**Coprinus cinereus** rad50 mutants reveal an essential structural role for Rad50 in axial element and synaptonemal complex formation, homolog pairing, and meiotic recombination

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Abstract

The Mre11/Rad50/Nbs1 (MRN) complex is required for eukaryotic DNA double-strand break (DSB) repair and meiotic recombination. We cloned the *Coprinus cinereus rad50* gene and showed that it corresponds to the complementation group previously named *rad12*, identified mutations in 15 *rad50* alleles, and mapped two of the mutations onto molecular models of Rad50 structure. We found that *C. cinereus rad50* and *mre11* mutants arrest in meiosis and that this arrest is Spo11 dependent. In addition, some rad50 alleles form inducible, Spo11-dependent Rad51 foci and therefore must be forming meiotic DSBs. Therefore, we think it likely that arrest in both *mre11-1* and the collection of *rad50* mutants is the result of unrepaired or improperly processed DSBs in the genome and that Rad50 and Mre11 are dispensable in *C. cinereus* for DSB formation, but required for appropriate DSB processing. We found that the ability of *rad50* mutant strains to form Rad51 foci correlates with their ability to promote synaptonemal complex formation and with levels of stable meiotic pairing, and that partial pairing, recombination initiation, and synapsis occur in the absence of wild-type Rad50 catalytic domains. Examination of single and double mutant strains showed that a *spo11* mutation that prevents DSB formation enhances AE formation for *rad50-4*, an allele predicted to encode a protein with intact hook region and hook-proximal coiled coils, but not for *rad50-1*, an allele predicted to encode a severely truncated protein, or for *rad50-5*, which encodes a protein whose hook-proximal coiled coil region is disrupted. Therefore, Rad50 has an essential structural role in the formation of axial elements, separate from the DSB-processing activity of the MRN complex.
INTRODUCTION

Meiosis is the unique cell division that results in the production of haploid gametes. During prophase I, homologs condense, pair and recombine. At least one recombination event per homologous pair becomes a crossover, which, in combination with sister-chromatid cohesion, creates a chiasma that holds homologs together as they attach to the metaphase I spindle. Release of sister-chromatid cohesion along chromosome arms allows anaphase I separation of homologs, and release of centromere cohesion allows anaphase II separation of sister chromatids.

Meiotic recombination events initiate with a Spo11-catalized DNA double-strand break (DSB), and some of the proteins required for meiotic recombination have been recruited from mitotic DNA repair pathways. A primary example is the complex of proteins referred to as MRN, for Mre11, Rad50, and Nbs1. Evidence from Saccharomyces cerevisiae, in which the Nbs1 ortholog is called Xrs2, showed that the MRN complex is necessary for meiotic DSB formation by Spo11 (ALANI et al. 1990; reviewed in BORDE 2007). However, in other species different results have been obtained. In Caenorhabditis elegans, Rad50 facilitates DSB formation by Spo11 but is apparently not strictly required for Spo11 activity (HAYASHI et al. 2007). In Schizosaccharomyces pombe (YOUNG et al. 2004), Arabidopsis thaliana (PUIZINA et al. 2004), and Drosophila melanogaster (MEHROTRA and McKIM 2006) the MRN complex is required for break processing but not break formation.

In all eukaryotic organisms studied, mre11, rad50, and xrs2/nbs1 mutants exhibit meiotic recombination defects and radiation sensitivity (AJIMURA et al. 1993; ALANI et al. 1990; CARNEY et al. 1998; CHAMANKHAH et al. 1998; COX and PARRY 1968; GALLEGÓ et
al. 2001; GAME et al. 1980; GERECKE and ZOLAN 2000; HARTSUIKER et al. 2001; HAYASHI et al. 2007; IVANOV et al. 1992; MALONE 1983; MALONE and ESPOSITO 1981; MATSUURA et al. 1998; PITTS et al. 2001; RAMESH and ZOLAN 1995; STEWART et al. 1999; TAVASSOLI et al. 1995; VARON et al. 1998; WILLIAMS et al. 2008 ). The members of the Mre11 complex have been grouped by epistasis analysis (AJIMURA et al. 1993; GAME et al. 1980; IVANOV et al. 1992; MALONE and ESPOSITO 1981; VALENTINE et al. 1995) and have been shown to directly interact (DOLGANOV et al. 1996; JOHZUKA and OGAWA 1995; PETRINI et al. 1995; TRUJILLO et al. 1998; VARON et al. 1998). Mammalian deletion mutants of the Mre11 complex exhibit embryonic lethality (LUO et al. 1999; XIAO and WEAVER 1997; YAMAGUCHI-IWAI et al. 1999; ZHU et al. 2001). Viable mutations in human homologs of the MRN complex invariably result in syndromes characterized by a loss of fertility, and cancer early in life (BENDER et al. 2002; CARNEY et al. 1998; MATSUURA et al. 1998; PITTS et al. 2001; STEWART et al. 1999; VARON et al. 1998). The biochemical properties of the individual proteins comprising this complex have been characterized in mammals and yeast (reviewed in WILLIAMS et al. 2007). Mre11 binds DNA and exhibits in vitro ssDNA endonuclease and Mn^{2+}-dependent 3'→5' double-stranded DNA exonuclease activities (PAULL and GELLERT 1998). Both Mre11 nuclease activity and homodimerization are important for DSB repair (WILLIAMS et al. 2008). Rad50 is an ATP-dependent DNA binding protein that forms a multimer (HOPFNER et al. 2000a; RAYMOND and KLECKNER 1993), and which has both ATPase and adenylate kinase activities that are important for protein function (BHASKARA et al. 2007). The Nbs1 protein serves to localize the complex in the nucleus (TAUCHI et al. 2001), modifies MRN
complex activity (PAULL and GELLERT 1999), and plays a primary role in signal transduction after DNA damage (LEE and PAULL 2004; 2005).

Partial Pyrococcus furiosus Rad50/Mre11 complexes have been crystallized by Hopfner et al. (2001; 2000b). Rad50 has two alpha-helical coiled coil domains separated by a flexible hinge (referred to as a hook) harboring a semi-zinc finger at the center. The N- and C-termini of the protein are globular in nature and together comprise a functional ATP-binding site. They are brought into contact with each other by an intertwining of the N- and C- terminal coils. The two interwoven coils form a flexible rod creating a binding site for Mre11 made up of acidic residues just adjacent to the globular domain (HOPFNER et al. 2001). Crystallographic and biochemical data (HOPFNER et al. 2002a) have confirmed a role in protein dimerization for the conserved semi-zinc finger in the hook region (SHARPLES and LEACH 1995). Current models (HOPFNER et al. 2002a; HOPFNER et al. 2002b; WILLIAMS and TAINER 2005) suggest hook interactions of Rad50 molecules allow bridging of either sister chromatids or DSB ends. These Rad50 molecules are also held together with the aid of two dimerized Mre11 molecules at each opposing head of the complex (WILLIAMS et al. 2008). Recent atomic force microscopy studies (MORENO-HERRERO et al. 2005) showed that MRN complexes change conformation upon DNA binding. The unbound complexes exhibit greater flexibility of the coiled coil regions, which may favor intracomplex interactions, whereas the coiled coils of DNA-bound complexes are more rigid and parallel, thus favoring intercomplex interactions that would facilitate complex ability to bridge and stabilize DNA ends (WILLIAMS and TAINER 2005).
Meiotic defects in *rad50* mutants (reviewed in BORDE 2007; CHERRY *et al.* 2007) include failure of Spo11-dependent DSB formation or processing (ALANI *et al.* 1990; HAYASHI *et al.* 2007; MEHROTRA and McKIM 2006; YOUNG *et al.* 2004) incomplete formation of axial elements (AE) and the synaptonemal complex (SC; ALANI *et al.* 1990), and the formation of non-viable products (ALANI *et al.* 1990; YOUNG *et al.* 2004). Thus, it has a central role in meiotic recombination and chromosome metabolism.

We cloned the Rad50 gene and examined members of the MRN complex in the basidiomycete *Coprinus cinereus*. *C. cinereus* has naturally synchronous meiosis (RAJU and LU 1970), well developed cytogenetics (LI *et al.* 1999a; PUUKILA and LU 1985; PUUKILA *et al.* 1984; SEITZ *et al.* 1996), and an annotated genomic sequence (STAJICH *et al.* 2006). In screens for mutants defective in both the survival of gamma radiation and meiosis (VALENTINE *et al.* 1995; ZOLAN *et al.* 1988), we identified four complementation groups, termed *rad3*, *rad9*, *rad11*, and *rad12*. These four genes are all part of the same gamma radiation survival pathway (CUMMINGS *et al.* 2002; VALENTINE *et al.* 1995). The gene *rad11* was cloned and found to encode the *C. cinereus* ortholog of Mre11 (GERECKE and ZOLAN 2000). Therefore, we reasoned that one of the other genes of the group would encode the *C. cinereus* ortholog of Rad50. Here we present our finding that the *C. cinereus* gene initially named *rad12* (RAMESH and ZOLAN 1995) encodes Rad50, and examine critical features of the role of Rad50 in meiosis.
MATERIALS AND METHODS

Strains and culture conditions: The wild-type dikaryon used for the Rad51 time course experiment shown in Figure 4 has been previously described (VALENTINE et al. 1995). rad50 mutant strains were generated in five separate mutant screens (RAMESH and ZOLAN 1995; VALENTINE et al. 1995; ZOLAN et al. 1993; ZOLAN et al. 1995), all starting with the strain Java-6 (BINNINGER et al. 1987). Strain Okayama-7 (WU et al. 1983) was used for all outcrosses and backcrosses. Of the rad50 strains examined in this study, rad50-1, rad50-2, rad50-3, rad50-4, rad50-5, rad50-6, rad50-8, rad50-14, and rad50-15 were outcrossed and then backcrossed four times, rad50-10, rad50-11, and rad50-16 were outcrossed and then backcrossed once, and rad50-7, rad50-9, rad50-12, and rad50-13 were outcrossed only.

