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Ctp1^{CtIP} and Rad32^{Mre11} Nuclease Activity Are Required for Rec12^{Spo11} Removal, but Rec12^{Spo11} Removal Is Dispensable for Other MRN-Dependent Meiotic Functions$^{\nabla}$

Edgar Hartsuiker,[1]* Kenichi Mizuno,[1] Monika Molnar,[2] Juerg Kohli,[2] Kunihiro Ohta,[3] and Antony M. Carr[1]

Genome Damage and Stability Centre, Sussex University, Brighton BN1 9RQ, United Kingdom[1]; Institute of Cell Biology, Baltzerstrasse 4, CH-3012 Bern, Switzerland[2]; and Department of Life Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan[3]

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The evolutionarily conserved Mre11/Rad50/Nbs1 (MRN) complex is involved in various aspects of meiosis. Whereas available evidence suggests that the Mre11 nuclease activity might be responsible for Spo11 removal in *Saccharomyces cerevisiae*, this has not been confirmed experimentally. This study demonstrates for the first time that Mre11 (*Schizosaccharomyces pombe* Rad32^{Mre11}) nuclease activity is required for the removal of Rec12^{Spo11}. Furthermore, we show that the CtIP homologue Ctp1 is required for Rec12^{Spo11} removal, confirming functional conservation between Ctp1^{CtIP} and the more distantly related Sae2 protein from *Saccharomyces cerevisiae*. Finally, we show that the MRN complex is required for meiotic recombination, chromatin remodeling at the *ade6-M26* recombination hot spot, and formation of linear elements (which are the equivalent of the synaptonemal complex found in other eukaryotes) but that all of these functions are proficient in a *rad50S* mutant, which is deficient for Rec12^{Spo11} removal. These observations suggest that the conserved role of the MRN complex in these meiotic functions is independent of Rec12^{Spo11} removal.

In meiosis, one round of DNA replication is followed by two nuclear divisions that divide the genetic material equally over four haploid daughter cells. Meiotic recombination contributes to genetic diversity and is essential for correct disjunction of the homologous chromosomes in the first meiotic division. In meiotic prophase, after meiosis-specific DNA replication, the homologous chromosomes pair and recombine. In the following two nuclear divisions, the homologous chromosomes (meiosis I) and the sister chromatids (meiosis II) are segregated. The study of meiosis in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has greatly contributed to our understanding of various meiotic processes. Because these model organisms are as distantly related to each other as to animals (35), detailed studies of similarities and differences between meiotic mechanisms in these yeasts are informative as to which mechanisms are conserved in higher eukaryotes.

The evolutionarily conserved Mre11/Rad50/Nbs1 (MRN) protein complex is involved in a wide range of early responses to DNA damage. Mutations in Nbs1 and Mre11 are responsible for the cancer-prone human disorders Nijmegen breakage syndrome and ataxia telangiectasia-like disorder. Central in this complex is the Mre11 nuclease, which is thought to be involved in double-strand break (DSB) end resection and DSB signaling (reviewed in reference 40).

The MRN complex is also involved in multiple aspects of meiosis. In *S. cerevisiae*, meiotic recombination is initiated by the topoisomerase-like protein Spo11 (17), which creates a DSB in the DNA. Spo11 remains covalently bound to the 5′ ends of the break (17) and is removed by endonucleolytic cleavage (28) to initiate subsequent DSB end resection and meiotic recombination. Meiotic DSB formation is abolished in *S. cerevisiae* MRN null mutants (6, 16). In an *S. cerevisiae rad50S* point mutant (a separation-of-function mutant with severe defects in meiosis but only mild defects in mitotic DNA repair) (2), meiotic DSBs are formed, but this mutant is unable to remove Spo11 from meiotic DSB ends (17). This observation has implicated the MRN complex in Spo11 removal. Since a nuclease-dead *mre11-D56N* mutant is defective in resecting meiotic DSBs (27), it has been proposed that the Mre11 nuclease activity is responsible for Spo11 removal, but this has not been confirmed experimentally. Also, in *S. pombe*, meiotic DSBs are formed by the Spo11 homologue, called Rec12 (7). An *S. pombe rad50S* mutant is defective in meiotic DSB repair, and this feature has been instrumental in the study of meiotic DSB formation in this organism (42). Although this phenotype is compatible with an involvement of the MRN complex in Rec12^{Spo11} removal, this has not been demonstrated experimentally.

Meiotic DSB formation in *S. cerevisiae* (30) is accompanied by an increase of micrococcal nuclease (MNase) sensitivity, suggesting that a more open chromatin structure could facilitate DSB repair. *S. cerevisiae* Mre11 is required for meiosis-specific chromatin remodeling, whereas Rad50 and Xrs2 (the *S. cerevisiae* Nbs1 homologue) are dispensable (29). Also in *S. pombe*, meiotic recombination hot spot activity at *ade6-M26* has been associated with increased MNase sensitivity (23), but a role of MRN in this process has not been reported.

In the great majority of sexually reproducing eukaryotes, a meiosis-specific tripartite structure is formed during meiotic

* Corresponding author. Mailing address: Genome Damage and Stability Centre, Sussex University, Brighton BN15 8HG, United Kingdom. Phone: 44 1273 873118. Fax: 44 1273 678121. E-mail: E.Hartsuiker@sussex.ac.uk.

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prophase; this is called the synaptonemal complex (SC) and is thought to be involved in various processes associated with chromosome pairing and recombination. Early in meiotic prophase, axial elements are formed along the sister chromatids of the individual chromosomes. Pairing and connection of the axial elements by transverse filaments lead to formation of the tripartite SC (in which the axial elements are now called lateral elements) (31). In *S. cerevisiae rad50*Δ and *rad50S* mutants, SC precursors are formed, but formation of a complete SC is blocked (2). In *S. pombe* wild-type (WT) cells, no fully formed SC is found, but instead linear elements (LEs) appear during meiotic prophase that show similarity to SC precursors in other organisms (4). It remains unknown if the MRN complex is involved in LE formation in *S. pombe*.

Mutants of *sae2* in *S. cerevisiae* have a *rad50S*-like phenotype in meiosis as well as in mitotic cells (32), and it has been shown that Sae2 is required for Spo11 removal in meiosis (28). Recently, a novel gene, *ctp1*, was identified in *S. pombe* (1, 21), and its product shows homology to the mammalian tumor suppressor CtIP (33) and the more distantly related Sae2 protein in *S. cerevisiae*. CtIP/Ctp1 has been shown to interact with the MRN complex (33) and is involved in DSB end resection (21, 33), but a role for $Ctp1^{CtIP}$ in $Rec12^{Spo11}$ removal in *S. pombe* has not been confirmed.

Whereas meiotic phenotypes of MRN and *sae2* mutants have been studied extensively in *S. cerevisiae*, much remains unknown about the role of the MRN complex and $Ctp1^{CtIP}$ in *S. pombe* meiosis. In this study, we characterize meiotic phenotypes of *S. pombe rad50* and $rad32^{mre11}$ null and separation-of-function mutants. First, we demonstrate, for the first time in any organism, that the nuclease activity of $Rad32^{Mre11}$ is required for $Rec12^{Spo11}$ removal. Second, we demonstrate that $Ctp1^{CtIP}$ is required for $Rec12^{Spo11}$ removal, confirming functional conservation between the distantly related *S. pombe* $Ctp1^{CtIP}$ and *S. cerevisiae* Sae2 proteins. Finally, we show that an *S. pombe rad50S* mutant has a defect in removing $Rec12^{Spo11}$ but is proficient for meiotic recombination (measured in surviving spores), chromatin remodeling at the *ade6-M26* recombination hot spot, and LE formation, whereas all of these functions are defective in the *rad50*Δ mutant.

