Cell, Vol. 103, 387–398, October 27, 2000, Copyright ©2000 by Cell Press

Disjunction of Homologous Chromosomes in Meiosis I Depends on Proteolytic Cleavage of the Meiotic Cohesin Rec8 by Separin

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Summary

It has been proposed but never proven that cohesion between sister chromatids distal to chiasmata is responsible for holding homologous chromosomes together while spindles attempt to pull them toward opposite poles during metaphase of meiosis I. Meanwhile, the mechanism by which disjunction of homologs is triggered at the onset of anaphase I has remained a complete mystery. In yeast, cohesion between sister chromatid arms during meiosis depends on a meiosis-specific cohesin subunit called Rec8, whose mitotic equivalent, Scc1, is cleaved at the metaphase to anaphase transition by an endopeptidase called separin. We show here that cleavage of Rec8 by separin at one of two different sites is necessary for the resolution of chiasmata and the disjunction of homologous chromosomes during meiosis.

Introduction

During mitosis, cohesion between sister chromatids generated during DNA replication (Uhlmann and Nasmyth, 1998; Skibbens et al., 1999; Toth et al., 1999) provides the means by which sister kinetochores attach to spindles that extend to opposite poles of the cell during prometaphase (Rieder and Salmon, 1998). When this occurs, sister chromatids come under tension as they are pulled in opposite directions (Nicklas, 1988). This tension lasts throughout metaphase, until sister chromatid cohesion is suddenly dissolved soon after congression of all chromosomes to the metaphase plate. Loss of cohesion triggers the segregation of sisters to opposite poles during anaphase (Nasmyth et al., 2000; Uhlmann et al., 2000 [this issue of *Cell*]).

During meiosis, two rounds of chromosome segregation following a single round of chromosome duplication give rise to haploid gametes from diploid germ cells. Remarkable changes in the behavior of chromosomes are required to produce this result. One is the pairing of homologous chromosomes and the subsequent recombination (or cross over) of the DNA strands and axes of homologous chromatids. Another is the monoorientation of sister kinetochores at the first meiotic division, which ensures their attachment to the same spindle pole and prevents the usual bipolar attachment. Due largely to these two properties, pairs of homologous chromosomes and not sister chromatids come under tension during metaphase of meiosis I, as kinetochores from homologs attach to spindles extending to opposite poles (Moore, 1998; Zickler and Kleckner, 1998; Orr-Weaver, 1999).

It has been proposed but never proven that sister chromatid cohesion within chromosome arms distal to chiasmata is responsible for holding homologs together until the onset of anaphase I (Maguire, 1974; Carpenter, 1994). If so, loss of this cohesion must be necessary for, and might even trigger, disjunction of homologs at the first meiotic division. Consistent with this hypothesis, sister chromatid arms remain closely connected throughout diakinesis and metaphase I, and invariably part from each other at the onset of anaphase I. Meanwhile, the segregation of sister chromatids at the second meiotic division depends on cohesion in the vicinity of their centromeres, which, unlike that along their arms, is maintained until metaphase of meiosis II and is dissolved only at the onset of anaphase II (Orr-Weaver, 1998).

Cohesion between sister chromatids during mitosis in yeast is mediated by a multisubunit complex called cohesin (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Nasmyth, 1999; Toth et al., 1999). The connections between sisters made by cohesin are dissolved at the onset of anaphase by proteolytic cleavage of one of its subunits, Scc1 (Uhlmann et al., 1999), by a specialized endopeptidase called separin, also known as Esp1 or separase (Uhlmann et al., 2000 [this issue of *Cell*]). Scc1 contains two sites recognized by separin and cleavage at either one of the two sites is necessary for sister separation. Remarkably, the best two matches to Scc1's separin cleavage sites within the entire yeast proteome are both found in the meiotic cohesin subunit Rec8 (Uhlmann et al., 1999), which is required for sister chromatid cohesion during meiosis but not mitosis (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). Rec8, along with other cohesin subunits, lines the entire longitudinal axes of pachytene chromosomes. It disappears from chromosome arms shortly before the first meiotic division, but persists in the vicinity of centromeres until the onset of anaphase II (Klein et al., 1999).

To address whether cleavage of Rec8 might be required for the resolution of chiasmata and hence, the disjunction of homologs, we generated noncleavable mutations in Rec8's potential separin cleavage sites. We show here that both sites are indeed substrates for separin in vitro and in vivo. We also show that their simultaneous mutation prevents Rec8's disappearance from chromosome arms during the first meiotic division without altering other meiosis I events, that the lack of

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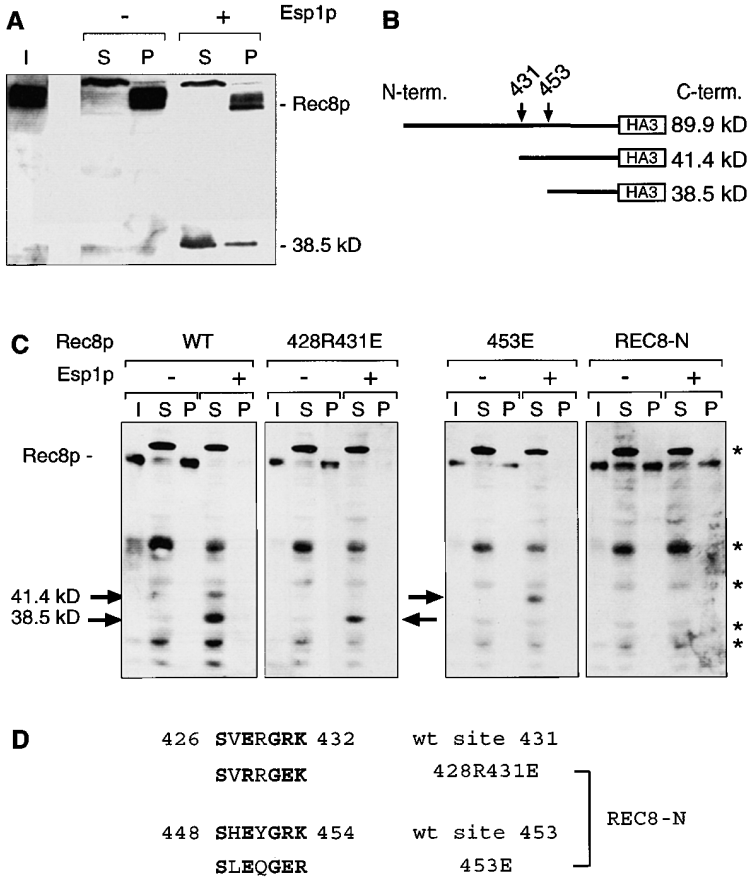

Figure 1. Rec8 Is Cleaved In Vitro by Separin at Residues 431 and 453

(A) Rec8 replacing Scc1 in mitotic cells is a substrate for separin cleavage in an in vitro reaction. A chromatin pellet was isolated from strain *K8811* (W303 prSCC1-REC8-HA3-LEU2::leu2 *scc1*Δ::URA3) and incubated in extracts of cells that had (lanes +) or had not (lanes −) been induced to overexpress Esp1 (*K7287:* W303 *esp1-1*, trp1::TRP1 Gal-ESP1) (Uhlmann et al., 1999). S: supernatant (soluble fraction) isolated after the in vitro reaction by centrifugation. P: corresponding chromatin pellet. I: input, total amount of Rec8 binding the chromatin before the in vitro reaction. Asterisk: antibody cross-reacting band.

(B) Scheme of full-length Rec8 protein and putative cleavage site positions, with the expected cleavage product sizes.