The rad50-1;spo11-1, rad50-4;spo11-1, rad50-5;spo11-1, rad50-8;spo11-1, and mre11-1;spoll1-1 strains were generated by crossing a rad50 or mre11 single mutant to R126-49 (spo11-1 (AmBm); CELERIN et al. 2000; CUMMINGS et al. 1999). Double mutants were identified by subjecting progeny to a radiation-sensitivity chunk test (ZOLAN et al. 1988) to test for the rad50 or mre11 mutation. All strains except those used to construct the mre11-1;spo11-1 double mutant were then mated to spo11-1 strains with \(A_{42}B_{42}\) or \(A_{43}B_{43}\) mating types. Strains that mated to spo11-1;\(A_{42}B_{42}\) only, fruited white (sporeless), and were radiation sensitive were scored as \(A_{42}B_{42}\) double mutants. These in turn were mated to strain Okayama-7 (\(A_{43}B_{43}\)) to generate double mutants, sibling single mutants and wild type controls with both \(A_{42}B_{42}\) and \(A_{43}B_{43}\).
mating types. Compatible monokaryons were mated and used for further analysis. For construction of the \textit{mre11-1; spo11-1} double mutant, progeny were screened for hygromycin resistance as a marker for the insertional mutation in \textit{spo11-1} (CELERIN \textit{et al.} 2000). Strain genotype was confirmed by sequencing and/or diagnostic restriction digests of PCR amplified fragments containing the mutated region. Mating pairs were identified as previously described (ZOLAN \textit{et al.} 1988).

\textbf{Spore viability:} Spore viabilities were determined using the spotted drop method described in CELERIN \textit{et al.} (2000). Spores were serially diluted and 10 µl drops were spotted onto a gridded plate. Five to seven mushroom caps were counted per strain and analyzed using the ANOVA test (SPSS 10.0 for the Macintosh, SPSS, Inc.).

\textbf{Degenerate PCR amplification of \textit{Ccrad50}:} Rad50 homologs from other organisms were aligned using ClustalX (THOMPSON \textit{et al.} 1997). Regions of high conservation were used as the basis for the design of degenerate primers. Rad50 was amplified from \textit{C. cinereus} genomic DNA using a degenerate primer pair: F1 = 5’ AAR ACN ACN ATH ATH GAR TGY YTN 3’; R1 = 5’ GGG NWS RTC YTC YTG RTG RCA RAA D 3’, corresponding to amino acids 38-46 and 158-165 of CcRad50. A product corresponding to \textit{rad50} was used to probe chromosome 8 and 9-enriched cosmid library of strain Okayama-7 (ZOLAN \textit{et al.} 1992). Three cosmids were isolated, 3B6, 5F8, and 23G1. The cosmid 23G1 was found to contain a complete copy of \textit{rad50} through subsequent sequencing analysis.

\textbf{RFLP analysis:} Strain Okayama-7 and a fourth generation backcrossed \textit{rad12-4} strain (\textit{rad12-4; 4-1}) were mated. Progeny were analyzed as described in Gerecke and Zolan
(2000) except that all genomic DNA was digested with EcoRI and probed with a 3B6 cosmid containing Cc\textit{rad}50.

**Transformation rescue assay:** Transformation rescue of oidial protoplasts of the \textit{C. cinereus rad}50-4 mutant was performed as described (BINNINGER \textit{et al}. 1987; ZOLAN \textit{et al}. 1992). A \textit{rad}50-4;\textit{trp}1-1,1-6;\textit{ade}8 strain was transformed with 5 \(\mu\)g of cosmid 23G1. This cosmid also contains the \textit{trp}1 gene, and tryptophan prototrophs were selected and assayed as described in Gerecke and Zolan (2000) except that the rescue of meiotic defects was assayed through a mating with a compatible \textit{rad}50-4 strain (\textit{rad}50-4;4-2).

**Sequencing of wild-type \textit{rad}50:** Plasmids containing subclones of genomic DNA of \textit{rad}50 were isolated from \textit{E. coli} and initially sequenced with M13F/R or T3/T7 vector primers. Primers for a sequencing walk were based upon the previous sequence data and designed using the OLIGO 5.0 program. Primers for internal sequencing and amplification were made by Genosys, Inc. or by MWG - Biotech. PCR products were sequenced using standard techniques. Sequences were edited and made contiguous by Sequencher v.3.1.1 (Gene Codes Corp.), and translated by DNASIS v.2.0 (Hitachi Software Engineering Co. Ltd.). Predicted proteins were identified using the blastx program in BLAST (ALTSCHUL \textit{et al}. 1990) and alignments were performed using Clustal X (THOMPSON \textit{et al}. 1997). Coiled structure of Rad50 was predicted using the MultiCoils program (WOLF \textit{et al}. 1997).

**Sequencing of \textit{rad}50 cDNA:** First-strand cDNA synthesis using reverse transcriptase (SuperScript II: GibCo-BRL Life Technologies) was performed on polyA\(^+\) mRNA isolated from mushroom caps at K+1. A gene-specific amplification was then performed using a \textit{rad}50 primer pair. The product was run on an agarose gel and Southern blotted. It was
then probed with an amplified piece of rad50. A band that hybridized to rad50 DNA was
gel extracted and cloned into a TOPO-TA vector. The resulting clones were sequenced
using vector primers and shown to correspond to a 1.1 Kb fragment of 3' rad50 cDNA.
A reverse primer was made extending across 10 bp of exon sequence immediately
following the 3' and 5' flank of intron 17. In this way we eliminated amplification of
genomic sequence. This primer was used to extend the oligo dT first-strand cDNA
synthesis reaction further. This first strand synthesis reaction was used as template for
an amplification with a forward primer across intron 15. This fragment was sequenced.
A new first-strand cDNA synthesis reaction was then performed using a reverse primer
across intron 15 and polyA+ mRNA isolated from mushroom caps at K+6. This first
strand reaction was amplified (Advantage 2.0 Kit: BD Biosciences Clontech) with a
forward primer from the beginning of the rad50 genomic sequence. This product was
also sequenced. Several primers in the 5'UTR were used to amplify the 5' end of rad50
with a reverse primer that spanned the 4th intron. These products were sequenced.
Sequences were made contiguous using the Sequencher program v.3.1.1. (Gene
Codes Corp.).

**Sequencing of mutant rad50 alleles:** Primers for internal sequencing and
amplification were based upon the wild-type sequence data and designed using the
OLIGO 5.0 program. Overlapping regions of DNA for sequencing original rad50 mutant
strains were amplified by PCR and directly sequenced. Template DNA for amplification
reactions consisted of rad50 mutant genomic DNA extracted from vegetative tissue
(ZOLAN and PUKKILA 1986) and treated for 5' at room temperature with 1 μl 0.1 mg/ml
RNase A (Amresco). PCR products were desalted using the QIAquick PCR Purification
Kit or extracted from agarose gels using the QIAEX II kit (QIAGEN INC.). Sequencing and analysis were performed using standard procedures.

**RT-PCR on Ccrad50 mutants:** RT-PCR was performed on RNA extracted from heterozygotes of rad50 mutant and wild type mushrooms. The two strains, rad50-1 and rad50-4, were mated to strain PJP 307, a meiotically wild-type strain. The resulting dikaryons were subcultured twice and polyA+ RNA was extracted from meiotic tissue at K+6 (RNeasy Plant Mini Kit: QIAGEN, PolyATtract® System 1000: Promega). First strand cDNA was made using the Superscript kit (GibCo-BRL Life Technologies). For rad50-1, primers were designed to be cDNA-specific and to span introns 3 and 5. For rad50-4, a cDNA-specific forward primer spanning intron 19 was used in combination with a 3’ UTR reverse primer or an intron 22-spanning cDNA-specific primer. PCR amplification and sequencing were performed by standard techniques.

**Protein modeling of CcRad50 mutants:** Protein images were based on the structure of the *Pyrococcus furiosus* Rad50 protein (HOPFNER et al. 2002a; HOPFNER et al. 2001; HOPFNER et al. 2000b). CcRad50, CcRad50-4, and CcRad50-5 were modeled using the MOLSCRIPT program written by Per J. Kraulis (KRAULIS 1991).