MATERIALS AND METHODS

Yeast strains and techniques. For strain construction and propagation, standard genetic methods and media were used (11). Strains used and constructed in this study are listed in Table 1.

Previously published procedures. Measurement of meiotic spore viability and recombination (11), synchronization of meiotic cultures (4, 7), pulsed-field gel analysis (7), preparation of chromosome spreads and electron microscopy (4), and analysis of meiotic nucleosome remodeling at *ade6-M26* (23) were described previously.

DNA-linked protein detection assay. We developed a DNA-linked protein detection assay based on previously published procedures (17, 34). Premeiotic or meiotic cells (25 ml) were washed in 1 ml lysis buffer (8 M guanidine HCl, 30 mM Tris, 10 mM EDTA, 1% Sarkosyl, pH 7.5), resuspended in 750 μl lysis buffer, and lysed using glass beads (±0.8 g). The cell extract was incubated at 70°C for 15 min; these strongly denaturing conditions remove noncovalently bound proteins from the DNA. After clarification (15 min at 13,000 rpm in an Eppendorf centrifuge), one aliquot of extract was set aside for DNA quantification (see below), while the rest was loaded on a CsCl gradient consisting of 1-ml layers with densities of 1.82, 1.72, 1.50, and 1.45 g/ml. The gradients were centrifuged for 24 h at 30,000 rpm in a Sorvall AH650 rotor to separate the free proteins from the DNA.

TABLE 1. *S. pombe* strains used in this study

Strain[a]	Genotype
265	*h⁻ smt0 leu1-32 ura4-D18*
251	*h⁺ ura4-D18 leu1-32*
260	*h⁻ smt0 rad50::kanMX6 leu1-32 ura4-D18*
284	*h⁺ rad50::kanMX6 leu1-32 ura4-D18*
258	*h⁺ rad50-K81I leu1-32 ura4-D18*
259	*h⁻ smt0 rad50-K81I leu1-32 ura4-D18*
263	*h⁺ rec12-152::LEU2 leu1-32 ura4-D18*
264	*h⁻ smt0 rec12-152::LEU2 leu1-32 ura4-D18*
261	*h⁺ rad50::kanMX6 leu1-32 ura4-D18 rec12-152::LEU2*
262	*h⁻ smt0 rad50::kanMX6 leu1-32 ura4-D18 rec12-152::LEU2*
266	*h⁺ leu1-32 ura4-D18 rec12-152::LEU2 rad50-K81I*
267	*h⁻ smt0 leu1-32 ura4-D18 rec12-152::LEU2 rad50-K81I*
805	*h⁻ smt0 ura4-D18 rad32-D65N*
814	*h⁺ rad32-D65N ura4-D18*
810	*h⁻ smt0 ura4-D18 rad32-D65N rad50::kan*
811	*h⁺ ura4-D18 rad32-D65N rad50::kan*
812	*h⁻ smt0 ura4-D18 rad32-D65N rad50S*
813	*h⁺ ura4-D18 rad32-D65N rad50S*
352/354	*h⁻/h⁺ ade6-M216/ade6-M210 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50-K81I/rad50-K81I*
611	*h⁻ smt0 ade6-M26 pat1-114 rec12-6HA:kanMX6*[†]
617	*h⁻ smt0 pat1-114 ade6-M26 rec12-6HA:kanMX6 rad50-K81I*
649	*h⁻ smt0 ade6-M26 rec12-6HA:kanMX6 rad50::kanMX6 pat1-114*
418	*h⁺ mat1PD17::LEU2 leu1-32 arg6-1*
417	*h⁻ smt0 ade7-152*
411	*h⁺ mat1PD17::LEU2 leu1-32 rad50-K81I arg6-1*
412	*h⁻ smt0 ade7-152 rad50-K81I*
407	*h⁺ mat1P::LEU2 leu1-32 rad50::kanMX6 arg6-1*
408	*h⁻ smt0 rad50::kanMX6 ade7-152*
421	*h⁺ smt0 lys7-1 leu1-32*
422	*h⁺ mat1PD17::LEU2 leu1-32 ade2-17 ura2-10*
405	*h⁻ smt0 lys7-1 leu1-32 rad50::kanMX6*
406	*h⁺ mat1PD17::LEU2 ade2-17 ura2-10 leu1-32 rad50::kanMX6*
415	*h⁻ smt0 rad50-K81I lys7-1 leu1-32*
416	*h+ mat1PD17::LEU2 rad50-K81I leu1-32 ade2-17 ura2-10*
157	*h⁻ smt0 ade6-M26 pat1-114* *h⁺ ade6-M26 pat1-114 leu1-32* *h⁺ ade6-M26 rad50-kanMX6 pat1-114 leu1-32* *h⁺ ade6-M26 rad32::ura4 pat1-114 leu1-32* *h⁺ ade6-M26 rad50-K81I pat1-114 leu1-32*
207/209	*h⁻/h⁻ smt0/smt0 pat1-114/pat1-114 ade6-M210/ade6-M216 ura4-aim/ura4-aim ura4-D18/ura4-D18*
203/205	*h⁻/h⁻ smt0/smt0 pat1-114/pat1-114 ade6-M210/ade6-M210 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50::kanMX6/rad50::kanMX6*
148/152	*h⁻/h⁻ pat1-114/pat1-114 ade6-M210/ade6-M216 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50-K81I rad50-K81I*
808	*h⁻ smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26*
834	*h⁻ smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26 rad50::kan*
832	*h⁻ smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26 rad50S*
824	*h⁻ smt0 ctp1::kan pat1-114 rec12-6HA:kan*

[a] Numbers are from the strain collection of E. Hartsuiker. The *rec12-6HA-kanMX* strains were created in the lab of K. Ohta. Other unpublished strains/constructs for this study were created by E. Hartsuiker.

To ensure equal DNA loading, the DNA concentration in the extract was measured and this value was used to adjust the volume of the fractions loaded on the slot blot. For this purpose, the aliquots of extract which were set aside for DNA quantification were treated overnight with RNase (0.5 μg/ml), and the DNA concentration was determined fluorimetrically using PicoGreen (Molecular Probes/Invitrogen detection technologies). After centrifugation, the gradients were fractionated into 0.5-ml fractions and adjusted amounts were loaded onto a slot blot.

To detect the presence of covalently bound hemagglutinin-tagged Rec12^{Spo11} in the DNA fractions, the membrane was probed with a monoclonal antibody (sc-7392; Santa Cruz). The membrane was processed using standard Western blot procedures and visualized using chemiluminescence. Using this procedure, control cultures of untagged strains showed only slight cross-hybridization with the top two fractions (fractions 9 and 10) from the CsCl gradient, which contained the free proteins. These fractions did not contain any DNA, were difficult to load on a slot blot as they tended to clog the membrane, and were therefore not loaded for most experiments. Slot blots of premeiotic cells showed no Rec12^{Spo11} signal in any of the DNA-containing fractions (data not shown).