(C) Six hours after the induction of meiosis, chromatin pellets were prepared from strains *K8806* (SK1 MATa/α REC8-HA3 wt -LEU2::*rec8*Δ::kanMX4), *K8968* (SK1 MATa/α REC8-HA3 428R431E-LEU2::*rec8*Δ::kanMX4), *K8805* (SK1 MATa/α REC8-HA3 453E-LEU2::*rec8*Δ::kanMX4), and *K8816* (SK1 MATa/α REC8-N-HA3-LEU2::*rec8*Δ::kanMX4) and incubated in extracts of cells that had (lanes +) or had not (lanes −) been induced to overexpress Esp1. S: supernatant. P: corresponding chromatin pellet. I: input. Asterisk: antibody cross-reacting bands.

(D) Amino acid changes in three Rec8 mutants. REC8-N is the mutant where both cleavage sites are modified simultaneously.

Rec8 cleavage blocks homolog disjunction, and that mutation of separin causes a similar phenotype.

Our observations imply that chiasmata are maintained until metaphase I by sister chromatid cohesion along chromosome arms, which is mediated by a version of cohesion complex containing Rec8, and is resolved at the onset of anaphase I by cleavage of Rec8 by separin. They also suggest that Rec8 in the vicinity of centromeres is protected from separin throughout the first meiotic division by an unknown mechanism. These results imply that proteolytic cleavage of Scc1-like cohesin subunits by separin may be a general mechanism for dissolving sister chromatid cohesion at metaphase to anaphase transitions.

Results

Mitotic Rec8 Is Cleaved by Separin In Vitro

To investigate whether Rec8 can serve as a substrate for separin, we took advantage of the discovery that Rec8 can substitute for Scc1 during vegetative growth. Rec8 is normally not produced during mitotic divisions and deletion of the *SCC1* gene is lethal at all temperatures (Uhlmann and Nasmyth, 1998). However, replacement of the *SCC1* gene by an HA-tagged version of *REC8* expressed from the *SCC1* promoter permits cells to proliferate at 25°C but not at 37°C (data not shown). Rec8 protein produced during mitotic divisions from this strain is cleaved by separin (Esp1) in vitro (Figure 1A). This suggests that Rec8 is a separin substrate, at least when presented on mitotic chromosomes.

Meiotic Rec8 Is Cleaved at Residues 431 and 453

To investigate whether meiotic Rec8 is also a separin substrate, we prepared chromatin from Rec8-HA3-expressing meiotic cells and treated it with extracts from mitotic cells. The chromatin was produced from cells that had completed premeiotic DNA replication but had not yet undergone the first meiotic division. Rec8-HA3 was both cleaved and removed from the pellet fraction by an extract from separin (Esp1) overproducing cells (Figure 1C, WT panel, lanes +) but not by one from *esp1-1* mutant cells (Figure 1C, WT panel, lanes −). Two different separin-dependent cleavage products (41.4 kDa and 38.5 kDa) were produced by this reaction (see Figures 1B and 1C, panel WT). Their sizes are consistent with cleavage at positions 431 and 453, the two sites within Rec8 closely related to Scc1 cleavage sites.

To confirm the identity of these cleavage sites, we mutated the proposed p1 arginine at site 453 to glutamic acid (Figure 1D, 453E). This mutation abolished production of the 38.5 kDa fragment (Figure 1C, panel 453E), suggesting that this product is generated by cleavage at position 453. An equivalent mutation of site 431 (431E) reduced but did not abolish production of the 41.4 kDa fragment (data not shown). We therefore produced a double mutant in which the p1 arginine at position 431 was replaced by glutamic acid and the conserved glu-

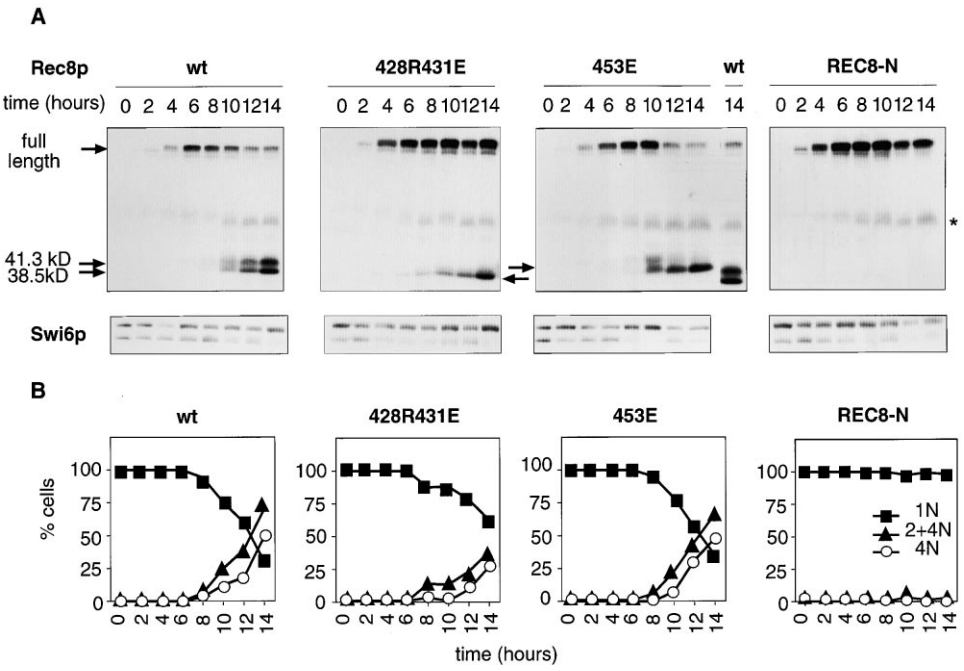

Figure 2. Meiotic Rec8 Is Cleaved In Vivo

(A) In vivo analysis of Rec8 cleavage during a meiotic time course for Rec8-HA3 wild type and the three mutants in *ubr1*Δ background. Both cleavage products (38.5 kDa and 41.4 kDa) are detectable around the first meiotic division in the wild type (strain *K9154:* SK1 MATa/α REC8-HA3 wt -LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1). In the mutant 428R431E (strain *K9156*: SK1 MATa/α REC8-HA3 428R431E-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) only the shorter product is generated (38.5 kDa), while the mutant 453E (strain *K9155*: SK1 MATa/α REC8-HA3 453E-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) originates only the longer cleavage product (41.4 kDa). In this particular experiment, the kinetic of meiotic progression of strain *K9156* is slightly slower than the other strains. REC8-N (*K9157:* SK1 MATa/α REC8-N-HA3-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) does not produce any of the expected cleavage products and full-length REC8-N protein levels do not decrease even at the latest stages. Swi6 was detected via specific antibody and used as loading control . Asterisk: antibody cross-reacting band.

(B) Analysis of nuclear division progression in DAPI-stained *ubr1*Δ cells allows detection of the effect of Rec8 mutant expression on meiotic progression. 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

tamic acid at position 428 was replaced by arginine. This double mutation (Figure 1D, 428R431E) abolished production of the 41.4 kDa fragment but did not affect the 38.5 kDa fragment (Figure 1C, panel 428R431E). Mutation of both sites (Figure 1D, REC8-N) abolished production of both 38.5 kDa and 41.4 kDa fragments and prevented removal of Rec8 from the chromatin "pellet" fraction (Figure 1C, panel REC8-N). These data suggest that separin made by mitotic cells removes Rec8 from meiotic chromatin in vitro by cleavage at sites 431 and 453.

The 38.5 kDa and (to a lesser extent) 41.4 kDa cleavage products are also produced by meiotic cells expressing Rec8-HA3 (data not shown). However, neither product ever accumulates to high levels, which hampers their reliable detection by Western blotting. Both products have N-terminal lysines, which are destabilizing amino acids for N-end rule ubiquitination, and their abundance is greatly elevated (data not shown) by inactivating the "N-end rule" Ubr1 ubiquitin protein ligase (Varshavsky, 1997). Degradation of Scc1 cleavage products is also mediated by Ubr1 (H. Rao, F. U., K. N., and A. Varshavsky, unpublished data). Fortunately, cells lacking Ubr1 still undergo meiosis efficiently, albeit somewhat more slowly than wild type, and this permitted us to follow Rec8 cleavage as *ubr1*Δ mutant cells undergo meiosis. Both 38.5 kDa and 41.4 kDa products appeared at the onset of meiosis I, which coincides with the reduction of full-length Rec8 (compare Figures 2A and 2B, panel wt). A similar reduction in Rec8's abundance also takes place at the onset of meiois I in *UBR1* cells (data not shown and Figure 4B, left panel). The 38.5 kDa fragment was abolished by the 453E mutation (Figure 2A, panel 453E) while the 41.4 kDa fragment was abolished by the 428R431E mutation (Figure 2A, panel 428R431E). Furthermore, mutation of both sites simultaneously (Figure 2A, panel REC8-N) abolished all cleavage and prevented the reduction of full-length Rec8, which normally occurs at the first meiotic division.