**Electron Microscopy (EM):** Chromosome spreads were performed as described by PUKKILA et al. (PUKKILA and LU 1985; PUKKILA et al. 1992b). Silver nitrate staining of meiotic nuclei was performed as per SEITZ et al. (1996). Grids were prepared as per PUKKILA et al. (1992b) and viewed with a JEOL-1010 electron microscope. Images of stained nuclei were scanned into a computer and analyzed using Adobe Photoshop 7.0 (Adobe Systems Inc.), NIH Image 1.63 (Wayne Rasband, NIH), and Microsoft Excel ’98 (Microsoft Corp.).
Epistasis analysis of late meiotic phenotypes: Slices of cap tissue were taken at various time points after karyogamy (as defined in Seitz et al. 1996), and veil cells were peeled and discarded. The tissue was stored in 70% ethanol, 30% NS buffer (20mM TRIS-HCl, pH 7.5, 0.25M mannitol, 1mM EDTA, 1mM MgCl₂, 0.1mM CaCl₂) at 4°C. Single gill layers of meiotic cells were dissected and mounted onto ProbeOn Plus (Fisher Scientific) microscope slides with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI; Vectashield, Vector Laboratories), and examined. For the rad50 experiments (Figures 3A, 3B, and 3C), slides were examined using a Nikon Microphot-FXA microscope with a Ludl filterwheel. Images of stained nuclei were captured using a Photometrics cooled digital CCD camera system and Quips XL SmartCapture/IP Lab Spectrum imaging software (Vysis). For the mre11-1 experiments (Figure 3D), images were taken using a Nikon E800 fluorescence microscope, driven by the Metamorph imaging system (Molecular Devices). Images were taken as z-series stacks using a step size of 0.09μm, capturing all planes in which the tissue sample was in focus. Each stack was then flattened into a single image using the maximum pixel brightness.

Rad51 protein expression and purification: We constructed a Rad51 protein expression construct by cloning the full-length rad51 cDNA (Stassen et al. 1997) into the pET-30 expression vector (Novagen), which was then transformed into BL21(DE3) cells for expression. Protein expression conditions are similar to Novagen company literature and alterations are described in full in supplemental methods.

Anti-Rad51 Antibody production: Purified Rad51 in SDS-PAGE gel slices was sent to Cocalico Biologicals, Inc. (Reamstown, PA) and used for immunization of rabbits according to their injection schedule, using the acrylamide as the adjuvant.
Affinity purification of anti-Rad51 antibody: To remove vector-encoded tags, serum was adsorbed using an affinity column made with a portion of Mre11 expressed in the same vector. Anti-Rad51 antibody was then purified using a second affinity column. A detailed description is in supplemental methods.

Commercial antibodies: Anti-Human Rad51 was obtained from Calbiochem (PC130). Purified non-specific IgG from rabbit serum was obtained from Sigma (18140).

Immunostaining of chromosome spreads: Staining of chromosome spreads was performed using the following protocol, which was adapted from Sym et al. (SYM et al. 1993). Chromosome spreads were obtained as described (PUKKILA et al. 1992a; SEITZ et al. 1996) except that we used uncharged slides (VWR), cleaned with Sparkle glass cleaner (A.J. Funk and Co. IL). Slides with chromosome spreads were wetted in PBS pH7.4 for 10 minutes. The slides were then blocked with 100μl of PBS containing 5% BSA and 1% goat normal serum (Vector laboratories Inc. cat#S-1000) twice for 15 minutes each at room temperature, under a parafilm coverslip. The slides were incubated with 100μl anti-Rad51 antibody diluted to a final concentration of 1μg/ml in PBS containing 5% BSA, covered with a parafilm coverslip, and incubated overnight protected from light in a humid environment. Slides were then washed three times for 10 minutes each with PBS+ 0.05% Tween-20 at room temperature. Secondary antibody was then added to the slides at a final concentration of 1.2μg/ml, in 5%BSA dissolved in PBS, for four hours at room temperature protected from light in a humid environment. Slides were then washed three times for 10 minutes each with PBS plus 0.05% Tween-20 at room temperature. Slides were mounted with DAPI in Vectashield mounting medium, sealed with nail polish, and stored at 4°C protected from light until
examined. For data analysis, an experiment was defined as the images taken from one slide, made using cells from one mushroom. Slides made from mushrooms that failed to complete fruiting body development were not used. For each combination of strain and time point examined, sufficient experiments were performed that at least two mushrooms were used and the total number of images combined across experiments was greater than or equal to 30. Antibodies from separate purifications gave statistically similar results; therefore, data obtained using antibodies from different purifications were pooled. For averages and statistical tests, the number of foci from images generated from different experiments was pooled.

**Immunostaining of tissue:** Meiotic spindles were detected in single layers of meiotic tissue with DM1A mouse anti-α-tubulin antibody (Accurate Chemical and Scientific Corp.) using the conditions described in Celerin *et al.* (2000).

**Irradiation of spo11-1:** At K+1 or K+3 *spo11-1* mushrooms were irradiated with 60 krad of gamma radiation, using a $^{137}$Cs irradiator (Mark 1 model 68-A, J.L. Shepard and Associates), at 1.2 krad/min. Chromosome spreads were made at K+2 or K+4, i.e., 10 minutes after samples were removed from the irradiator.

**Fluorescence in situ hybridization (FISH):** FISH was performed as described (Li *et al.* 1999b). Probes numbered 3 and 6 described in Li *et al.* (Li *et al.* 1999b) were used, representing telomeric and interstitial sites respectively, of chromosome 13.

**Statistical analyses:** For spore viability comparisons and for data from immunostraining of chromosome spreads using anti-Rad51 antibody, comparisons between mean numbers for two groups were performed using a student’s t-test, while multiple pairwise comparisons were performed using an ANOVA test and, where
appropriate, a Tukey post hoc test. All tests were performed using Minitab (Minitab Inc.), or SPSS (SPSS Inc.)

For FISH and EM data, statistical comparisons between strains were performed using chi-square tests, using the Excel program (Microsoft). For the analysis of EM data, the categories AE only and SC were pooled.
RESULTS

Cloning of *C. cinereus* rad50 and its identification as rad12: We aligned Rad50 protein sequences from humans, mice, *S. cerevisiae*, and *A. thaliana*, and designed primers to amplify a fragment within the relatively conserved 5' region of the gene. Using *C. cinereus* genomic DNA as template, we amplified and sequenced a 363 bp fragment, which BLAST (ALTSCHUL et al. 1997) analysis showed was likely orthologous to Rad50. Previous restriction fragment length polymorphism (RFLP) mapping had shown that the *C. cinereus* rad12 locus is on chromosome 8 in strain Okayama-7 (ZOLAN et al. 1993). Therefore, the rad50 PCR fragment was used to probe colony blots of a cosmid library enriched for chromosomes 8 and 9 from the Okayama-7 strain of *C. cinereus* (ZOLAN et al. 1992). Three cosmids were isolated, and the Cc_rad50 gene was sequenced from subclones of these cosmids. Because Rad50 orthologs are strongly conserved at the N- and C- termini, start and stop sites were defined by alignment to other Rad50 proteins. The *C. cinereus* genomic rad50 gene from strain Okayama-7 is 5091 bp in length, and the sequence we determined exactly matches the sequence reported for the same strain by the Broad Institute [Coprinus cinereus Sequencing Project, Broad Institute of MIT and Harvard (www.broad.mit.edu)].

To determine rad50 gene structure, we used RT-PCR to isolate overlapping portions of rad50 cDNA, and we compared cDNA sequence with genomic sequence to identify introns. The Cc_rad50 coding region is 3930 bp in length and is interrupted in the genome by 22 introns (Figure 1A; Table 1; Genbank accession number 1017584). The
predicted protein is 1309 amino acids in length, with a molecular weight of 150.7 kDa (Figure 1B).

To test whether the rad50 gene maps to the rad12 genetic locus, we analyzed progeny of a cross between a rad12-4 strain (rad12-4;4-1) and the backcross parent (Okayama-7; VALENTINE et al. 1995). Strains were assayed for radiation sensitivity, genomic DNA was isolated and cleaved with EcoRI, and fragments were separated on agarose gels. A Southern blot of the digested DNA was probed with a cosmid containing the rad50 sequence. The enzyme digest revealed a polymorphism between the radiation-sensitive (rad12) and radiation-resistant (wild-type) parent. No recombinants were found in 115 isolates examined, indicating that rad50 is tightly linked to rad12.

A cosmid containing the complete rad50 gene and was used in transformation rescue of the rad12-4 allele. The cosmid complemented both the radiation sensitivity and spore formation defects of 34/97 (35%) of the transformants, and 6/97 (6.2%) were complemented for one defect but not both.