RESULTS

***S. pombe rad50S* mutant is temperature sensitive for meiotic spore viability and DSB repair and deficient for Rec12^{Spo11} removal.** We previously created a *rad50S* mutant (*rad50-K81I*) which has been instrumental in the study of meiotic DSB formation in *S. pombe* (42). As previously reported (10), we found that the *rad50S* mutant is temperature sensitive for meiotic spore viability (Fig. 1a). At 34°C, the spore viability was 0.6% (similar to that of the *rad50*Δ mutant), and at 25°C, it was 14.6%. As shown previously for the *rad50*Δ strain (41), the low spore viability of the *rad50S* strain at 34°C was rescued by deletion of *rec12^{spo11}*, suggesting that the reduced viability is due to a DSB repair defect in this mutant. To test if the temperature-sensitive spore viability phenotype of the *rad50S* mutant indeed reflects a defect in DSB repair, we looked at meiotic DSB formation and repair in a meiotic time course with the *rad50S* strain at permissive and restrictive temperatures, using pulsed-field gel electrophoresis (Fig. 1b). We found that at 34°C, the intact chromosomes were transformed into broken DNA fragments that remained unrepaired. At 25°C, most DNA was transformed into broken fragments, but a significant fraction of chromosomal DNA was intact near the end of the time course. After approximately 8 h, the intensity of the intact chromosomal DNA bands started to diminish. At this time, the majority of the cells had sporulated (Fig. 1c) (spore wall formation makes spores resistant to lysis, preventing the DNA from entering the gel). We concluded that the *rad50S* mutant is deficient for meiotic DSB repair at 34°C but partially proficient at 25°C.

We next asked if the low spore viability and inability to repair meiotic DSBs in *rad50*Δ and *rad50s* mutants was due to a defect in removing covalently bound Rec12^{Spo11} from the DSB ends. Based on previously published procedures (17, 34), we developed an assay (DNA-linked protein detection assay) to detect the presence of covalently bound Rec12^{Spo11} on the DNA (see Materials and Methods). As shown in Fig. 1d, both *rad50*Δ and *rad50S* mutants showed a strong presence of covalently bound Rec12^{Spo11} 6 h after the initiation of meiosis, whereas in WT cells Rec12^{Spo11} was removed from the DNA. We consistently found higher covalently bound Rec12^{Spo11} levels in the *rad50S* strain than in the *rad50*Δ strain (please see Discussion for possible explanations). We concluded from these experiments that the *rad50S* mutant is temperature sensitive and, like the *rad50*Δ strain, is defective in Rec12^{Spo11} removal, leading to the inability to repair meiotic DSBs and a strong reduction in spore viability at restrictive temperature.

The *rad50S* strain is a separation-of-function mutant which is proficient for meiotic recombination functions independent of Rec12^{Spo11} removal. To find out if the *rad50S* mutant is deficient only for Rec12^{Spo11} removal or also for other recombination-related functions which are defective in the *rad50*Δ mutant, we compared different meiotic phenotypes between the *rad50*Δ and *rad50S* strains.

The repair of meiotic DSBs results in genetically detectable recombination when the homologous chromosome is used as a repair template, while recombination with the sister chromatid is usually silent. We determined meiotic recombination levels (in surviving spores) at three genetic intervals in *rad50*Δ and *rad50S* mutants at both 25°C and 34°C. As shown in Fig. 2a, meiotic intergenic recombination levels in the *rad50*Δ mutant were strongly reduced at all intervals tested at both 25°C and 34°C (a 28-fold reduction on average). Surprisingly, recombination levels in the *rad50S* mutant were similar to those of WT cells at both temperatures (1.0-fold on average).

The *S. pombe ade6-M26* mutation creates a hot spot for meiotic recombination. This hot spot activity has been associated with the creation of a meiosis-specific MNase hypersensitive site through chromatin remodeling (23). To see if this meiosis-specific MNase sensitivity is affected in MRN mutants, we performed an MNase assay on a synchronized meiotic time course. As shown in Fig. 2b, meiosis-specific chromatin remodeling at *ade6-M26* (see black arrow for the WT at 3 h) was defective in both *rad50*Δ and *rad32^{mre11}*Δ mutants, whereas remodeling was proficient in the *rad50S* strain.

S. pombe LEs are similar to the axial elements of the SC in other eukaryotes and are believed to play a role in meiotic chromosome organization and recombination (4). To assess the role of the MRN complex in LE formation, we looked at LEs in meiotic spread preparations of *rad50*Δ and *rad50S* mutants (Fig. 3). We found that LEs in *pat1-114* meiosis (used for its high degree of synchrony and relative stability of *rad50*Δ diploids [see Discussion]) (Fig. 1a) are shorter and lack the networks, bundles, and long elements normally found in *pat1^{+}* meiosis (4). We therefore classified *pat1-114* LEs into classes 1a (short LEs) and 1b (longer LEs). LEs in the *rad50S* mutant were slightly longer and more abundant than those in the WT (signified by the increase of class 1b LEs in this mutant compared to the WT) (Fig. 3b), but LEs were totally absent in the *rad50*Δ mutant. We quantified the position of the spindle pole body (SPB) relative to the nucleolus (which is associated with the ribosomal DNA near both ends of chromosome 3) in *rad50*Δ cells to confirm that these cells went into meiosis. In mitotic cells, the SPB is found next to the centromeres, but upon induction of meiosis, the centromeres and telomeres switch positions and the telomeres associate with the SPB (9). As shown in the bottom left panel of Fig. 3b, the majority of the *rad50*Δ cells showed a meiotic SPB configuration, confirming that these cells went into meiosis.

We concluded from this set of experiments that the *rad50S* strain is a separation-of-function mutant which is deficient only for Rec12^{Spo11} removal but proficient for other Rad50-dependent functions related to meiotic DSB repair.

