-42* and *scc1-73* cells were sporulated at 34°C after an initial incubation at 23°C in the experiment shown in Figure 1. In order to obtain binucleate cells in the experiment described in Table 1, *smc3-42* cells were sporulated at 25°C. In order to reduce bleaching of GFP signals in the *scc1-73* cells, the experiment described in Table 1 was done at 30°C. Spore formation and viability did not change considerably for both mutant alleles for temperatures between 25°C and 35°C (no viable spores formed in *smc3-42* and 40%, 50%, and 60% viability at 25°C, 30°C, and 35°C for *scc1-73*).

Light and Electron Microscopic Analysis of Meiotic Spreads and Whole Cells

Yeast meiotic spreads and electron microscopic analysis were performed as described previously (Nairz and Klein, 1997; Loidl et al., 1998). Sera from rabbits against Zip1p, Red1p were obtained from Shirleen Roeder. These proteins were detected using a CY3-conjugated goat anti-rabbit serum from Amersham. Whole cells were prepared for indirect immunofluorescence according to Piatti et al. (1996). Myc- and HA-tagged proteins were detected with mouse monoclonal antibodies and a CY3-conjugated secondary antibody. For Myc and HA double labeling, a rabbit anti-Myc antibody was combined with a mouse anti-HA antibody to avoid cross-reaction of secondary antibodies. Spindles were detected with a rat antiserum to yeast tubulin and a fluorescein isothiocyanate–conjugated secondary antibody. Pictures were taken with a Photometrics charge-coupled device camera mounted on a Zeiss microscope.

Spore Viability, Double-Strand Breaks, and Recombination

Spore viability was assayed either by Tetrad dissection or, in cases of very low spore viability, by plating of spores to rich medium, followed by killing of unsporulated cells using ether (Prinz et al., 1997). Ascus frequencies were assayed under phase contrast. Gene conversion between *arg4* (or *his4*) heteroalleles was determined in a return to growth experiment as previously described (Prinz et al., 1997), using strains derived from NKY1303, NKY1543 (Xu and Kleckner, 1995).

For physical assay of DNA, DSBs or recombination cells were sporulated at 25°C and aliquots taken at different time points of which genomic DNA was prepared by alkaline lysis of spheroplasted yeast cells (Prinz et al., 1997). After electrophoresis, blotting, and hybridization, the resulting signals were quantified on a Storm Phosphoimager. In order to separate recombinant from parental fragments far enough, the XhoI-digested genomic DNA was subjected to pulsed-field electrophoresis using a Bio-Rad FIGE system. Fragments were separated at room temperature for 30 hr in a 1% agarose gel in 0.5×TBE, with 180 V forward voltage, 120 V reverse voltage, and an initial switching time of 0.1 s, which changed with a linear gradient to a final switching time of 0.2 s. This resulted in high resolution, which enabled us to detect an additional DSB site (DSBIII) in both the PstI and XhoI digests.

Western Blotting

To obtain protein extracts, cells were broken with glass beads in 10% trichloroacetic acid, pelleted, and resuspended in loading buffer. Separation and blotting was according to standard procedures; Myc-tagged proteins were detected using 9E10 mouse anti-Myc antibody followed by the ECL method (Amersham).

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