Separin Is Needed for Rec8 Cleavage, for Removal of Rec8 from Chromosomes, and for the First Meiotic Division

The disappearance of separin's inhibitor, securin (Pds1), at the onset of anaphase I, followed by its reappearance between divisions and disappearance once again at the onset of anaphase II (Salah and Nasmyth, 2000) suggests that separin might be transiently activated twice during meiosis: once shortly before the first meiotic division and a second time shortly before the second division. To investigate separin's role during meiosis, we isolated *esp1* DNAs from several *ts esp1* mutant strains and transferred them into the SK1 background (Experimental Procedures). All mutant strains sporulated effi-

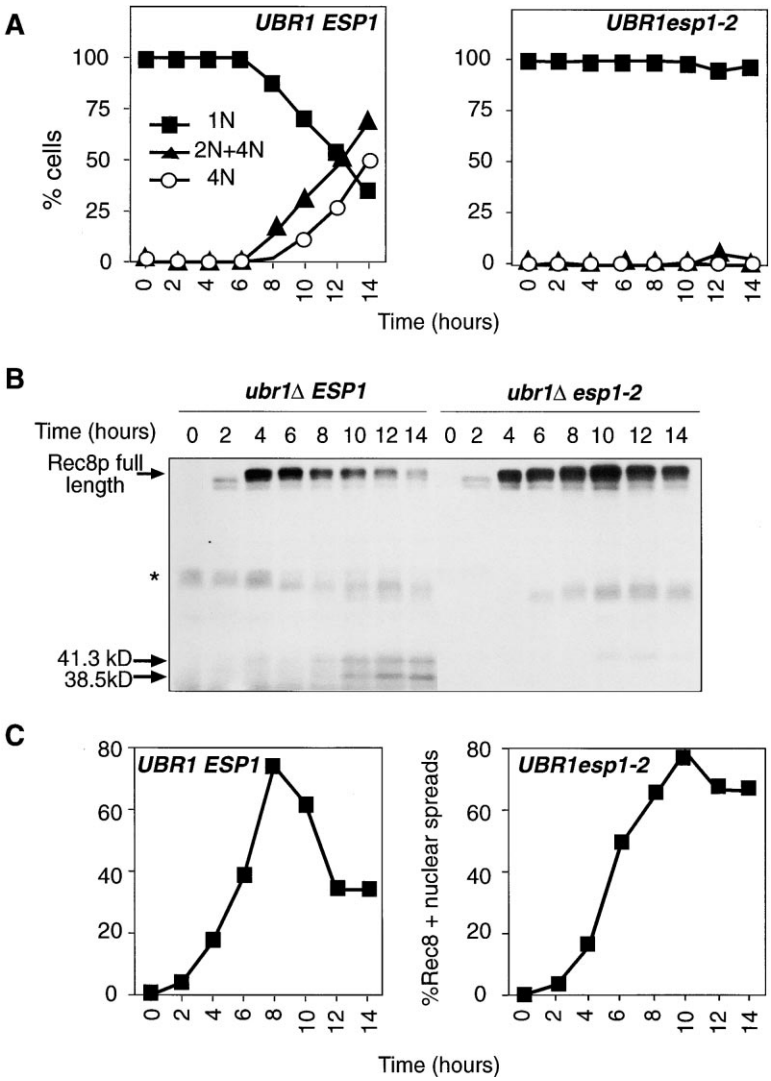

Figure 3. Rec8 Protein Level Decrease at Metaphase–Anaphase I Transition Requires In Vivo Separin Activity

(A) Nuclear division (scored by DAPI staining) is blocked at 34°C in strain *K8684* (SK1 MATα/a *UBR1 esp1-2* REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4), carrying the thermosensitive *esp1* allele *esp1-2*. 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) Western blot analysis of Rec8-HA3 protein levels at 34°C in an *ESP1 ubr1*Δ (*K9154*) and in an *esp1-2 ubr1*Δ background (*K9158*: SK1 MATa/α *esp1-2* REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1). In *esp1-2*, full-length Rec8 accumulates even at the latest time points, while in the *ESP1* background, Rec8 protein levels start to decrease in correspondence of first meiotic division. Both cleavage products are generated in the wild type, while only a putative longer product is detectable at low levels after overexposure in *esp1-2*. Asterisk: antibody cross-reacting band.

(C) Chromosome spreads from strain *K8806* (*UBR1 ESP1*) and *K8684* (*UBR1 esp1-2*) sporulated at 34°C were immunostained for Rec8-HA3 and positive nuclei were counted for each time point.

ciently at 25°C but did so poorly, if at all, at 34°C (the highest temperature at which wild type sporulates efficiently). Some mutants, for example *esp1-2* and *esp1-4*, failed to sporulate at 34°C, whereas others, for example *esp1-1*, did so inefficiently and produced (at least on plates), a high proportion of two-spored asci (data not shown).

We analyzed in further detail the phenotype of an *esp1-2* diploid strain expressing an HA-tagged Rec8 protein. Premeiotic DNA replication, formation and dissolution of synaptonemal complex (monitored by Zip1 immunostaining of chromosome spreads [Sym et al., 1993; Dong and Roeder, 2000]), and assembly of meiosis I spindles (scored by in situ immunofluorescence using anti-tubulin antibody) took place with similar kinetics in wild-type and *esp1-2* cells at 34°C (data not shown). However, the mutant cells failed both to divide their nuclei (Figure 3A) and to elongate their spindles at the first division (data not shown). Western blotting showed that the disappearance of full-length Rec8 protein was greatly delayed and that the production of 38.5 kDa and 41.4 kDa cleavage fragments was greatly reduced in the mutant cells (Figure 3B). Furthermore, analysis of chromosome spreads immunostained for Rec8-HA3 showed that Rec8 failed to dissociate from chromosomes in *esp1-2* mutant cells (Figure 3C). Despite these defects, the lack of separin activity in *esp1-2* mutants does not seem to arrest the meiotic process, as the meiosis I spindles eventually break down and cells attempt to form two, albeit abnormal, meiosis II spindles and eventually form abnormal spores, which often lack DNA (data not shown). The mutant cells never form viable spores. Our data indicate that both Rec8's site-specific cleavage and its dissociation from chromosomes during meiosis I depend on separin activity.

Rec8 Cleavage Is Required for Homolog's Disjunction

To address whether the failure of separin mutants to undergo meiosis I might be due to their failure to cleave Rec8, we investigated the meiotic phenotypes of strains expressing cleavage site mutant proteins. Meiosis appeared unaffected by mutations that abolished cleavage solely at the 431 site (428R431E) or at the 453 site (453E) (Figure 2B, panels 428R431E and 453E), even when the mutations were homozygous; that is, when the mutant proteins were the only form of Rec8 made by the cell.