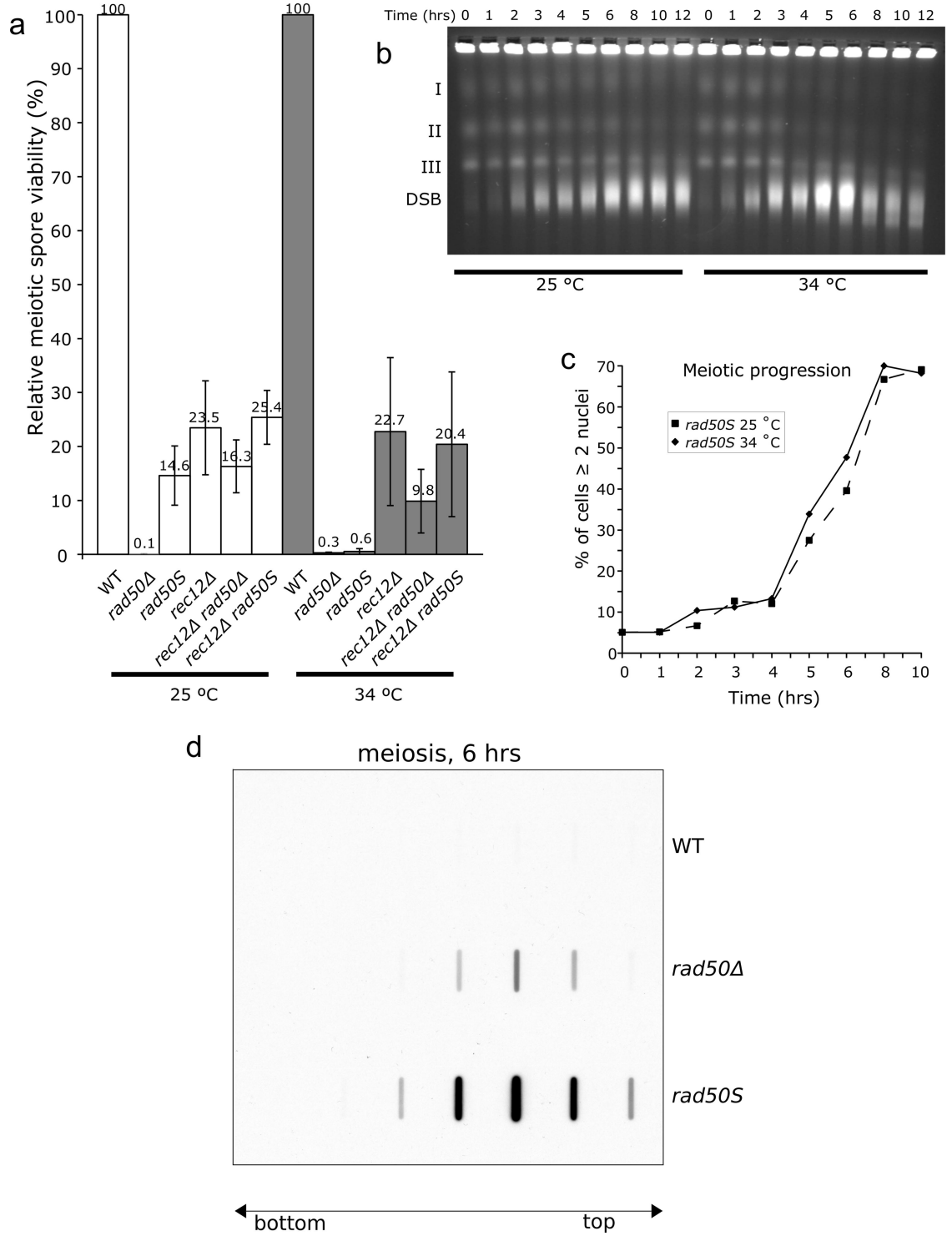

FIG. 1. The *rad50S* mutant is temperature sensitive for meiotic spore viability and defective for meiotic DSB repair and $Rec12^{Spo11}$ removal. (a) Meiotic spore viability relative to that of the WT in different strains at 25°C and 34°C. Error bars show standard deviations, and values are averages for three independent experiments. (b) Pulsed-field gel electrophoresis of a synchronized meiotic *pat1*$^+$ *rad50S* culture at 25°C and 34°C. The bands labeled I, II, and III correspond to the intact chromosomes, and the smears labeled DSB correspond to broken DNA fragments. (c) Meiotic progression of the time course presented in panel b, expressed as numbers of cells that have completed meiosis I at different time points. (d) Slot blot showing the presence of covalently bound $Rec12^{Spo11}$ on the DNA 6 h after meiotic induction at 34°C. At time point 0, no $Rec12^{Spo11}$ signals were visible (data not shown). The arrow indicates where the top and bottom fractions of the CsCl gradient were loaded. The bulk of the DNA was found in fractions 5, 6, and 7, which showed the strongest $Rec12^{Spo11}$ signals for *rad50* mutants.

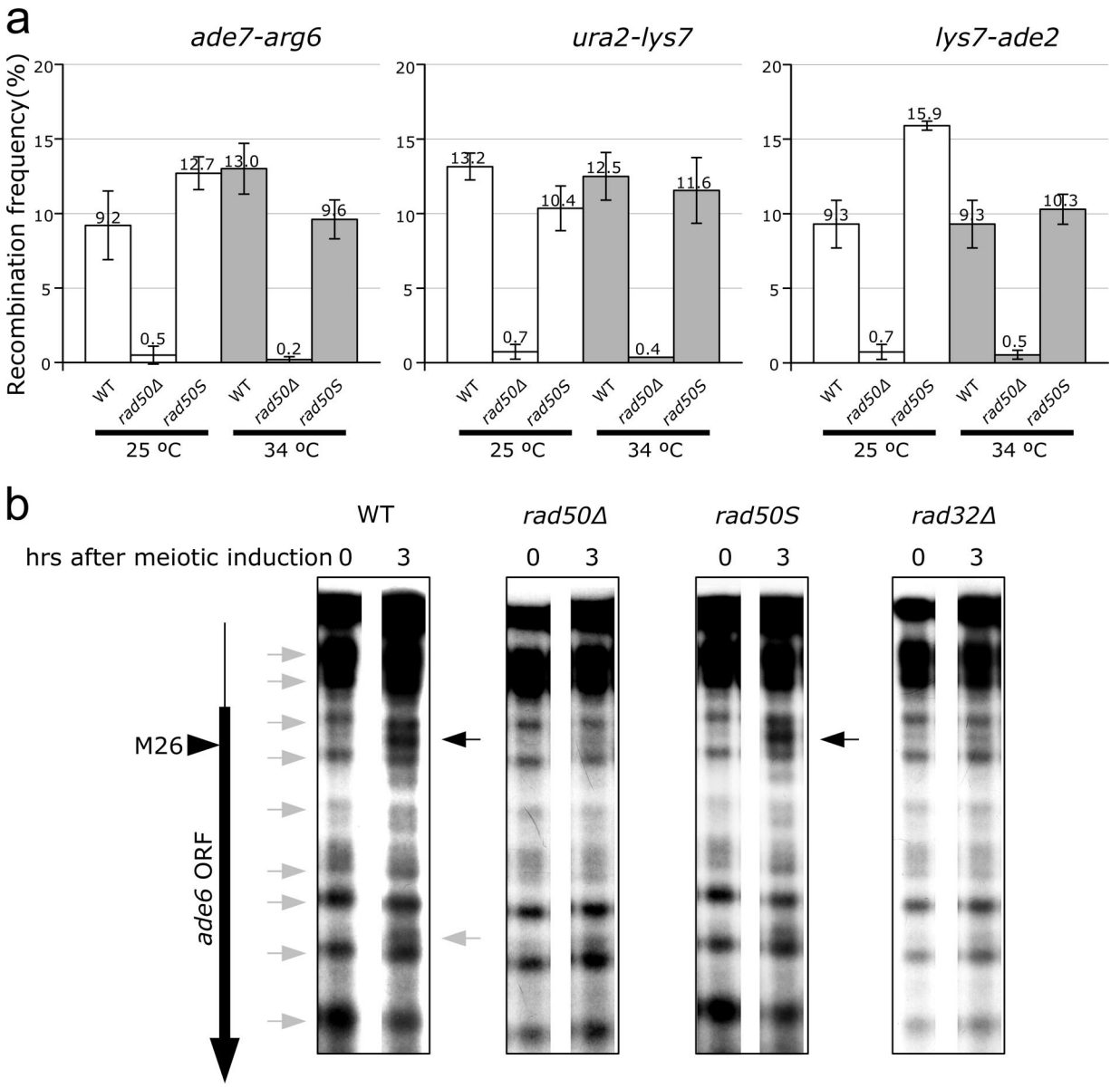

FIG. 2. The *rad50S* mutant is proficient for meiotic recombination and meiosis-specific nucleosome remodeling, but both functions are defective in the *rad50*Δ mutant. (a) Meiotic intergenic recombination levels in surviving *rad50*Δ spores were strongly reduced at different genetic intervals at both 25°C and 34°C. However, the *rad50S* strain was proficient for recombination at both temperatures. (b) Meiosis-specific nucleosome remodeling at *ade6-M26* (see black arrow for the WT at 3 h) was defective at 34°C in the *rad50*Δ strain (and the *rad32*^{mre11}Δ mutant), whereas the *rad50S* strain was proficient.