Previous work had identified 16 mutants in the rad12 complementation group (VALENTINE et al. 1995; Table 1). Overlapping fragments of the rad50 gene were amplified from DNA isolated from all 16 alleles, and mutations were found within the gene for all but one allele. Based on the RFLP mapping, complementation, and DNA sequence data, we renamed the C. cinereus rad12 gene rad50, and will henceforth refer to the gene as rad50.
The predicted structures of mutant *C. cinereus* Rad50 proteins: Most *C. cinereus* rad50 mutations (Table 1; Figures 1B and 1C) are single base pair changes resulting in stop codons that truncate the Rad50 protein in the first half of the molecule. The shortest of these predicted mutant proteins is Rad50-11, with a premature stop after amino acid 195, and the longest is Rad50-12, predicted to be 722 amino acids. We found that two alleles, *rad50-1* and *rad50-4*, have mutations in intron splice acceptor sites. For *rad50-1*, genomic sequencing revealed a G to A transition mutation in the AG' splice acceptor site of intron 4 (Table S1). We performed RT-PCR using RNA isolated from mushroom caps of a heterokaryotic (*rad50-1* x wild type) strain, cloned the products, and sequenced multiple clones. We found only two types of clones, either wild-type sequences (28/36 clones sequenced) or sequences representing RNAs that had been spliced at the first available AG (italics in Table S1) after the mutated splice junction (8/36 sequenced clones). The predicted result of the mutation is a Rad50 protein that is normal for 360 amino acids, followed by a four-amino acid sequence in which three of four positions are incorrect and then truncation; the resulting protein would be 364 amino acids in length.

Genomic sequencing of *rad50-4* revealed an A to C transversion mutation in the splice acceptor site of intron 20 (Figures 1B and S1; Tables 1 and S1). We sequenced a total of 81 clones from RT-PCR products amplified three separate times from caps of *rad50-4* x wild type crosses, and found that in 24 clones intron 20 was correctly spliced; these clones were expected because the heterokaryotic crosses contained one wild-type allele of *rad50*. For 20 clones, the intron had not been spliced out at all, although upstream and/or downstream introns were spliced in those molecules. The remaining
clones revealed three alternatively spliced products. Twenty-one clones had been spliced at the first AG after the normal splice site, six had been spliced at the second AG, and seven had been spliced at the third AG (Figure S1). Therefore, rad50-4 is predicted to encode a mixture of mutant proteins. If intron 20 is not spliced at all, the resulting protein is identical to wild type for 1189 amino acids and then has 24 incorrect amino acids before a premature stop. If splicing is at the first AG after the wild-type splice site, the resulting protein has a deletion of five amino acids (‘VVMTK’, aa 1190-94), if splicing is at the second AG, the protein has a deletion of seven amino acids (‘VVMTKDKDQ’, aa 1190-96), and if splicing is at the third AG, the protein has 1190 correct amino acids, 11 incorrect amino acids, and then a stop.

Three rad50 strains have the same mutation, a single T to C transition that results in the substitution of a proline for a leucine at amino acid 742 (Figure 1B; Table 1). The mutants have at least two independent origins; the first, rad50-5, was isolated from a screen that was separate from the mutagenesis that generated the other two (VALENTINE et al. 1995). However, it is possible that rad50-9 and rad50-10 are actually derived from the same mutagenic event and that the isolate was sampled twice in the screen.

Two other substitution (missense) mutations were found, in rad50-8 and rad50-14, both of which lead to predicted changes within the Walker B box of Rad50 (Figure 1B; Table 1). Rad50-8 has a leucine to serine change at amino acid 1234, and Rad50-14 has an aspartic acid to asparagine change at position 1235. One DNA deletion allele (rad50-6) and one insertion allele (rad50-15) were also found; rad50-6 results in an in-frame deletion of eight amino acids within the second coiled coil domain of Rad50,
and rad50-15 has an insertion of 10 base pairs (TAC GCA AGG G) at position 2929 of the cDNA (Figure 1B; Table 1); the predicted protein is wild type in sequence until amino acid 976 and then has 25 missense amino acids before it ends after amino acid 1001, thus deleting the entire carboxy terminal globular domain of the protein.

**Molecular modeling:** Diagrams of predicted Rad50 proteins for alleles studied in depth are shown in Figure 1C. To analyze structural repercussions of mutant Rad50 proteins in more detail, we modeled the predicted effects of the seven amino acid deletion in Rad50-4 and of the amino acid substitution in Rad50-5 based on crystal structures of *P. furiosus* Rad50 (Figure 2; HOPFNER et al. 2000b). Sequence alignments show the equivalent region encompassing the *C. cinereus* Rad50-4 deletion maps to *P. furiosus* Rad50 beta sheet β9 (Figures 2A and 2B). In the ATP bound structure of *P. furiosus* Rad50, sheets β9 and β10, and the neighboring Rad50 ABC signature motif directly participate in formation of the ATP binding pocket and the nucleotide-mediated Rad50 dimerization interface. When deleted in the rad50-4 mutant, removal of β9 likely causes destabilization of the β8-β9-β10 sheet, disruption of the signature motif, and distortion of the ATP binding pocket (Figures 2A and 2B). The signature motif is critical for Rad50 dimerization, and for MRN complex DNA binding (HOPFNER et al. 2002b). A signature motif mutation in *P. furiosus* Rad50 protein was found to dramatically lower ATP binding (MONCALIAN et al. 2004); however, equivalent mutations in the *S. cerevisiae* and human proteins are defective in adenylate kinase activity but not ATP binding or hydrolysis (BHASKARA et al. 2007).
The Rad50-5 mutation, a Leucine to Proline substitution, occurs at the beginning of the second coiled coil domain of the protein (Table 1, Figures 1B and 2D). This is predicted to result in partial or complete destabilization of the portion of the coil just after the hook domain (Figure 2C).

**Meiotic defects in rad50 mutants**

**Spore viability of rad50 mutants and heterozygotes:** Because Rad50 functions as a multimer (HOPFNER et al. 2000b), the rad50-1, rad50-4, rad50-5, rad50-8, and rad50-15 mutants were assessed for dominance using a spore viability assay of homozygous (homokaryotic) versus heterozygous (heterokaryotic) strains. Five experiments were performed for each strain, at least two hundred spores were scored for each experiment, and spore viabilities were averaged. Spore viability for the wild type strain, J6;5x4, was 87.8%. Mutant strains rad50-1, rad50-4, rad50-5, rad50-8 and rad50-15 and had average spore viabilities of 2.0%, 4.4%, 3.8%, 18.7%, and 16.7%, respectively. The heterokaryons of rad50-1, rad50-4, rad50-5, rad50-8 and rad50-15, in each case crossed to wild type, gave spore viabilities of 78.8%, 80.4%, 79.9%, 89.3% and 80.1%, values which exhibited no statistically significant difference from wild-type mushrooms (ANOVA analysis; p value > 0.05).

**Diffuse diplotene arrest of rad50 mutants:** Meiosis in *C. cinereus* is synchronous, and takes about 12 hours from karyogamy through the second meiotic division (PUKKILA et al. 1984; RAJU and LU 1970). At six hours after karyogamy (K+6), essentially all meiotic cells in the mushroom cap are in pachytene (PUKKILA et al. 1984; SEITZ et al. 1996); homologous chromosomes are fully condensed and paired, and the SC is fully
developed. At about eight hours after karyogamy (K+8), cells enter diffuse diplotene. At this stage, the SC has dissipated, and chromosomes are diffuse in appearance. Nine to ten hours after karyogamy (K+10) chromosomes undergo a second condensation and enter metaphase I, and by 12 hours after karyogamy (K+12) all basidia have completed both meiotic divisions and contain a tetrad of haploid nuclei (Figure 3A).

Previous analysis of chromosome spreads of rad50-1, rad50-4, and rad50-15 (RAMESH and ZOLAN 1995) showed that they undergo karyogamy 30-60 minutes later than wild-type strains but are similar to wild-type strains in the timing of the formation and dissolution of AE and SC during meiosis. That is, although rad50 mutants do not achieve complete SC formation (i.e., pachytene), the timing of maximal AE and SC formation is comparable to that of wild type, and the AE and SC that do form then disperse at times similar to that of wild type. However, we also found that rad50 mutants arrest in diffuse diplotene. We extended this study within our collection of rad50 mutants, and examined single-layer gill segments taken from mushrooms at K+12, relative to karyogamy for (Table 1). We found that all rad50 mutants examined apparently arrest at diffuse diplotene except for rad50-8 (Figure 3A). The majority of basidia in rad50-8 arrest at metaphase I in a spindle-like shape, which implies that this mutant progresses further through meiosis. To examine rad50 arrest in more detail, we stained arrested tissue from rad50-4 with both DAPI and an antibody against α tubulin (Figure 3B). We found that 43% of the cells arrested with larger discs of chromatin, and that these were without organized spindles, while 57% of cells had more compact DAPI-staining discs and spindle-shaped microtubule organization (n=60). Therefore, the
The *rad50-4* arrest phenotype is actually a mixture of two distinct, arrested cell types, probably corresponding to diffuse diplotene and condensed chromatin entering metaphase I. The *rad50-8* allele allows all cells to progress to the metaphase I-like state.