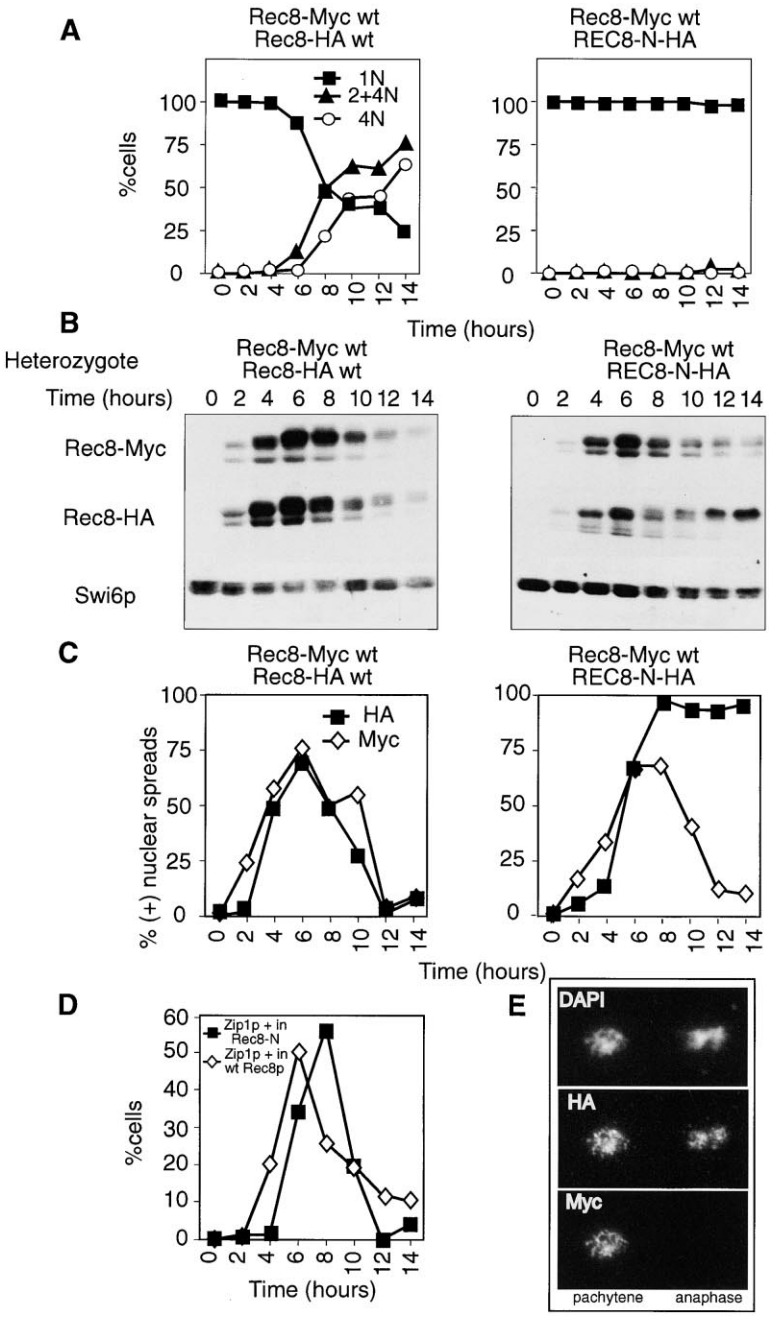

Figure 4. Uncleavable Rec8 (REC8-N) Causes a Dominant Segregation Block

(A) Nuclear division during a meiotic time course (scored by DAPI staining) in a strain heterozygous for wild-type Rec8 tagged with Myc and HA epitopes (*K8971*: SK1 MATa/α REC8 wt-Myc9::ura3 REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4) and in a strain heterozygous for wild-type Rec8-Myc9 and REC8-N-HA3 (*K8970*: SK1 MATa/α, REC8wt-Myc9::ura3 REC8-N-H3-LEU2::*rec8*Δ::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) Western blot analysis of Rec8 protein levels during a meiotic time course in strains *K8970* and *K8971*. Swi6 was used as loading control.

(C) Chromosome spreads were immunostained for Rec8-Myc9 wild type and for REC8-N HA3. Chromosomes positive for Myc and for HA were counted for each time point.

(D) Synaptonemal complex formation was assayed on chromosome spreads by immunostaining for Zip1 (see Experimental Procedures).

(E) Chromosome spreads on strain *K8970* were double-immunostained for Rec8-Myc9 wild type (FITC) and for REC8-N-HA3 (Cy3, see Experimental Procedures).

Neither "single" mutation had any significant effect on kinetics of meiotic divisions (Figure 2B), efficiency of sporulation, or viability of spores produced (data not shown). The somewhat sluggish division of the 453E mutant (Figure 2B, 453E) was not seen in cells with a wild-type *UBR1* gene and was probably due to slow entry into the meiotic program. In contrast, mutation of both sites (428R431E + 453E, called *REC8-N*) completely blocked both meiotic divisions (Figure 2B, panel REC8-N), even when heterozygous (i.e., when one *REC8* gene in diploids was mutant and the other was wild type; see Figure 4A). We conclude that proteolysis at either one of the two cleavage sites is both necessary and sufficient for meiotic chromosome segregation.

We next compared diploid cells in which a noncleavable Rec8 protein was tagged with HA epitopes and a wild-type Rec8 protein was tagged with Myc to diploid cells expressing HA- and Myc-tagged wild-type proteins. HA- and Myc-tagged wild-type proteins were degraded and disappeared from chromosomes with similar kinetics (Figures 4B and 4C, panels Rec8-Myc wt/Rec8-HA wt). In contrast, the noncleavable HA-tagged Rec8 failed both to be degraded and to dissociate from chromosomes, even when Myc-tagged wild-type protein expressed by the same cells had done so with normal kinetics (Figures 4B and 4C, panels Rec8-Myc wt/REC8-N-HA; Figure 4E). These data imply that cleavage of Rec8 is needed for its disappearance from chromo-

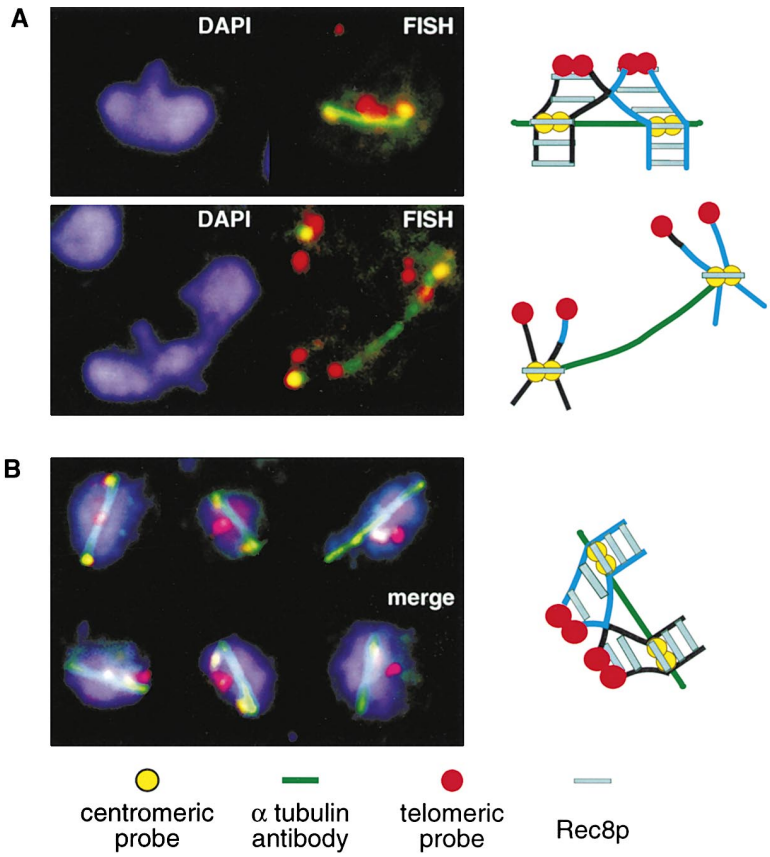

Figure 5. Uncleavable Rec8 Blocks Homologous Chromosomes' Disjunction

(A) FISH analysis of meiotic chromosome *XI* in a wild-type background (*K8806*) at 5 hr. The upper panel shows a metaphase I cell: the homologous centromeres (yellow signals) have already reached the spindle pole bodies, but the homologous telomeres still cluster in the medial region. The lower panel shows an anaphase I cell. Homologous telomeres are now separated. In addition, sister telomeres are apart too, confirming that homologs' separation is coincident with loss of cohesion along sister chromatid arms. A schematic model of this interpretation is drawn at the side of the pictures.