Rad32^{Mre11} nuclease activity is required for Rec12^{Spo11} removal. Although various observations suggest that the nuclease activity of Mre11 is responsible for Spo11 removal in *S. cerevisiae*, these experiments could not distinguish between a role for Mre11 in Spo11 removal and the subsequent exonucleolytic resection, and an involvement of the Mre11 nuclease activity in Spo11 removal has not been confirmed experimentally (19). The possibility that a nuclease other than Mre11 could be responsible for Spo11 removal is illustrated by the observation in *S. pombe* that the nuclease activity that degrades the C-rich strand at the telomeres (to create a 3′ G-rich strand overhang) is dependent on but not provided by the MRN complex and that a second single-stranded-DNA-specific endonuclease, Dna2 (3), is recruited by MRN and provides the nuclease activity (37, 38).

To distinguish between the possibilities that Rec12^{Spo11} is removed by Rad32^{Mre11} or by another nuclease recruited and/or controlled by MRN, we created a *rad32*^{mre11}*-D65N* nuclease-dead mutant. This mutant is the equivalent of the well-characterized *S. cerevisiae mre11-D56N* mutant, which has been shown to be deficient for nuclease activity and proficient for MRN complex formation (18). Also, in *S. pombe*, the *rad32*^{mre11}*-D65N* mutant is proficient for MRN complex formation (Nick Rhind, personal communication).

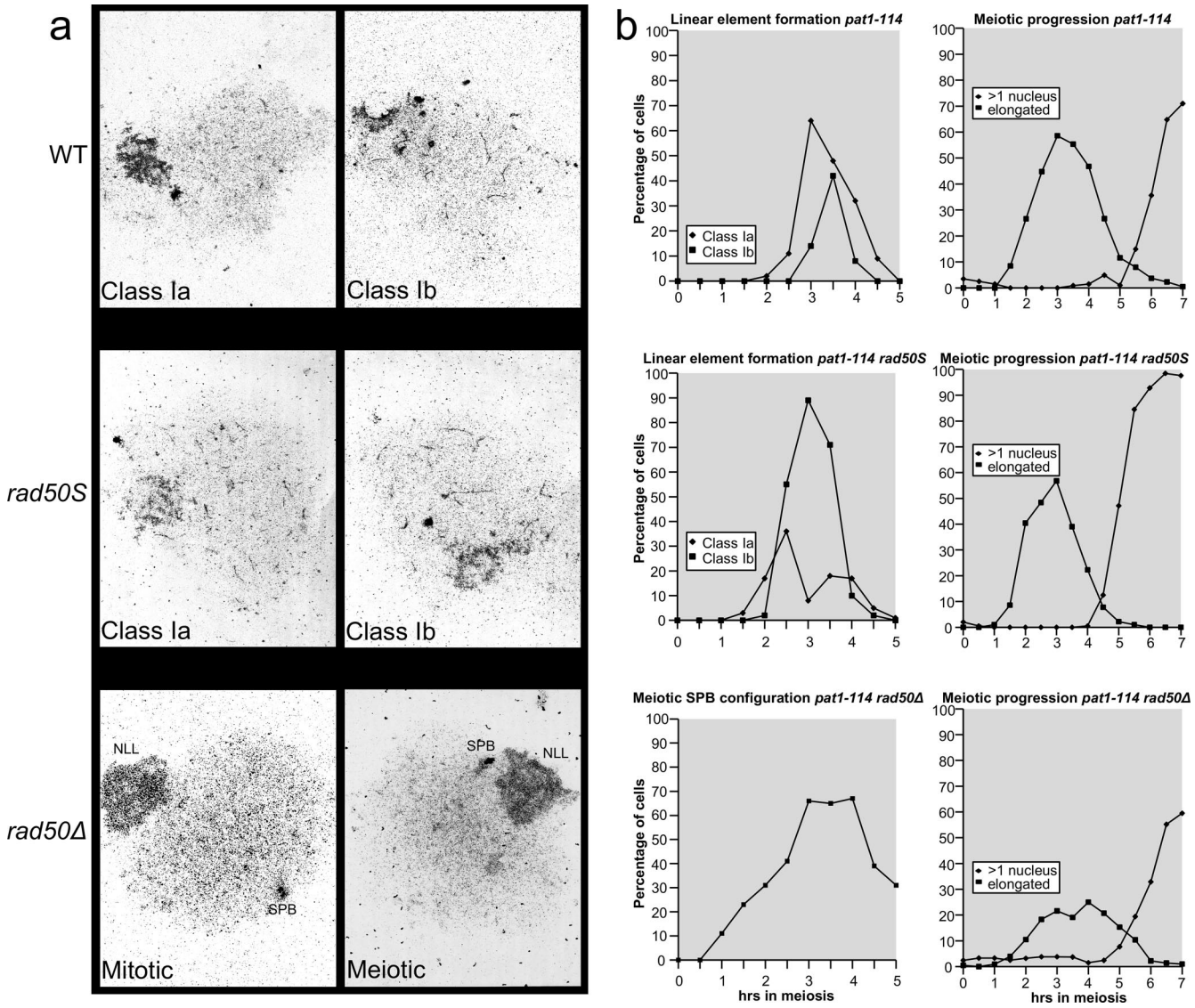

FIG. 3. The *rad50S* mutant is proficient for linear element formation, whereas LEs are absent in the *rad50*Δ mutant. (a) Electron micrographs of lysed and spread meiotic nuclei. LEs in *pat1-114* meiosis (used for its high degree of synchrony) were shorter than those in the WT (*pat1*$^{+}$) (4), whereas networks and bundles were not detected. LEs in *pat1-114* meiosis were classified into classes 1a (short LEs) and 1b (long LEs). LEs in *rad50S* cells were slightly longer and more abundant than those in the WT. LEs were absent in *rad50*Δ cells. The bottom two panels (*rad50*Δ) illustrate typical SPB orientations in mitotic (opposite the nucleolus [NLL]) and meiotic (next to the nucleolus) cells. This allows the distinction between mitotic and meiotic cells and confirms that *rad50*Δ cells underwent meiosis in the absence of LEs. (b) (Left top and middle) Quantification of LE classes 1a and 1b at different time points. Class 1b was more abundant in *rad50S* cells than in the WT. (Bottom left) Quantification of *rad50*Δ cells (without LEs) containing an SPB configuration indicative of meiosis. At later time points, cells started to form ascus and spore walls, making the cells resistant to lysis, which led to an artifactual underrepresentation of meiotic cells. (Right) Quantification of DAPI (4′,6-diamidino-2-phenylindole)-visualized elongated (horsetail) nuclei indicative of meiotic prophase. The percentage of cells with more than one nucleus indicates progression through the first and second meiotic divisions. All quantifications are based on at least 100 cells per time point.

We first studied the meiotic spore viability of the *rad32*^{mre11}*-D65N* mutant in combination with *rad50*Δ and *rad50S* mutations (Fig. 4a). The viability of *rad32*^{mre11}*-D65N* and *rad32*^{mre11}*-D65N rad50S* spores was strongly reduced compared to that of *rad50*Δ spores, whereas *rad32*^{mre11}*-D65N rad50*Δ spore viability was rescued to approximately *rad50*Δ strain levels. We believe that these observations might reflect MRN complex stability in *rad50S* and *rad32*^{mre11}*-D65N* mutants versus instability in the *rad50*Δ mutant. An intact but nuclease-deficient complex might block access of DSB ends to (as yet unknown) alternative removal activities, further decreasing spore viability. The double mutants with *rad50S* (and *rad50*Δ) did not show a lower spore viability than the single *rad32*^{mre11}*-D65N* mutant, suggesting that they are all defective in the same Rec12^{Spo11} removal pathway.