**The *spo11-1* mutation is epistatic to *rad50* arrest:** In *S. cerevisiae*, most *rad50* mutants are phenotypically similar to *spo11* null mutants, in that meiotic DSBs are not formed. However, in *rad50S* mutants (ALANI et al. 1990), DSBs are made but not processed; Spo11 protein remains bound to the 5’ ends of the DSB (KEENEY et al. 1997). In *C. cinereus*, the *spo11-1* mutant is likely defective at DSB formation, because gamma irradiation of mushrooms partially suppresses the mutant’s meiotic defects (CELERIN et al. 2000); in addition, Rad51 foci are not induced during meiosis in *spo11-1* (Figures 4F, S3 and S4). In *spo11-1*, meiotic cells progress into an aberrant, metaphase I-like stage and then undergo programmed cell death (PCD; Figure 3C; CELERIN et al. 2000). In contrast, *rad50* mutants remain arrested in either diffuse diplotene or a metaphase I-like state for several hours (RAMESH and ZOLAN 1995; Figure 3A).

We thought it possible that the *C. cinereus rad50* mutants were all permissive for meiotic DSB formation but defective in DSB processing. If this were the case, then *spo11-1*, which does not make meiotic DSBs, would be epistatic to *rad50* mutations. To test this prediction, we constructed *rad50;spo11-1* double mutants, and found that they underwent signature PCD chromatin changes characteristic of the *spo11-1* single mutant (data for the *rad50-4* and *rad50-8* mutants are shown in Figure 3C; similar results were obtained for *rad50-5*). This result indicated that Spo11 has activity in *rad50*...
mutants, and that this activity is required for the rad50 mutant arrest. In a previous study, we found that mre11-1 arrests variably at diffuse diplotene or in a metaphase I-like state (GERECKE and ZOLAN 2000). We constructed an mre11-1; spo11-1 double mutant and found that it also exhibited a spo11-1 PCD phenotype, indicating that spo11-1 PCD is epistatic to the mre11-1 arrest phenotype (Figure 3D). Therefore, Spo11 likely functions before both Mre11 and Rad50 in C. cinereus meiosis; this observation also implies that Spo11-catalyzed DSB formation may be MRN complex independent in C. cinereus.

**Rad51 foci are induced during meiosis for some rad50 alleles:** We reasoned that if Spo11 forms DSBs in C. cinereus MRN complex mutants, then the array of Rad50 mutant proteins in our alleles might vary in their ability to process those breaks and succeed with recombination-dependent events of prophase I. As shown in S. cerevisiae (reviewed in (KEENEY 2008)), break formation is followed by MRN complex-dependent processing, which removes Spo11 from the 5' ends of the breaks and asymmetrically forms single-strand ends that bind Rad51 and Dmc1 and invade a non-sister chromatid. To investigate recombination events in C. cinereus, we raised and purified antibody against full-length Rad51 protein (Figures S2, S3, and S4) and used it to examine the time course of Rad51 focus formation during meiosis in a wild-type dikaryon (Figure 4). We found that Rad51 foci are induced after karyogamy and peak in number at K+4 and K+6, corresponding to the zygotene and pachytene stages of prophase I, and our results are similar to those obtained previously using immunofluorescence staining of C. cinereus tissue sections with an independently derived anti-Rad51 antibody (NARA et al. 2001). In contrast, Rad51 foci are not induced during meiosis in spo11-1 (Figures 4F,
S3 and S4), in which the number of foci was not significantly different from that seen in wild type before and during karyogamy (in two-sample t-tests: K-1, p=0.830; K+0, p=0.258), and remained essentially constant throughout prophase I. Our results with spo11-1 are similar to those reported for Dmc1 by Holzen et al. (Holzen et al. 2006), who found a low, residual number of foci in an S. cerevisiae spo11 mutant.

To compare wild-type and mutant strains, we examined Rad51 foci at K+4 in mre11-1 and an array of rad50 mutants. We found that mutant strains fell into two clear classes (Figure 5A). The first, which includes mre11-1, rad50-1, rad50-4, and rad50-5, formed numbers of foci that were not significantly different from that found in spo11-1. The second class of mutants, rad50-8, rad50-14, and rad50-15, formed significantly greater numbers of Rad51 foci than spo11-1, although significantly fewer foci than wild type. These three strains were examined further for the timing of Rad51 focus formation. In rad50-14, the number of foci increased at later meiotic stages, to levels greater than that of wild type by K+8 (Figure 5B). For both rad50-8 and rad50-15, early meiotic time points showed higher than wild-type type levels of foci at K+1 and then a decrease in foci to an intermediate level by K+4 (data for rad50-15 are shown in Figure 5B). For rad50-15, we further examined its time course by staining samples taken before karyogamy (K-1 and K+0), to examine the initial appearance of foci, and at K+6 and K+8, to determine whether the number of foci remains constant at these later time points (Figure 5B). At K-1 and K+0, the numbers of Rad51 foci in a rad50-15 strain were not significantly different from those in either spo11-1 or wild type (ANOVA p=0.107 at K-1 and p=0.330 at K+0). This indicates that the increase in foci seen in rad50-15 at K+1 is occurring in response to breaks formed during meiotic prophase and
not during the karyogamy or pre-meiotic S phases. Examination of the later time points showed a persistent level of foci, likely indicating that unresolved recombination events remained.

If Rad51 foci in rad50 mutants are meiosis-specific, they should be Spo11-dependent. As predicted, a rad50-8; spo11-1 double mutant examined at K+6 had significantly fewer foci (22.9 ± 3.0) than that observed in rad50-8, rad50-14, or rad50-15 at any time point (p<.001).

**Synaptonemal complex formation in rad50 mutants:** To characterize SC formation in rad50 mutants, we examined electron micrographs of silver-stained surface-spread nuclei. The three rad50 alleles found to be defective for Rad51 focus formation, rad50-1, rad50-4, and rad50-5 (Figure 5A) were also severely deficient for SC formation (Figures 6 and 7), and our results for rad50-1 and rad50-4 (which had been examined previously; RAMESH and ZOLAN 1995). In contrast, the three rad50 alleles with significantly more Rad51 foci than spo11-1, rad50-8, rad50-14, and rad50-15, formed more AE and SC than the severely defective alleles (Figure 6). The allele rad50-15 had previously been examined after one outcross generation (RAMESH and ZOLAN 1995), and our results were similar to those previously obtained.

**Homolog pairing:** The concordance between elevated (relative to spo11-1) numbers of Rad51 foci and elevated SC formation for rad50-8, rad50-14, and rad50-15 led us to ask whether stable homolog pairing correlates with these indicators of recombination activity. We used FISH (LI et al. 1999a) to examine the extent of meiotic pairing on two sites on chromosome 13, one telomeric and one interstitial, for rad50-1, rad50-5, rad50-8, and rad50-14 (Table 2). The data for rad50-1 were similar to those obtained
previously for \textit{mre11-1} (GERECKE and ZOLAN 2000) and those we obtained for \textit{spo11-1}; approximately 25\% of nuclei showed pairing for both probes at K+6. In contrast, \textit{rad50-5} had less pairing than any other strain; only 13\% of nuclei had both loci paired at K+1, and only 6\% had both loci paired at K+6.

The \textit{rad50} strains that had some partial recombination activity exhibited a higher amount of pairing at K+6 (\textit{rad50-8}, 36\%; \textit{rad50-14}, 40\%) than \textit{rad50-1} (24\%), although with our dataset this difference is not statistically significant (p=0.202 for the \textit{rad50-8} comparison with \textit{rad50-1} and p=0.096 for the \textit{rad50-14} comparison).

Because we used both a telomere-proximal probe (LI \textit{et al.} 1999a) and an interstitial probe we were able to examine differences in pairing on the same chromosome. For the \textit{rad50-1} strain we observed a greater amount of pairing with the interstitial probe as compared to the telomeric probe. This difference was significant at K+1 (70\% to 33\%; chi square test p<0.001), but was not significant at K+6 (p=0.342). In addition, none of the other \textit{rad50} mutants examined showed a significant difference in percent pairing between the two probes at any time point.

\textbf{Analysis of AE formation in \textit{rad50} mutants:} Most studies of Rad50 have focused on mutations that affect the catalytic activities of the MRN complex. In addition, the hook portion of the Rad50 protein has been shown to be of critical importance in both intra-complex and inter-complex interactions (HOPFNER \textit{et al.} 2002a) and in the in vivo functions of the complex (WILTZIUS \textit{et al.} 2005). We were interested in separating the functions of Rad50 in meiotic DSB formation and processing from any structural role the
protein may have in meiosis. Therefore, we investigated AE formation by rad50 mutants in the spo11-1 background, in which DSBs are not made.

Using rad50;spo11-1 double mutants and sibling progeny that were single mutants for each gene or wild type, we generated electron micrographs of silver-stained, surface-spread meiotic nuclei and scored each strain for AE and SC formation (Figure 7; Table 3). AE and SC lengths were measured from nuclear spreads taken from one mushroom of each strain (data for rad50-4 and rad50-5 are shown in Figures 7B and 7D).