(B) FISH analysis of meiotic behavior of chromosome *XI* in a REC8-N background (*K8816*) at 12 hr. All cells show a single DNA mass (blue) or metaphase I or anaphase I A spindles. Homologous centromeres have separated (yellow signals), but telomeres (red signals) stay clustered.

some arms at the first meiotic division. However, the persistence of noncleavable Rec8 protein on chromosomes does not affect cleavage and dissociation of wild-type protein from the very same chromosomes. The presence of noncleavable protein also had little or no effect on the formation and dissolution of synaptonemal complexes, as monitored by Zip1 staining of chromosome spreads (Figure 4D), on formation of meiosis I spindles, or on the production of recombinant DNA molecules at the *LEU2* locus, which was measured using a diploid heterozygous at this locus for a restriction fragment polymorphism (data not shown) (Storlazzi et al., 1995; Xu and Kleckner, 1995).

Diploids expressing noncleavable Rec8-HA3 formed metaphase I spindles with similar kinetics to those expressing wild-type protein (data not shown). Fluorescence in situ hybridization (FISH) with probes specific for the centromere and telomere of the left arm of chromosome *XI* showed that centromeric regions of homologs had usually segregated to the poles in cells with metaphase spindles (Figure 5A, upper panel, yellow signals). In contrast, the distal portion of chromosome arms remained tightly associated, usually in the vicinity of the midline between the spindle poles (Figure 5A, upper panel, red signal). In wild-type cells, cohesion between sister chromatids along chromosome arms is lost soon after disjunction of homologs and extension of the meiosis I spindle (Figure 5A, lower panel, red signals). However, this process did not take place in cells expressing noncleavable Rec8. While centromeric regions (yellow) usually segregated to the spindle poles, sister chromatid arms not only remained paired but also failed to disjoin from their homologous partner (Figure 5B, single red signal). In summary, the phenotype of cells expressing noncleavable Rec8 (even in the presence of wild-type protein) resembles that of cells lacking separin activity. This raises the possibility that one, if not the only, crucial function of separin during meiosis I is to cleave Rec8, either at site 431 or at site 453.

Mutation of *SPO11* Restores Meiosis I in *esp1-2* Mutants and in Mutants Expressing Noncleavable Rec8

If cleavage of Rec8 by separin were necessary for separating sister chromatid arms and thereby for resolving chiasmata, then the lack of chromosome segregation at meiosis I in mutants either lacking separin activity or expressing noncleavable Rec8 should be suppressed by eliminating recombination. To test this, we analyzed the consequences of deleting *SPO11*. The endonuclease encoded by this gene generates the double strand breaks that initiate recombination during prophase (Bergerat et al., 1997; Keeney et al., 1997). *spo11*Δ mutants neither pair nor synapse homologous chromosomes during pachytene (Giroux et al., 1989; Loidl et al., 1994; Weiner and Kleckner, 1994; Rockmill et al., 1995; Cha et al., 2000) nor produce the chiasmata that hold homologs together during their alignment on the metaphase I spindle. Despite this crucial deficiency, *spo11*Δ mutants nevertheless form an apparently normal meiosis I spindle

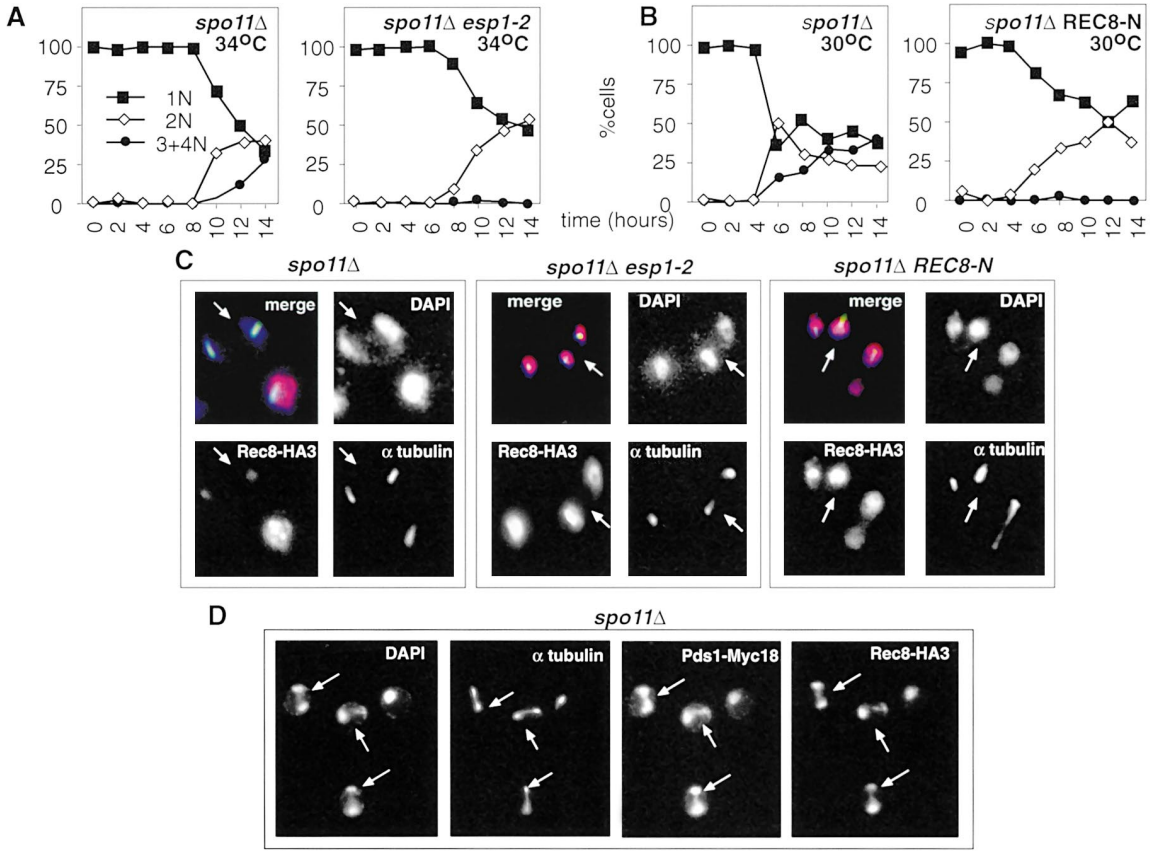

Figure 6. *spo11*Δ Rescues First Nuclear Division Block in REC8-N and in *esp1-2* Background

(A) The left panel shows progression of nuclear division (scored by DAPI staining of cells) in a *spo11*Δ *ESP1* background (*K8975*: SK1 MATa/α *spo11*Δ:: ura3 REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4*)* at 34°C. The right panel shows the same in *spo11*Δ *esp1–2* background (*K8976*: SK1 MATa/α *esp1–2 spo11*Δ::URA3 REC8-HA3wt-LEU2::*rec8*Δ::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) The left panel shows nuclear division progression (scored by DAPI staining) in a *spo11*Δ *REC8* background (*K8980*: SK1 MATa/α *spo11*Δ::URA3 heterozygous REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4, *rec8*Δ::kanMX4*)* at 30°C. The right panel shows the same in *spo11*Δ *REC8-N* (*K8979*: SK1 MATa/α *spo11*Δ::URA3 heterozygous REC8-N-HA3-LEU2::*rec8*Δ::kanMX4, *rec8*Δ::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(C) In situ immunofluorescence to visualize Rec8-HA3 (mouse anti-HA 16B12, see Experimental Procedures) and spindles (rat α tubulin, see Experimental Procedures). The left panel shows a metaphase II cell (indicated by the arrow) in *spo11*Δ (*K8975)*; at this stage, the majority of the cells (71%) retain only centromeric Rec8 (see Rec8-HA3 panel). In *spo11*Δ *esp1-2* (*K8976*, central panel) the arrow indicates metaphase II cells still positive for Rec8 staining (89.7% of the cells, see Rec8-HA3 panel). The right panel shows a metaphase II cell (indicated by the arrow) in *spo11*Δ *REC8-N* (*K8979*, right panel); 94% of metaphase II cells (one example is indicated by the arrow) are still positive for Rec8 staining (see Rec8-HA3 panel)