The extremely low spore viability of the *rad32-D65N* mutant precluded a reliable measurement of meiotic recombination. Among 280 survivors, not a single recombinant was detected. However, prolonged snail enzyme digestion (which is used to kill vegetative cells) further reduced the number of survivors

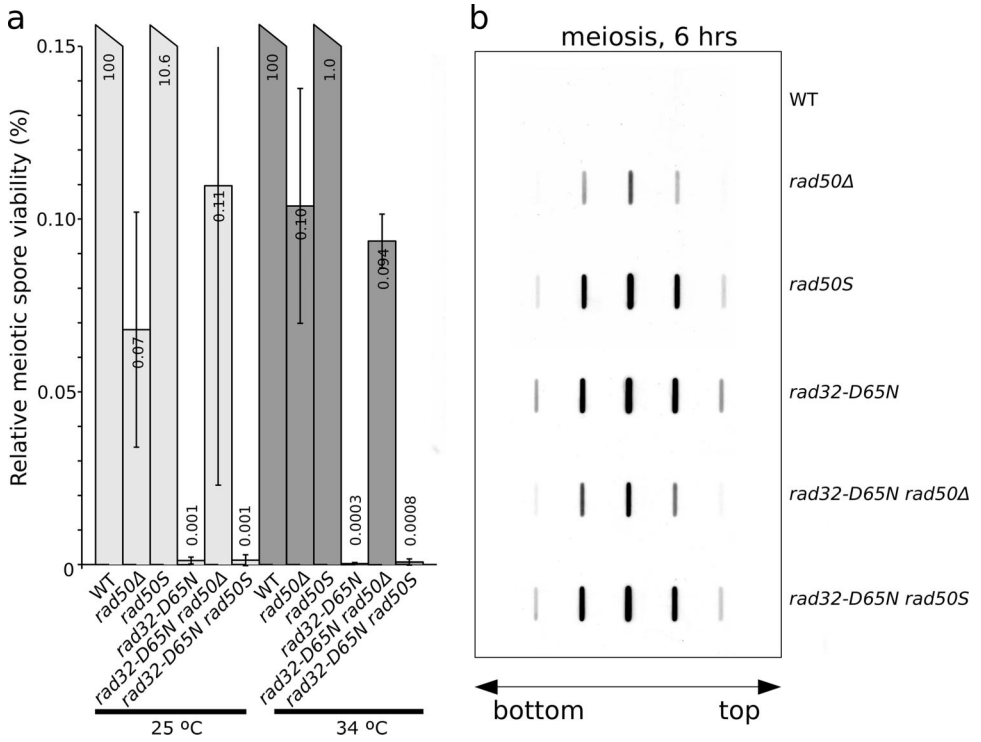

FIG. 4. The *rad32*^{mre11}*-D65N* mutant is defective for Rec12^{Spo11} removal. (a) Analysis of spore viability epistasis between different *rad32*^{mre11}*-D65N* mutants. Note that the graph shows only the lower range (<0.15%) of spore viability. Error bars show standard deviations, and values are averages for three independent experiments. (b) Analysis of Rec12^{Spo11} removal in different mutants at 34°C. Levels of covalently bound Rec12^{Spo11} were increased in *rad32*^{mre11}*-D65N* mutant strains. The arrow indicates where the top and bottom fractions of the CsCl gradient were loaded. The bulk of the DNA was found in fractions 5, 6, and 7, which showed the strongest Rec12^{Spo11} signals in *rad50* mutants.

(unpublished observation), and it is therefore likely that these survivors did not result from meiotic spores but represent a small fraction of vegetative cells that resisted snail enzyme treatment.

Using the DNA-linked protein detection assay to detect covalently bound Rec12^{Spo11} (Fig. 4b), we found that the *rad32*^{mre11}*-D65N* mutant was indeed defective in Rec12^{Spo11} removal, to a degree similar to that of the *rad50S* mutant. As in the spore viability assay, the defect was less pronounced in the *rad50*Δ and *rad32*^{mre11}*-D65N rad50*Δ mutants, whereas the defect in the *rad32*^{mre11}*-D65N rad50S* strain was comparable to that of the *rad32*^{mre11}*-D65N* and *rad50S* mutants. Taken together, these data show that the Rad32^{Mre11} endonuclease activity is required for Rec12^{Spo11} removal in meiosis.

Ctp1^{CtIP} is required for Rec12^{Spo11} removal. Sae2 shows only weak homology to Ctp1^{CtIP} (21). *S. cerevisiae sae2* mutants exhibit a *rad50S* mutant-like phenotype and are deficient for Spo11 removal (28, 32). Like the *rad50S* strain, the *sae2*Δ strain is only mildly methyl methanesulfonate sensitive (21). In contrast, the sensitivities of the *S. pombe ctp1*Δ mutant to ionizing radiation (21) and methyl methanesulfonate (13) are identical to those of MRN null mutants and much higher than those of the *rad50S* strain (13). Using pulsed-field gel electrophoresis, it was previously shown that a *ctp1*Δ mutant is deficient for meiotic DSB repair (1). To test for functional conservation between Sae2 and Ctp1^{CtIP}, we analyzed the *ctp1*Δ strain for the ability to remove covalently bound Rec12^{Spo11} from the DNA. As shown in Fig. 5a, the *ctp1*Δ strain was as defective in Rec12^{Spo11} removal as the *rad32*^{mre11}*-D65N* strain, suggesting a functionally conserved role for Ctp1/Sae2 homologues in Spo11 removal. Whereas covalently bound Rec12^{Spo11} levels were similar in the *ctp1*Δ mutant and a *ctp1*Δ *rad32-D65N* double mutant, these levels were comparatively lower in the *rad50*Δ mutant and a *ctp1*Δ *rad50*Δ double mutant.

As shown in Fig. 5b, meiotic spore viability in the *ctp1*Δ strain was reduced to below *rad50*Δ strain levels, similar to that of the *rad32-D65N* strain. The spore viability was rescued to *rad50*Δ levels in a *ctp1*Δ *rad50*Δ double mutant but not in a *ctp1*Δ *rad32-D65N* double mutant, possibly reflecting the fact that the MRN complex remains intact in *ctp1*Δ strains. As explained above for the *rad32-D65N* strain, the extremely low viability of the *ctp1*Δ spores precluded a reliable measurement of meiotic recombination.

We also studied meiosis-specific chromatin remodeling and found that in the *ctp1*Δ mutant the *ade6-M26* MNase hypersensitive site was present during meiosis (Fig. 5c) and thus that Ctp1^{CtIP} is not required for this chromatin remodeling event.