As expected and as previously reported for other spo11-1 strains (CELERIN et al. 2000), nuclei from the spo11-1 single mutants showed either no structure or AE formation (Figure 7; Table 3). The number of AE segments and their lengths in those spo11-1 nuclei that formed AE were variable, with from 5 to 42 AE segments forming per nucleus, individual AE lengths ranging from 0.3 µm to 20 µm, per nucleus lengths ranging from 12 to 173 µm, and overall AE length per nucleus ranging from 12 to 206 µm. The average AE lengths per nucleus for the two spo11-1 strains shown in Figure 7 were 73 and 126 µm, for the strains shown in Panels A and C, respectively. For comparison, a congenic wild-type strain consistently formed 26 AE, with an average AE length per nucleus of 124 µm. Although the rad50-1, rad50-4, and rad50-5 single mutants were similar to one another, the rad50-4;spo11-1 double mutant was strikingly different from the rad50-1;spo11-1 and rad50-5;spo11-1 double mutants (Figure 7; Table 3). We found that the presence of Rad50-4 was sufficient for the formation of long AE’s in a spo11-1 mutant background (Figures 7A and 7B). For those nuclei that formed AE, the number of AE segments ranged from 17 to 40, and the average AE
length per nucleus was 153 µm. However in both the rad50-1;spo11-1 and rad50-5;spo11-1 double mutants, little AE was found.

Chi square analysis showed that for rad50-4;spo11-1, the distribution of nuclei into classes with chromosome structures (AE and/or SC) and without structure was the same as for spo11-1 (p=0.116). In contrast, for both rad50-1;spo11-1 and rad50-5;spo11-1, the distributions of nuclei in the two categories were significantly different from that of the sibling spo11-1 strain and in all but one case not significantly different from that of the sibling rad50 single mutant. For rad50-1, progeny from one cross to spo11-1 were examined (Table 3, Cross B), and the rad50-1;spo11-1 double mutant was different from spo11-1 (p=0.018) and the same as rad50-1 (p=0.322). For rad50-5, two different crosses were examined (Table 3, Crosses C and D). For progeny from the first cross, the rad50-5;spo11-1 double mutant was significantly different from spo11-1 (p<0.001) and not different from the rad50-5 single mutant (p=0.322). For Cross D, two different rad50-5;spo11-1 double mutants were examined. The first (48x6) was significantly different from spo11-1 (p<0.001) and the same as rad50-5 (p=0.754). The second (40x70) was significantly different from spo11-1 (p=0.047) but also significantly different from the sibling rad50-5 strain examined (p=0.007). Overall, our data show that the disruption (in Rad50-5) or absence (in Rad50-1) of the Rad50 hook-proximal coiled coil makes the formation of stable AE unlikely. Our results indicate that there is a structural role for Rad50, separate from the DSB-processing activity of the MRN complex, which is required for the formation of meiotic chromosome structures.

DISCUSSION
**C. cinereus rad50 mutations are recessive:** For proteins that normally function as multimers, mutants that can assemble into complexes often exhibit dominant negative phenotypes in heterozygotes (HERSKOWITZ 1987). Because of the known structure of the Mre11/Rad50 complex, we expected that at least some rad50 alleles would behave as dominant negatives. However, heterokaryon spore viabilities did not show statistically significant differences from wild-type spore viabilities, indicating that the rad50 mutations examined (rad50-1, rad50-4, rad50-5, rad50-8, and rad50-15) are not dominant. A simple explanation for this would be instability of all mutant Rad50 proteins. However, the phenotypes of a number of rad50 mutants are distinct (e.g., Figure 4; RAMESH and ZOLAN 1995); therefore at least some of them make stable protein. In addition, recent studies have shown that Rad50-5 forms foci on meiotic chromosomes (A. Many, C. Melki, D. Maillet, S. Acharya, and M. Zolan, manuscript submitted). Therefore, although it is possible or even likely that some alleles (e.g., rad50-1) encode unstable protein, the recessive nature of all rad50 mutations examined cannot be explained by a simple absence of stable protein. It is possible that none of the mutant proteins can form sufficiently stable dimers with wild-type Rad50 to become incorporated into complete MRN complexes or to diminish the pool of wild-type complexes. In any case, the pool of fully functional MRN is sufficient in the heterokaryons for the production of viable meiotic products, although subtle effects on meiosis (e.g., on crossover frequency or distribution) would not have been detected by our analysis.

**C. cinereus rad50 mutants form meiotic DSBs:** In rad50;spo11-1 and mre11-1;spo11-1 double mutants, the spo11-1 PCD phenotype is epistatic to the rad50 or
mre11-1 phenotype, in all allele combinations examined (Figure 3 and data not shown). Therefore, our data imply that Spo11 has activity in rad50 and mre11 mutants. Although Spo11 has meiotic functions that are independent of its DSB-forming activity (e.g., CHA et al. 2000), the spo11-1 single mutant forms essentially no SC (CELERIN et al. 2000), whereas rad50 and mre11 mutants form SC in variable amounts (RAMESH and ZOLAN 1995; GERECKE and ZOLAN 2000; this work). As discussed recently by Borde (2007), the partial SC formation in C. cinereus rad50 and mre11 mutants is similar to that seen in those S. cerevisiae MRN mutants (such as rad50S; ALANI et al. 1990) in which Spo11 forms but does not process meiotic DSBs (KEENey et al. 1997). In addition, like S. cerevisiae rad50S (GASIOR et al. 1998), some of the C. cinereus rad50 alleles form Rad51 foci at levels lower than that observed in wild-type strains but significantly higher than that found for spo11-1 (Figure 5). Because the Rad51 foci correlate in the rad50 alleles with enhanced levels of stable homolog pairing and SC formation (relative to spo11-1; Figure 6 and Table 2), and because they are Spo11-dependent, at least a portion of them must represent progression through a normal meiotic program of Spo11-dependent DSB processing, leading to synapsis (KLECKNER 2006).

For the rad50 alleles that formed Rad51 foci, the time courses of focus formation and loss were different from that of the wild-type strain examined (Figure 5B). For rad50-14, the number of Rad51 foci increased at time points corresponding to late prophase in wild-type strains. The predicted Rad50-14 protein has an aspartate to asparagine substitution in the Walker B domain (Figure 1B; Table 1), which might result in DSB processing that is stalled or delayed compared to that in wild-type cells. An
alternative possibility is that these late events may not be related to Spo11-induced DSBs but may be repair events resulting from the accumulation of DNA damage as the cells attempt to proceed through meiotic prophase. For rad50-8 and rad50-15, we found that a large number of foci are observed at K+1 and decrease thereafter. One simple interpretation of this is that these foci mark recombination events in which an aberrant processing step occurs soon after the formation of meiotic DSBs, followed by disassociation of the MRN complex.

Because all of the rad50 alleles, and mre11-1, arrest in meiosis, because this arrest is Spo11-dependent (Figure 3), and because some of the alleles form inducible, Spo11-dependent Rad51 foci (Figure 5) and therefore must be forming DSBs, we think it likely that arrest in both mre11-1 and the collection of rad50 mutants is the result of unrepaired or improperly processed DSBs in the genome and that Rad50 and Mre11 are dispensable for DSB formation in C. cinereus, but required for appropriate DSB processing. This interpretation assumes that truncation alleles (e.g., rad50-1; mre11-1), in fact encode unstable protein. Alternatively, the Walker A box alone of Rad50 and the N-terminal 40% of Mre11 are sufficient to allow formation, but not complete and/or proper processing, of meiotic DSBs. Although S. cerevisiae rad50 and mre11 null or deletion mutants show no meiotic DSB formation, as assayed by pulsed field gel analysis of genomic DNA (CAO et al. 1990; NAIRZ and KLEIN 1997), rad50 deletion mutants of S. pombe do form meiotic DSBs (YOUNG et al. 2004). In addition, Spo11 has DSB-forming activity in mre11 mutants of S. pombe (YOUNG et al. 2004), and A. thaliana (PUIZINA et al. 2004). In Caenorhabditis elegans, Rad50 facilitates DSB formation by Spo11, but Rad50 is apparently not strictly required for Spo11 activity, because a defect
in a cohesin protein, Rec8, allows Spo11-dependent DSB formation in the absence of Rad50 function (Hayashi et al. 2007). Thus, S. cerevisiae may be the exception, rather than the rule, in its requirement for the MRN complex in meiotic DSB formation.

**Rad50’s structural roles in meiosis:** The C. cinereus rad50 mutants undergo varying amounts of AE and SC formation, and a comparison of the predicted Rad50 structures (Figures 1 and 2) with the meiotic phenotypes of the mutants (Ramesh and Zolan 1995; Figure 4) is informative. Three alleles, rad50-1, rad50-4, and rad50-5, are comparably severe as single mutants (except in homolog pairing, discussed below). Because rad50-1 encodes a severely truncated protein, it is not surprising that little function is retained, and this allele is most likely effectively null. For Rad50-4, modeling revealed that the loss of a β-strand (Figure 2A) likely distorts the ATP binding pocket and neighboring signature motif. The signature motif (in the C-terminal globular domain) of Rad50-4 may not be in the correct orientation for contact with the ATP bound to the N-terminal domain of another Rad50-4. This may result in a loss of Rad50 head domain dimerization (Moncalian et al. 2004). In addition, the signature motif is essential for the recently reported and essential adenylate kinase activity of S. cerevisiae Rad50 (Bhaskara et al. 2007) and may be abrogated in Rad50-4 molecules. Also, because the deletion is at the junction where the Rad50 globular ATPase domain adjoins the coiled-coil, it may affect coil stability in this region, and therefore Mre11 binding (Hopfner et al. 2001). An unspliced rad50-4 transcript is predicted to make a protein similar to Rad50-15, and transcripts containing an unspliced intron comprised about one-third of the mutant cDNAs examined. Given that there is a mix of aberrantly spliced transcripts in
the rad50-4 mutant, it is perhaps surprising that only the severe phenotype prevails. It may be that there is insufficient truncated, Rad50-15-like protein to allow SC formation in rad50-4.