(D) Pds1-Myc18 levels were scored by in situ immunofluorescence (FITC, see Experimental Procedures) in a *spo11*Δ background (right panel, *K9096*: SK1 MATa/α *spo11*Δ:: ura3 REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4, heterozygous for PDS1-Myc18::TRP1). The arrows indicate anaphase I cells. Rec8-HA3 (Cy3, see Experimental Procedures) and α tubulin staining (Cy5, see Experimental Procedures) are shown too.

and segregate homologous chromosomes at random to the two spindle poles (Klapholz et al., 1985). Having segregated homologs at random at the first meiotic division, *spo11*Δ mutants proceed with an apparently normal second meiotic division, during which they segregate sister chromatids to opposite poles.

We found that deletion of *SPO11* fully relieved the lack of chromosome segregation during meiosis I of mutants either lacking separin activity (*esp1-2*) (compare Figures 3A and 6A, right panels) or expressing noncleavable Rec8 protein (REC8-N) (compare Figure 2B, REC8-N panel and Figure 6B, right panel). FISH with probes for the centromere and left telomere of chromosome *XI* confirmed that *spo11*Δ *esp1-2* and *spo11*Δ *REC8-N* double mutants segregated homologs randomly during meiosis I with efficiencies and kinetics that resembled that of *spo11*Δ single mutants (data not shown). However, despite forming meiosis II spindles, the double mutants neither separated sister chromatids nor segregated chromosomes during what should have been meiosis II.

Deletion of *SPO11* did not, however, relieve the failure of *esp1-2* or *REC8-N* mutants to remove Rec8 from chromosomes at the first meiotic division. In situ immunofluorescence of fixed cells shows that Rec8-HA3 is reduced in abundance in most (i.e., 71%) *spo11*Δ metaphase II cells and is predominantly associated with centromeric regions (Figure 6C, left panel). In contrast, high

levels of Rec8 persisted throughout the nuclei of most metaphase II *spo11Δ REC8-N* (94%) (Figure 6C, right panel) and *spo11Δ esp1-2* (89.4%) (Figure 6C, central panel) double mutant cells.

During these studies, we discovered that most (75%) *spo11Δ* cells undergo the first meiotic division before destruction of Pds1 securin (Figure 6D). Furthermore, FISH analysis showed that sister telomeres separated during anaphase I in no more than 23% of *spo11Δ* mutant cells (data not shown). This contrasts with wild-type cells in which meiosis I is invariably associated with loss of arm cohesion (see Figure 5A) and where securin destruction always precedes the onset of anaphase I (Salah and Nasmyth, 2000). Because securin is a potent inhibitor of separin, this finding confirms that cleavage by separin is only needed for disjoining homologs if chiasmata have previously been produced by Spo11. Finally, our experiments on *spo11Δ* mutants imply that the failure of *esp1-2* and *REC8-N* mutants to segregate homologs at meiosis I cannot be due to a defective meiosis I spindle.

Discussion

It has long been suspected that sister chromatid cohesion along chromosome arms might have a crucial role in holding homologous chromosomes together following reciprocal exchange between maternal and paternal chromatids (Moore and Orr-Weaver, 1998). Cytological studies in a wide variety of organisms have shown that sister chromatids remain tightly paired throughout diakinesis and metaphase I but suddenly separate at the onset of anaphase I. Loss of cohesion along chromosome arms might even be the trigger that resolves chiasmata and thereby promotes segregation of homologous chromosomes to opposite poles at the first meiotic division. This hypothesis has, however, been difficult if not impossible to test in a rigorous manner without knowing about the molecules that mediate sister chromatid cohesion during meiosis I.

The starting point of the work described in this paper was the recent recognition that meiotic sister chromatid cohesion depends on a meiotic variant of the cohesin complex required for mitotic sister chromatid cohesion. Sister separation during mitosis depends on cleavage of cohesin's Scc1 subunit (Uhlmann et al., 1999) by an endopeptidase called separin or separase. During meiosis, Scc1 is replaced by a variant called Rec8 (Klein et al., 1999), which contains two potential separin cleavage sites. Our current work shows that both of these sites are indeed recognized by separin in vitro and are cleaved around the time of the first meiotic division in vivo. Mutant diploids expressing *REC8* genes lacking either one or the other cleavage site undergo meiosis normally but diploids expressing even only a single copy of a *REC8-N* gene (coding for uncleavable Rec8) fail to segregate chromosomes at either division, despite forming normal-looking meiotic spindles. The phenotype of these mutants resembles that of separin (*esp1-2*) mutants, which also fail to resolve chiasmata at meiosis I. The lack of chromosome segregation during meiosis I due to the expression of noncleavable Rec8 or due to separin inactivation is largely if not completely bypassed by eliminating recombination through deletion of the *SPO11* gene. Indeed, anaphase I takes place precociously in *spo11Δ* mutants and does so in the presence of high levels of separin's inhibitor, securin (Pds1). Thus, Rec8 cleavage and separin activity are only required for chromosome segregation during meiosis I if maternal and paternal chromatids have recombined and formed chiasmata. The simplest interpretation of these results is that sister chromatid cohesion (mediated by a Rec8-containing cohesin complex) distal to crossovers does indeed hold homologs together during metaphase of meiosis I and that this chiasmata linkage is resolved by cleavage of Rec8 by separin (Figure 7).

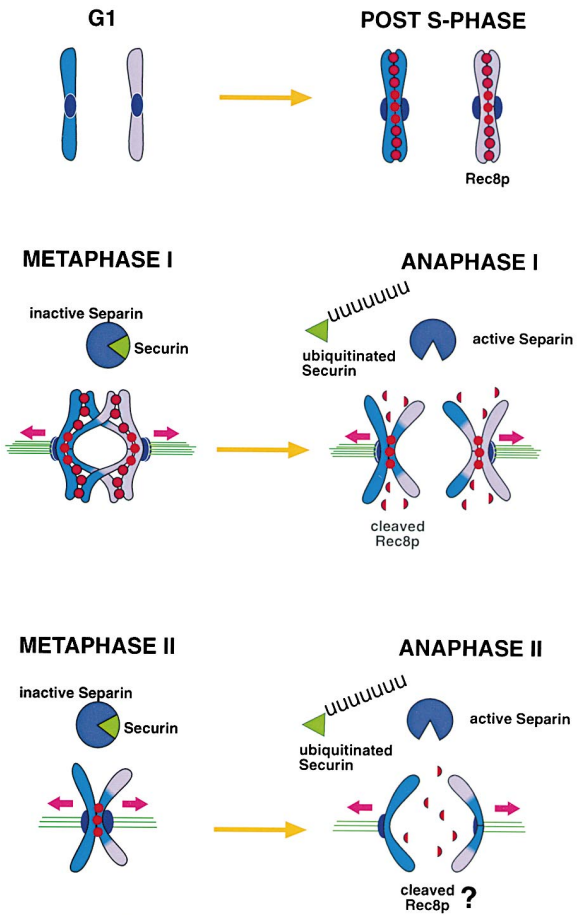

Figure 7. Separin-Dependent Rec8 Cleavage Is Required for First Meiotic Division

Sister chromatid cohesion is established during S-phase. During prophase, recombination takes place. As a consequence of this process, homologous chromosomes are held together from the sister chromatid cohesion complex (red spheres). At metaphase I, the securin Pds1 (green triangle) is ubiquitinated in a Cdc20-dependent manner. Its degradation activates the separin Esp1 (blue sphere). Esp1 cuts Rec8 in a site-specific manner along the arms, leaving homologous chromosomes free to move towards the opposite pole of the spindle. Centromeric Rec8 is protected from the cleavage by an unknown mechanism and persists at the centromere until metaphase–anaphase II transition. The mechanism of Rec8 removal from the centromere is still unknown; it is reasonable to hypothesize the involvement of Esp1 in the metaphase–anaphase II transition, too.