DISCUSSION

Rad32^{Mre11} nuclease activity and Ctp1^{CtIP} are required for Rec12^{Spo11} removal. Several studies have suggested that Mre11 nuclease activity might be responsible for Spo11 removal. Moreau et al. (27) found that an *S. cerevisiae mre11* nuclease-dead mutant was deficient in meiotic DSB end resection and proposed that Mre11 is responsible for removing Spo11. However,

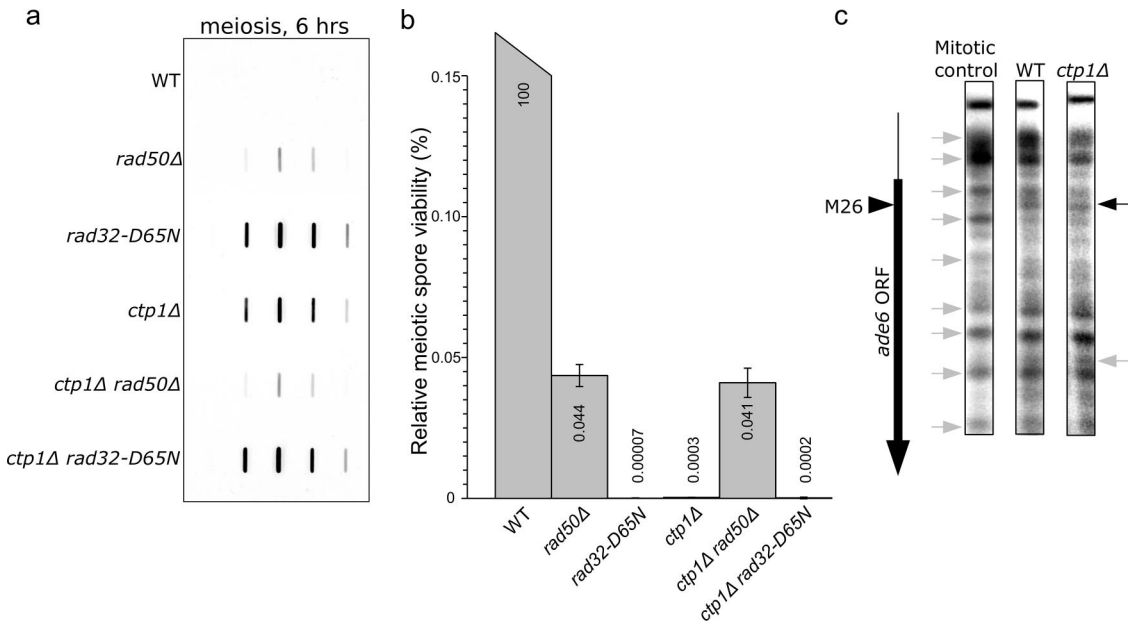

FIG. 5. (a) A *ctp1* deletion mutant is deficient in removing $Rec12^{Spo11}$ from the DNA in meiotic cells. The defect is comparable to that of *rad50S* and *rad32^{mre11}-D65N* strains. (b) Meiotic spore viability is strongly reduced in the *ctp1*Δ strain, similar to that of the *rad32-D65N* strain. (c) The *ctp1*Δ strain is proficient for *ade6-M26* chromatin remodeling (black arrow).

this study could not distinguish between a role of Mre11 in (endo)nucleolytic Spo11 removal and a role in (exo)nucleolytic resection downstream of Spo11 removal. Similarly, the presence of the Spo11 removal product (Spo11 with a covalently attached nucleotide) has not been studied with an *mre11* nuclease-dead mutant (28). In this study, we thus provide the first direct demonstration that the $Rad32^{Mre11}$ nuclease activity is indeed required for $Rec12^{Spo11}$ removal in meiosis.

We have shown that *rad50*Δ, *rad50S*, *rad32^{mre11}-D65N*, and *ctp1*Δ mutants are defective in removing $Rec12^{Spo11}$ from the DNA. We consistently found higher levels of covalently bound $Rec12^{Spo11}$ in *rad50S*, *rad32^{mre11}-D65N*, and *ctp1*Δ mutants than in the *rad50*Δ mutant (e.g., see Fig. 1d, 4b, and 5a). This might be (partially) due to a reduced viability of *rad50*Δ cells (approximately 25% of *rad50*Δ cells were dead) (12). However, this reduced viability is unlikely to account fully for the threefold reduction in meiotic DSB formation in the *rad50*Δ strain (41). Also, levels of covalently bound $Rec12^{Spo11}$ in the *ctp1*Δ strain were higher than those in the *rad50*Δ strain and only very slightly reduced compared to those in the *rad50S* and *rad32^{mre11}-D65N* strains, while the growth defect of the *ctp1*Δ strain was comparable to that of MRN null mutants (1, 21). These observations suggest that Rad50 is required for WT levels of meiotic DSBs. In *S. cerevisiae*, *RAD50* is absolutely required for meiotic DSB formation (6, 16).

The almost identical $Rec12^{Spo11}$ removal defects of the *rad50S* and *rad32^{mre11}-D65N* mutants suggest that Rad50 somehow controls the $Rad32^{Mre11}$ nuclease activity. Based on structural studies, it has previously been proposed that ATP-driven directional switching of Rad50 controls the Mre11 nuclease activity (14). Interestingly, the *rad50S* mutation is found in a putative protein interaction site, and based on structural studies, it has previously been proposed that this site might interact with Sae2 (15). A recent study (33) showed that CtIP interacts directly with the MRN complex. Since the $Rec12^{Spo11}$ removal defect in the *ctp1*Δ mutant is also similar to that of the *rad32^{mre11}-D65N* mutant, this opens up the possibility that CtIP/Sae2 controls the Mre11 nuclease activity through its interaction with Rad50.

The most straightforward interpretation of our data is that the $Rad32^{Mre11}$ nuclease is directly responsible for $Rec12^{Spo11}$ removal. However, a recent study (20) showed that purified *S. cerevisiae* Sae2 possesses a nuclease activity which cleaves hairpin DNA structures in vitro, cooperatively with the MRN complex (called MRX in *S. cerevisiae*). Purified MRX promotes cleavage by enlarging a single-strand gap in the DNA opposite the Sae2 cleavage site. This raises the possibility that the coordinated action of Mre11 and Sae2 nuclease activities might be required and that Sae2 is ultimately responsible for Spo11 removal.

MRN null mutants are defective for meiosis-specific chromatin remodeling, LE formation, and recombination. We found that meiotic recombination in the *rad50*Δ mutant was reduced approximately 28-fold, in line with the previously reported reduction in meiotic recombination in a *rad32^{mre11}*Δ mutant (36). This reduction might be partially due to reduced DSB formation in this mutant (see the previous section). We showed that in *rad50*Δ and *rad32*Δ mutants, *ade6-M26* chromatin remodeling is almost completely abolished. In contrast, in *S. cerevisiae*, only Mre11 is required for meiosis-specific chromatin remodeling at meiotic recombination hot spots, whereas Rad50 is dispensable for this process (29). The role of meiosis-specific chromatin remodeling and the role of MRN therein are not well understood, but they are probably involved in meiotic DSB formation and/or subsequent recombinational repair. *S. cerevisiae* Mre11 has also been implicated in chromatin remodeling during mitotic DSB repair (39).

We found that LE formation was totally abolished in the *rad50*Δ mutant. A potential caveat is that these experiments