The rad50-5 mutation causes a leucine to proline change at the beginning of the second coil (Figures 1, 2C and 2D). From modeling, it is predicted to result in either an interruption or distortion of the second coil. The stability of coiled-coil structures and their segmented structure (M. Oakley, personal communication) predict that only the beginning of the coiled coil region, and probably the hook region, are affected in Rad50-5. Therefore, it is striking that the meiotic defects in rad50-5 strains are as severe as those caused by the rad50-1 allele, and in fact, that homolog pairing is worse in rad50-5 than in rad50-1 or spo11-1 (Table 2). The analysis of Wiltzius et al. (2005) showed that the hook region of S. cerevisiae Rad50 is essential for DNA repair and meiotic DSB formation. Our data show that this region is also essential for the formation of stable AE and SC.

The requirement for the hook-proximal coiled coil of Rad50 in stable AE formation is shown dramatically by our analysis of AE formation in rad50;spo11-1 double mutants (Figure 7). For rad50-4, the spo11-1 mutation seems to be fully epistatic; the rad50-4;spo11-1 double mutant is the same as spo11-1 for both PCD (Figure 3) and AE formation (Figure 7; Table 3). In contrast, the spo11-1 phenotype of variable AE formation is masked, in rad50-1 and rad50-5 strains, by the inability of those mutants, even in the spo11-1 background, to form stable AE. Therefore, Rad50 has a structural role in AE formation that must be independent of its role in processing Spo11-catalyzed DSBs.
In contrast with rad50-1, rad50-4, and rad50-5, the rad50-15 mutant makes extensive AE and SC. This allele contains an insertion that leads to the production of a protein with 25 incorrect amino acids after position 976, followed by a stop (Table 1 and Figures 1B and 1C). The Walker A box is intact and the coiled domains presumably fold normally, but the globular domain containing the Walker B motif is missing. Since the Walker B motif is absent, and this mutant is radiation sensitive, it is unlikely that Rad50-15 is catalytically active. Similarly, rad50-8 and rad50-14 have mutations within the Walker B region, which should abrogate or at least diminish function. The phenotypes of these mutants imply that AE and SC formation are to a large extent independent of Rad50’s ATPase or adenylate kinase functions. Instead, the main role of Rad50 in DSB processing may be via its interaction with Mre11. Recent biochemical data from S. cerevisiae and S. pombe implicate Mre11 as the nuclease that removes the covalently bound Spo11 from meiotic DSBs (Neale et al. 2005; Williams et al. 2008). Based upon their predicted structures (Figures 1C and 2), we expect that Rad50-8 and Rad50-14 retain the ability to interact with Mre11. Although Rad50-15 is missing the N-terminal portion of the Mre11 binding site, its phenotype predicts that Rad50-15 does interact with Mre11 as well.

Although our data show that catalytic activity by the Rad50 molecules themselves cannot be essential for AE formation or DSB processing, none of the Rad50 mutant proteins allows the formation of wild-type levels of SC, and all mutant strains arrest in meiosis. Therefore, enzymatic activity conferred by intact C and N terminal globular domains is necessary for complete synapsis and meiotic progression.
What is the role of Rad50 in AE formation? AE formation results from structural changes in meiotic chromatin that may require Rad50, or require Rad50 for stability. In comparison to wild-type *S. cerevisiae* meiotic cells, *rad50Δ* strains show increased sensitivity of DNA to micrococcal nuclease treatment (OHTA *et al.* 1998). It is possible that in the absence of Rad50, or of its coiled coil regions, structural changes of the chromatin do not occur, and AE cannot form. It is additionally possible that Rad50 has a structural role either in the AE core itself, or in mediating appropriate sister-chromatid interactions that are necessary for AE formation. In *S. pombe* *rad50Δ* mutants, sister chromatid recombination is reduced and homologous recombination frequencies are increased (HARTSUIKER *et al.* 2001). The same is true of homologous recombination frequencies in *S. cerevisiae* *rad50Δ* strains (MALONE *et al.* 1990). Haploid *rad50Δ* G2 strains are more sensitive to radiation than diploid *rad50Δ* G1 strains, suggesting that Rad50 is associated with sister chromatid interactions (FABRE *et al.* 1984; IVANOV *et al.* 1992; PAQUES and HABER 1999). Additionally, a *C. cinereus* *rad50-4; msh5-22* double mutant is rescued for SC formation (MERINO *et al.* 2000). Therefore, the role of Rad50 in AE formation could also be due to its requirement in sister chromatid interactions. Although a recent study found no loss of mitotic sister-chromatid cohesion in *rad50* mutants of *S. cerevisiae* (WILTZIUS *et al.* 2005), there are several lines of study that argue that meiotic recombination imposes additional requirements for sister-chromatid cohesion. First, DSB repair requires the recruitment of additional cohesin to sites of DNA damage (STROM *et al.* 2004; UNAL *et al.* 2004). Cohesin recruitment to DSB sites requires Mre11, and although it is unknown whether the entire MRN complex is necessary, Mre11 does not localize to chromatin in the absence of Nbs1 (TAUCHI *et al.*
2001), arguing that complex formation is necessary. Second, a spo11 mutation partially suppresses the need for Rec8 and Spo76 in Sordaria macrospora (STORLAZZI et al. 2008), meaning that chiasma formation imposes an increased requirement for cohesion. Finally, the rad9-1 mutant of C. cinereus has no obvious mitotic growth defect but is almost completely defective in meiosis (ZOLAN et al. 1988). Rad9 is the C. cinereus ortholog of S. cerevisiae Scc2 (MICHAELIS et al. 1997), S. pombe Mis4 (FURUYA et al. 1998), D. melanogaster NippedB (ROLLINS et al. 1999), and the human delangin protein (KRANTZ et al. 2004; TONKIN et al. 2004) and is required for cohesin localization to chromatin (CIOSK et al. 2000). The difference in mitotic and meiotic phenotypes for rad9-1 argues for an enhanced role of sister-chromatid cohesion in meiosis. Because Rad50 is an SMC protein, structurally similar to members of condensin and cohesin complexes (LOSADA and HIRANO 2005; NASMYTH and HAERING 2005) its role in meiotic sister-chromatid interactions may be as a member of the MRN complex, required for cohesin recruitment (STROM et al. 2004; UNAL et al. 2004), or the Rad50 protein may be structurally required for appropriate sister-chromatid interactions. These two roles are not mutually exclusive, and it is also possible that the modification of chromatin structure by Rad50 may be a key factor in cohesin recruitment to sites of recombination.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grants GM43930 (to MEZ), CA117638 and CA092584 (to JAT), and Training Grant T32 GMO07757 (to SNA). We thank Claire Walczak and Joel Ybe for assistance in developing antibody purification techniques, Ellen Quardokus for immunofluorescence assistance and advice, and Jim Bever for statistics advice. We also thank F. Rudolph Turner for
preparation of electron micrographs, Katy Van Hook for preliminary antibody purification work, Martha Oakley for helpful discussion, Pat Pukkila and Elizabeth A. Sierra for helpful discussion and critical reading of the manuscript, and Megan Kingslover for clerical support. In addition, we thank Dr. Kengo Sakaguchi and his laboratory for their generous gift of the Dmc1 expression construct.
LITERATURE CITED


FURUYA, K., K. TAKAHASHI and M. YANAGIDA, 1998 Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G1 phase. Genes Dev 12: 3408-3418.


KRANTZ, I. D., J. MCCALLUM, C. DESCIPIO, M. KAUR, L. A. GILLIS et al., 2004 Cornelia de Lange syndrome is caused by mutations in NIPBL, the human homolog of Drosophila melanogaster Nipped-B. Nat Genet 36: 631-635.


MALONE, R. E., 1983 MULTIPLE MUTANT ANALYSIS OF RECOMBINATION IN YEAST. Molecular & General Genetics 189: 405-412.


PUKKILA, P. J., C. SKRZYNIA and B. C. LU, 1992a The rad3-1 mutant is defective in axial core assembly and homologous chromosome pairing during meiosis in the basidiomycete Coprinus cinereus. Developmental Genetics 13: 403-410.


STASSEN, N. Y., J. M. LOGSDON, JR., G. J. VORA, H. H. OFFENBERG, J. D. PALMER et al., 1997 Isolation and characterization of rad51 orthologs from Coprinus cinereus.