Rec8 Cleavage by Separin?

The crux of this paper is the claim that Rec8 is cleaved by separin during meiosis I. The evidence can be sum-

marized as follows: (1) Rec8 protein is degraded around the time of meiosis I. (2) Rec8 contains two sites with strong resemblance to Scc1's separin cleavage sites (indeed, these are the best two matches in the entire yeast proteome). (3) Both sites are indeed cleaved by separin in vitro, when presented either on mitotic or meiotic chromosomes. (4) Rec8 cleavage products are produced in large amounts around the time of Rec8 degradation during meiosis I. (5) Mutation of each site individually blocks cleavage at that site but not at the other, whereas mutation of both sites blocks degradation of the mutated Rec8 but not wild-type protein expressed in the same cell. (6) Separin mutants also fail to cleave and degrade Rec8 at meiosis I. (7) Securin (Pds1), which is known to inhibit separin activity, is degraded at around the same time that Rec8 degradation commences, shortly before the onset of anaphase I. (8) In the absence of recombination, cells undergo the first meiotic division prematurely and do so in the presence of high levels of securin. These data are all consistent with the notion that Rec8 degradation during meiosis I is due to cleavage by separin, which is activated by the destruction of securin. The products of Rec8 cleavage never accumulate to high levels in a *UBR1* background but are stabilized in *ubr1*Δ mutant cells, indicating that Rec8 cleavage products are rapidly targeted to the 26S proteosome destruction via the N-end rule ubiquitination pathway.

Rec8 Cleavage by Separin Is Needed for Chromosome Segregation

Expression of a form of Rec8 mutated at both cleavage sites (428R431E + 453E, called REC8-N) completely blocks meiotic chromosome segregation even when cells express equal amounts of wild-type Rec8 protein. A crucial question is whether this dominant phenotype is due to a lack of proteolytic cleavage or to an unforeseen side effect on an as yet unknown function of Rec8. Rec8 does indeed have multiple functions. It is required for sister chromatid cohesion, for the formation of synaptonemal complex (Klein et al., 1999; Parisi et al., 1999), for efficient recombination between homologs (Ponticelli and Smith, 1989; Krawchuk et al., 1999), and possibly even for rapid premeiotic DNA replication (Cha et al., 2000). *REC8-N* 's complete dominance contrasts with the lack of any phenotype caused by mutation of each "cleavage" site individually even when these single mutations are homozygous. Both the dominance of *REC8-N* and the silence of single site mutations are readily explained if the function of the mutated sequences is merely to serve as cleavage sites and if cleavage at either one of the two sites is necessary to destroy the cohesin connections that link sister chromatids. In contrast, neither phenomenon can be readily explained by the notion that the two sequences are in fact required for some other Rec8 activity needed for chromosome segregation. As far as we can tell, no function of Rec8 other than its susceptibility to separin cleavage is detectably altered by the *REC8-N* mutation. We therefore suggest that the main if not sole effect of the *REC8-N* mutation is to prevent proteolytic cleavage of those molecules expressed from the mutant locus and that these molecules persist in holding sister chromatids together even when an equivalent number of wild-type Rec8 molecules are degraded on schedule.

Mutation of both Rec8 cleavage sites has at least three effects on chromosome behavior. In wild-type cells, dissociation of Rec8 from chromosome arms, chiasmata resolution, and loss of arm sister chromatid cohesion all occur around the time that cells extend meiosis I spindles. However, none of these three events take place in cells that express noncleavable Rec8 nor, indeed, in *esp1-2* mutant cells.

Neither *REC8-N* nor the *esp1-2* mutations affect the attachment of sister centromeres to meiotic spindles and the segregation of maternal and paternal centromere pairs to opposite poles. In contrast, both mutations prevent the disjunction of homologous arm sequences. Disjunction of centromeres, but not of chromosome arms, suggests that mutants expressing REC8-N cannot resolve chiasmata. This suggests that the lack of chromosome segregation cannot be attributed to a defect in spindle function. Indeed, when recombination is eliminated by deleting *SPO11*, the spindles of *REC8-N* and *esp1-2* mutant cells are capable of segregating homologous chromosomes to each pole, albeit in a random manner. We therefore propose that the resolution of chiasmata (at least in yeast) is mediated by cleavage of Rec8 by separin. Rec8 is necessary for sister chromatid cohesion during meiosis and can even substitute Scc1 in this function during mitosis. It is therefore reasonable to suppose that homologs are held together from prophase until metaphase I primarily, if not exclusively, by cohesion between sisters distal to crossovers, which is mediated by a Rec8-containing cohesin complex. If so, cleavage of Rec8 by separin would trigger homolog disjunction by destroying sister chromatid cohesion. We cannot, however, exclude at this juncture the alternative possibility that the Rec8 cohesin complex mediates the linkage between homologs by participating in a special structure (Maguire's chiasma binder [Maguire, 1974]), which is situated not on chromosome arms but at crossover sites themselves.

Is Cleavage of Rec8 along Chromosome Arms Needed for Meiosis I in Animal Cells?

There is currently some uncertainty whether cleavage of Scc1 during mitosis triggers the separation of sister chromatids at the metaphase to anaphase transition in animal cells. Cleavage of something (possibly of residual cohesins) is presumably required because separin is conserved in all eukaryotes, but it is still unclear whether Scc1 is its target. The reason for this uncertainty is that the bulk of cohesin dissociates from animal cell chromosomes during prophase and prometaphase (Losada et al., 1998) and only small amounts remain associated with metaphase chromosomes (Waizenegger et al., 2000 [this issue of *Cell*]). Furthermore, when securin destruction is inhibited either by triggering the Mad2 chromosome alignment surveillance mechanism (Rieder and Palazzo, 1992) or by inactivating APC/cdc20 (Rieder and Cole, 1999), chromosome arms, though not centromeres, fully separate, presumably in the absence of any separin activity (Nasmyth et al., 2000). The implication is that sister chromatid cohesion along chromosome arms, though possibly not that at centromeres, can be dissolved by dissociation of cohesin from chromosomes by a process that does not involve proteolysis of Scc1. This raises the question whether a similar separin-inde-

pendent pathway exists in meiotic cells and, if so, whether it and not separin mediates homolog disjunction during meiosis I. It will therefore be of some interest to establish whether cleavage of Rec8 by separin is also needed for meiosis I in animal cells.

What Is the Fate of Rec8 at Centromeres?

Our data suggest that the bulk of Rec8 is cleaved at the first meiotic division and that this event triggers Rec8's dissociation from chromosome arms. Meanwhile, a small pool of Rec8 protein persists in the vicinity of centromeres until the second meiotic division and disappears at the onset of anaphase II (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). The fraction of Rec8 persisting at centromeres is too low and the synchrony of meiosis too poor to address directly whether cleavage of centromeric Rec8 is delayed until anaphase II. However, persistence on chromosomes very possibly reflects lack of cleavage. In which case, we propose that Rec8 protein in the vicinity of centromeres is specifically protected from separin during the first meiotic division, loses this protection soon after the reaccumulation of securin following meiosis I, and is cleaved by separin upon securin's destruction at the onset of anaphase II (Figure 7). Because cleavage of Scc1 is sufficient to trigger anaphase during mitosis (Uhlmann et al., 2000 [this issue of *Cell*]), we suggest that cleavage of Rec8 on chromosome arms triggers anaphase I, whereas cleavage of Rec8 at centromeres triggers anaphase II. Indeed, the notion that both meiotic divisions might be triggered by the same enzyme, namely separin, is consistent with observations in grasshoppers, showing that meiosis I bivalents transferred to the spindles of meiosis II cells disjoin at the same time as endogenous sister chromatids and that sister chromatids from meiosis II cells separate at the same time as meiosis I bivalents when transferred to the spindles of meiosis I cells (Nicklas, 1977).