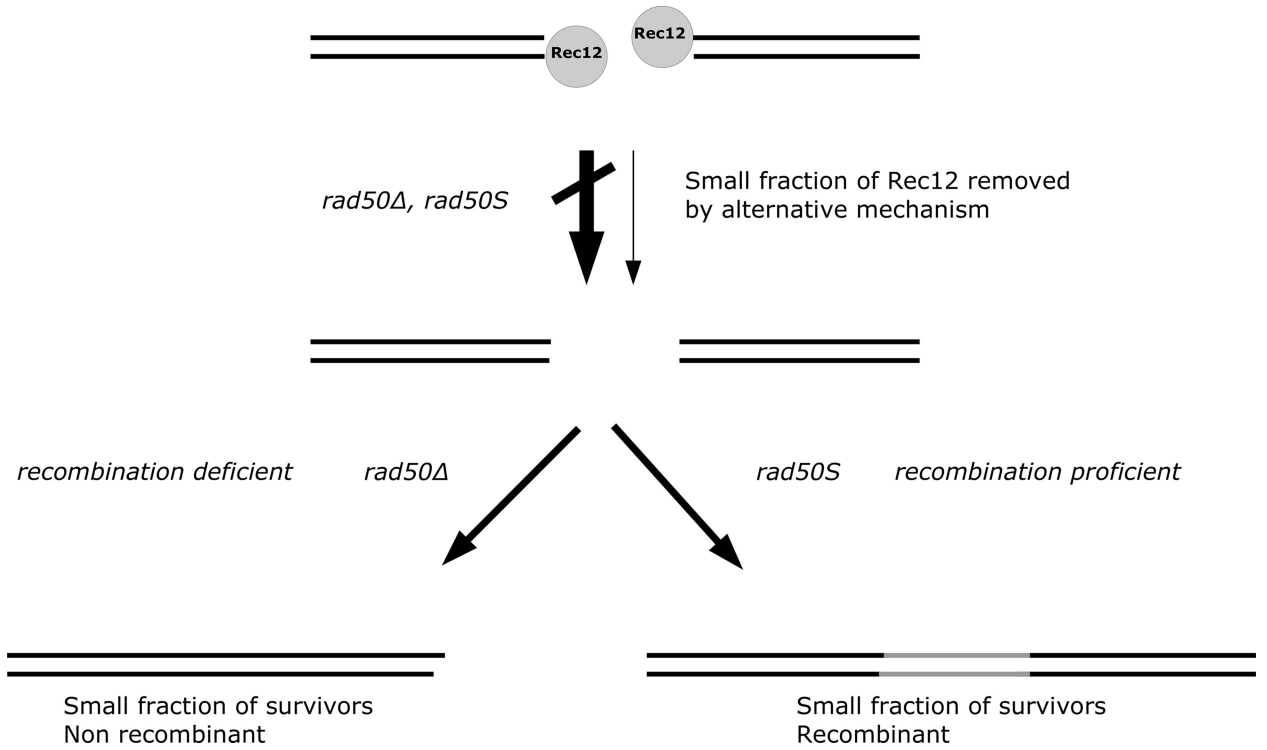

FIG. 6. Interpretation of the observed meiotic phenotypes of *rad50*Δ and *rad50S* mutants. Both *rad50*Δ and *rad50S* mutants (at a restrictive temperature) are deficient for the removal of covalently bound $Rec12^{Spo11}$ after meiotic DSB formation, leading to low spore viability. However, a small fraction of cells are able to remove $Rec12^{Spo11}$ (through an unknown mechanism), allowing repair of the DSBs and viable spore formation. For the *rad50*Δ mutant, these survivors show a strong reduction in recombination rates, suggesting that they survive through a nonrecombinogenic survival mechanism. The *rad50S* cells are proficient for meiotic recombination (once $Rec12^{Spo11}$ has been removed), and the surviving spores therefore show normal meiotic recombination levels.

were performed with a *pat1-114* mutant, which shows shortened LEs, while networks, bundles, and longer LEs (as found in a $pat1^+$ strain) (4) are absent. Because of the extreme instability of h^+/h^- *rad50*Δ/*rad50*Δ diploids (12), we were not able to perform these experiments for $pat1^+$ meiosis. In an *S. cerevisiae rad50*Δ mutant, shortened axial cores are formed, but they never form a tripartite SC structure (2). Most recombination-defective mutants studied so far do form (often aberrant) LEs (24, 25, 26). LE formation is also abolished is the *rec10*Δ mutant (26), and it has been shown that Rec10 is an LE component (22). Our observations raise the possibility that Rad50 fulfils a structural role or might regulate an early step in LE formation.

$Rec12^{Spo11}$ removal is not required for meiosis-specific chromatin remodeling, LE formation, and recombination. Whereas the *rad50S* strain is defective in $Rec12^{Spo11}$ removal, we found that it is proficient for meiotic recombination, meiosis-specific chromatin remodeling, and LE formation, which are all defective in the *rad50*Δ mutant.

Meiotic recombination levels and levels of MNase sensitivity at *ade6-M26* are very similar in the *rad50S* mutant and the WT. However, we found that LEs in *rad50S* cells were elongated compared to those in the WT. We speculate that this might be related to the prolonged presence of meiotic DSBs in the *rad50S* strain, maybe allowing more time for the LEs to mature. In an *S. cerevisiae rad50S* mutant, as in the *rad50*Δ mutant, no fully formed SC is found. However, axial cores in the *rad50S* strain are longer than those in the *rad50*Δ strain, and sometimes short stretches of tripartite structure are formed (2).

Figure 6 shows a diagram which explains our interpretation of the relationships between $Rec12^{Spo11}$ removal, meiotic spore viability, and meiotic recombination in *rad50*Δ and *rad50S* mutants. Both the *rad50*Δ and *rad50S* mutants (at a restrictive temperature) are deficient for the removal of covalently bound $Rec12^{Spo11}$ after meiotic DSB formation, leading to low spore viability. However, the presence of viable spores of these mutants suggests that a small fraction of cells are able to remove $Rec12^{Spo11}$ (through an as yet unknown alternative mechanism), allowing repair of the DSB and viable spore formation. For the *rad50*Δ strain, these survivors show a strong reduction in recombination rates, suggesting that they survive through a nonrecombinogenic survival mechanism (possibly nonhomologous end joining or recombinational repair using the sister chromatid as a template). The *rad50S* cells are proficient for meiotic recombination (once $Rec12^{Spo11}$ has been removed), and the surviving spores therefore show normal meiotic recombination levels.

Conclusions and outlook. Although it has been predicted that *S. cerevisiae* Mre11 nuclease activity is responsible for Spo11 removal, this has not been confirmed experimentally. This study demonstrates for the first time that the $Rad32^{Mre11}$ nuclease activity is required for $Rec12^{Spo11}$ removal. The $Rec12^{Spo11}$ removal defect in the *ctp1*Δ strain suggests a func-

tional conservation between the distantly related Sae2 and Ctp1^{CtIP} proteins. We also confirmed that the *S. pombe rad50S* mutant is defective in Rec12^{Spo11} removal. Our finding that the temperature-sensitive *rad50S* mutant is proficient for meiotic recombination, meiosis-specific chromatin remodeling, and LE formation, functions which are all defective in the *rad50*Δ strain, suggests that the involvement of MRN in these functions is independent of Rec12^{Spo11} removal.

The conservation of the involvement of the MRN complex and Ctp1^{CtIP} in Rec12^{Spo11} removal, SC/LE formation, and meiosis-specific chromatin remodeling in the distantly related yeasts *S. cerevisiae* and *S. pombe* suggests that these MRN functions might be conserved throughout the eukaryotic kingdom. The analysis of MRN and CtIP functions in meiosis of higher eukaryotes has been hampered by the inviability of relevant mutants. Whereas a mouse *Rad50^{R83I}* mutant (with an allele equivalent to the *rad50-K81I* allele used in this study) is inviable, a *Rad50^{K22M}* mutant (equivalent to the less-well-characterized *S. cerevisiae* R20M *rad50S* mutant) (2) is viable but shows only mild meiotic phenotypes (5). These observations might reflect either that the similar but not identical amino acid change (K22M in the mouse versus R20M in *S. cerevisiae*) might not confer a Spo11 removal defect or that the MRN complex is not involved in Spo11 removal in mice. Another study (8) suggests that the mouse MRN complex is involved in meiotic prophase progression, chromosome synapsis, and recombination.

The findings presented in this study have important implications for our understanding of the role of the MRN complex and CtIP in meiotic recombination, as their defects lead to chromosome nondisjunction, infertility, and chromosomal abnormalities.

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