Williams, R. S., Moncalian, G., Williams, J. S., Yamada, Y., Limbo, O., Shin, D. S., Groocock, L. M., Cahill, D., Hitomi, C., Guenther, G., Moiani, D., Carney, J. P.,


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<tr>
<th>Allele</th>
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<th>Predicted Mutant Protein</th>
<th>Meiotic Arrest Stage</th>
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<tr>
<td>rad50-1</td>
<td>intron 4</td>
<td>See text</td>
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<td>Diffuse diplotene</td>
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<tr>
<td>rad50-2</td>
<td>644 TTG&gt;TAG</td>
<td>L215*</td>
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<tr>
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<td>Q254*</td>
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<tr>
<td>rad50-11</td>
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<td>K195*</td>
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<tr>
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<td>L742P</td>
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<td></td>
<td></td>
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</table>
aShaded rows denote alleles studied in depth and shown in Figure 1C.
bAll positions refer to nucleotides of the coding strand of the open reading frame except where indicated.
cNucleotide changes are in boldface.
dSingle-letter amino acid designations are used. An asterisk denotes a stop codon.
eThe end of intron 4’s sequence is changed from CAG to CAA in rad50-1.
fThe end of intron 20’s sequence is changed from TAG to TCG in rad50-4.
gn.d., not determined
TABLE 2. Homolog pairing

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<th>Telomeric probe</th>
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<th>Either probe</th>
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<td>78</td>
<td>72</td>
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<td>90</td>
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<tr>
<td>Wild type</td>
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<td>92</td>
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<td>56</td>
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<td>70</td>
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<td>20</td>
<td>13</td>
<td>33</td>
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<tr>
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<td>24</td>
<td>15</td>
<td>6</td>
<td>32</td>
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<tr>
<td>rad50-8</td>
<td>K+6</td>
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<td>43</td>
<td>43</td>
<td>36</td>
<td>51</td>
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<td>93</td>
<td>52</td>
<td>49</td>
<td>40</td>
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</table>

1Data are arrayed by time point and strain. For each combination of time point and strain at least two experiments, examining two different mushrooms, were performed. Data shown combine images from all experiments on a given time point and strain.
Table 3. Effect of hook-proximal coiled coil structure on AE and SC formation

<table>
<thead>
<tr>
<th>Straina</th>
<th>% SCb</th>
<th>% AE</th>
<th>% No Structure</th>
<th>Total (N)</th>
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<td></td>
<td></td>
<td></td>
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<td>rad50-4 11x30</td>
<td>6</td>
<td>15</td>
<td>79</td>
<td>79</td>
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<tr>
<td>spo11-1 93x44</td>
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<td>43</td>
<td>57</td>
<td>62</td>
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<tr>
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<td>49</td>
<td>44</td>
<td>120</td>
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<td><strong>Cross B: rad50-1 x spo11-1</strong></td>
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<td></td>
<td></td>
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<td>75</td>
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<td>55</td>
<td>55</td>
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<td>79</td>
<td>34</td>
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<td>248</td>
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<tr>
<td>rad50-5;spo11-1 40x70</td>
<td>7</td>
<td>14</td>
<td>78</td>
<td>222</td>
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</table>

aStrains used were dikaryons formed by crosses between the indicated, numbered, sibling monokaryons.

bFor some crosses, percentages do not sum to 100 due to rounding.
FIGURE LEGENDS

**Figure 1.** *C. cinereus rad50 gene, protein, and mutants.* (A) Gene structure. Solid areas represent exons and open areas represent introns. The translational start site is represented by ATG, and the translational stop is represented by TGA. (B) Rad50 protein and mutations. Stars denote the Walker A and Walker B Boxes. Asterisks denote nonsense mutations (stop codons). The Rad50-15 insertion is shown above the protein diagram and the Rad50-1, Rad50-6 and Rad50-4 deletions are shown below. Details of the changes in Rad50-1, Rad50-4, and Rad50-15 are given in Table 1, Figure S1, and the text. (C) Diagrams of predicted Rad50 mutant proteins. Crosses represent sites of single amino acid substitution, or, in the case of Rad50-4, its internal deletion and the altered position of the signature motif. Multiple predicted structures of Rad50-4 are summarized as the two classes that encompass the mutant proteins. Also show is a model of the MRN complex, after (Hopfner *et al.* 2002b) and (De Jager *et al.* 2004).

**Figure 2.** *Molecular models of C. cinereus Rad50.* (A) The Rad50-4 deletion removes sheet β9, and the β9-β10 connecting loop, thereby disrupting the Rad50 ATP binding and dimerization interaction site. Ribbon models were constructed using Pymol (www.pymol.org), and are based upon homology with the *Pyrococcus furiosus* Rad50 protein. (B) Alignment of the β9 and signature motif regions of *P. furiosus*, *C. cinereus*, *S. pombe*, and *H. sapiens* Rad50. The green-highlighted amino acids are deleted in one splicing variant of Rad50-4; in another, the five amino acids VVMTK are deleted. The ABC signature motifs are boxed in purple. (C) Rod model of the hook and part of
the coiled coil regions of wild-type Rad50 and Rad50-5. (D) A portion of the Rad50 protein sequence, showing the region mutated in Rad50-5.

**Figure 3.** *rad50* mutant and *mre11-1* arrests are Spo11 dependent. (A-C) Samples were taken from mushrooms 12 hours after karyogamy, fixed, stained with DAPI, and photographed. (A) Wild type, and *rad50-4* and *rad50-8* single mutants. (B) *rad50-4* stained with DAPI and with spindles detected using mouse anti-α-tubulin antibody, as described in Celerin et al. (2000). (C) A *spo11-1* single mutant compared with *rad50-4;spo11-1* and *rad50-8;spo11-1* double mutants. (D) Wild type, *spo11-1*, and *mre11-1* single mutants compared to *mre11-1;spo11-1*. Tissue samples, taken at K+10, were stained with DAPI and photographed as a z-stack series of images. Image stacks were flattened into a single image as described in the text. Scale bars represent 2 µm.

**Figure 4.** Anti-Rad51 staining in wild type and *spo11-1*: (A-E) Representative images of wild-type meiotic time course (A) Pre-leptotene image taken one hour after karyogamy (K+1). (B) Leptotene image taken at K+2. (C) Zygotene image taken at K+4. (D) Pachytene image taken at K+6. (E) Diplotene image taken at K+8. Scale bars represent 2µm for all images. (F) Mean number of Rad51 foci in *spo11-1* (♦) and in wild type (□). Error bars show 95% confidence intervals.

**Figure 5.** Mutants fall into two classes for Rad51 focus formation. (A) Mean number of Rad51 foci observed at K+4. (B) Time course of Rad51 foci in *rad50-14* and
rad50-15. Wild-type data (□) are the same as in Figure 4 and shown for reference. rad50-14 is shown by (●) and rad50-15 is shown by (▲). Error bars show 95% confidence intervals.

**Figure 6.**---SC formation in wild type, rad50-8, rad50-14, and rad50-15: Surface spreads of wild type (A) and rad50-14 (B) nuclei from the K+6 time point were stained with silver nitrate and photographed using transmission electron microscopy. Scale bars represent 2 µm for all images. (C) Percent SC and AE formation for each strain. The number of images categorized was as follows: wild type, 40; rad50-8, 78; rad50-14, 57; rad50-15, 100.

**Figure 7.**---The coiled coil of Rad50 proximal to the hook is required for AE formation: For A and C, samples were taken at 6 hours after karyogamy, fixed, spread, and stained and photographed using EM as described (SEITZ et al. 1996). For B and D, data from nuclear spreads from a single mushroom were plotted according to increasing SC length. Each graph corresponds to the genotype shown in the image above it. Images were chosen to represent the major class of nuclear spreads observed for each genotype. Scale bars represent 2 µm.
A. Rad50 Molecule 1 interacts with ATP and has a deletion in Rad50-4. Molecule 2 has a signature motif.

B. Helices β8, β9, and β10 are labeled, with an αF helix. The sequences for Rad50 in S. pombe, H. sapiens, P. furiosus, and C. cinereus are shown with deletions highlighted.

C. Rad50 monomer A contains a Zn²⁺ ion and interacts with monomer B.

D. The sequence shows a semi-zinc finger motif with sequences for coil 1 ending and coil 2 beginning, labeled with amino acid positions and mutations such as L742P.
Figure 3

A

WT  rad50-4  rad50-8

B

rad50-4  spo11-1  rad50-4; spo11-1  rad50-8; spo11-1

D

WT  spo11-1  mre11-1  mre11-1; spo11-1
Figure 4

A B C D E

F

Mean number of foci

Timepoint
Figure 5

A

B

Mean number of foci

Timepoint

Wild type  spo11-1  mre11-1  rad50-1  rad50-4  rad50-5  rad50-8  rad50-14  rad50-15

Strain

k-1  k+0  k+1  k+2  k+4  k+6  k+8
**Figure 6**

Table: Phenotype percentage

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<th></th>
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<th>rad50 -1</th>
<th>rad50 -8</th>
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<td>89.0</td>
<td>66.7</td>
<td>64.9</td>
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Figure 7

A

rad50-4  spoll-1  rad50-4;spoll-1

B

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C

rad50-5  spoll-1  rad50-5;spoll-1

D

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