Cleavage of Cohesins by Separins: A General Mechanism for Triggering Sister Separation

Despite the conservation of separins and Scc1-like cohesin subunits, there has thus far been no direct evidence that proteolytic cleavage of cohesin subunits might be a universal mechanism for separating sister chromatids. It has been hard to spot separin cleavage sites in animal Scc1 proteins. Furthermore, cohesin's dissociation from chromosomes during prophase in animal cells suggests that only small amounts remain on metaphase chromosomes where they might be subject to cleavage by separin. The discovery that cleavage of Rec8 is crucial for the resolution of chiasmata therefore represents concrete evidence that cleavage of cohesins by separin might be a universal mechanism for separating sister chromatids at the metaphase to anaphase transition. Most, if not all, the major hallmarks of meiotic cell divisions are conserved among eukaryotic organisms. In which case, the use of Scc1 and Rec8 cleavage to separate sister chromatids during mitosis and meiosis, respectively, may have existed in the common ancestor of all meiotic organisms.

Experimental Procedures

Plasmids and Yeast Strains

REC8-HA3 gene and its promoter (333 bp upstream of start codon) were amplified from genomic DNA of strain K8033 (SK1 MATa/α REC8-HA3::URA3) (Klein et al., 1999) and were cloned into YIplac128 (Gietz and Sugino, 1988). All *REC8* mutants were obtained by exchanging restriction fragments from *REC8* (NcoI-BglII sites) with PCR fragments obtained using primers containing the desired nucleotide changes.

All strains are derivatives of SK1, *rec8*Δ (K8079 SK1 MATa and K8081 MATα, *rec8*Δ::kanMX4) (Klein et al., 1999). After linearization, using the MluI site within the promoter, the constructs were integrated into the *REC8* locus, upstream of the deletion. Diploids were obtained by performing independently the integration into MATα and MATa strains and subsequently crossing them, or by diploidizing by transforming the haploid with an *HO*-expressing plasmid.

The *ubr1*Δ was obtained by PCR-mediated gene replacement (Wach et al., 1994), replacing the complete sequence of the ORF (positions +20 to 23 bp upstream the stop codon) with the *TRP1* marker.

The *esp1-2* allele was recovered from strain K8493 (*esp1-2*) using a gap repair strategy (Guthrie and Fink, 1991). The recovered allele was subcloned into the URA3-integrating vector pRS306 (Sikorski and Hieter, 1989). Following plasmid linearization the allele was transferred into the SK1 strain K8812 (SK1 MATα REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4) by transformation and 5-FOA counter-selection (Guthrie and Fink, 1991). The resultant temperature-sensitive strain K8674 (SK1 MATα *esp1-2* REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4) was diploidized by transformation with a plasmid containing the *HO* gene, generating the *esp1–2* homozygous diploid K8684. Transformation with a plasmid containing the wild type *ESP1* gene (c3944) rescued both the mitotic and meiotic temperature-sensitivity of this strain.

Sporulation Procedures

Strains were streaked onto YPG (glycerol) plates from stocks in 15% glycerol stored at −80°C and grown for 60 hr at 25°C. A single colony was patched on YPD and grown for 48 hr. Cells were inoculated in liquid YEPA 2% (2% bactopeptone, 1% yeast extract, and 2% potassium acetate) and grown for 10 hr to stationary phase, then inoculated into YEPA 1% (2% bactopeptone, 1% yeast extract, and 1% potassium acetate) and grown overnight, to ~3 OD/ml. They were subsequently washed with potassium acetate 2% (SPO medium) and incubated for 14–24 hr in SPO medium to a density of ~3–5 OD/ml. All the meiotic experiments were conducted at 30°C, with the exception of *esp1-2* experiments (shifted at 34°C after 2 hr in SPO medium at 25°C).

Chromosome Spreading

Chromosome spreading was performed according to procedures described previously (Nairz and Klein, 1997; Loidl et al., 1998). To detect Rec8-HA3, mouse 12CA5 or 16B12 (Babco) antibodies were used at 1:1200 and 1:600. The secondary antibody was anti-mouse Cy3 at 1:1000 (CHEMICON). Zip1 has been detected by rabbit antibody kindly provided by Shirleen Roeder. Goat anti-rabbit FITC 1:100 (CHEMICON) was used as secondary antibody. Rec8-Myc9 was detected by rabbit anti-Myc 1:200 (Gramsch) and goat anti-rabbit-FITC 1:50 (CHEMICON).

In Vitro Assay for Rec8 Cleavage by ESP1

In vitro cleavage of Rec8 was performed as described for Scc1 (Uhlmann et al., 1999). The chromatin substrate was prepared from 40–60 OD of meiotic cells after 6 hr in SPO medium.

Western Blotting

Cell extracts were prepared by cell breakage with glass beads into 2× protein loading buffer, preceded and followed by 5 min boiling. Equal amounts of protein were analyzed by SDS-PAGE and blotting according to standard procedures (Sambrook et al., 1989). The HA-epitope tag was detected by 16B12 mouse antibody 1:10000 and the Myc-tag was detected by mouse 9E10 antibody 1:200. Rabbit anti-Swi6 antibody has been diluted 1:100000 (Klein et al., 1999).

In Situ Immunofluorescence

The in situ immunostaining was performed according to Piatti et al. (1996). α-tubulin staining was obtained using a rat antibody (Serotec) 1:100. The secondary antibody was either goat anti-rat FITC 1:100 or donkey anti-rat Cy5 (CHEMICON) 1:50. Pds1-Myc18 was detected by rabbit anti-Myc 1:200 (Gramsch) and goat anti-rabbit-FITC 1:50 (CHEMICON).

Immunostaining and FISH

Cells were prepared according to the semispreading procedure described previously in Jin (2000). Spindles were immunolabeled with YOL1/34 monoclonal rat anti-yeast tubulin antibody (Kilmartin et al., 1982) (Serotec) and FITC-conjugated secondary antibody (SIGMA) according to a standard protocol (Pringle et al., 1991). For subsequent fluorescent in situ hybridization (FISH), the cells were postfixed for 10 min in 4% paraformaldehyde at room temperature. For labeling the centromeric region of chromosome XI, two overlapping cosmid clones (pUKG041, pEKG021) covering a 47 kb tract (including the centromere) were chosen. The telomere of the left arm of the same chromosome was marked by two overlapping cosmid clones (pUKG040, pEKG086) covering a region of 48 kb three kilobase pairs away from the physical end. All cosmid clones were kindly provided by Bernard Dujon (Thierry et al., 1995). The centromeric probe was labeled with Cy5-dUTP (Amersham) and the telomere-proximal probe with Cy3-dUTP (Amersham) using a standard nick translation protocol (see Loidl et al., 1998). FISH was performed according to Jin (2000).

Acknowledgments

We are grateful to Franz Klein for yeast strains and many helpful discussions, to Mark Winey who kindly provided the *esp1-2* allele, to Alex Varshavsky, whose unpublished data on the role of Ubr1 in mediating proteolysis of Scc1 cleavage fragments encouraged us to test its role during meiosis, and to J. M. Peters for his critical comments on the manuscript. We thank Shirleen Roeder for the Zip1 antibody, H. Tkadletz for help with figures, and P. Steinlein for the microscopy support. S. C. B. B. is supported by the TMR Network; J. L. and J. F. are supported by FWF project S8202; F. U. is supported by an EMBO long-term fellowship; and R. K. C. is supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship. This research was supported by Boehringer Ingelheim and by the Austrian Industrial Research Promotion Fund.

Received June 21, 2000; revised July 31, 2000.

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