

# Meiotic recombination protein Rec12: functional conservation, crossover homeostasis and early crossover/non-crossover decision

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## ABSTRACT

In fission yeast and other eukaryotes, Rec12 (Spo11) is thought to catalyze the formation of dsDNA breaks (DSBs) that initiate homologous recombination in meiosis. Rec12 is orthologous to the catalytic subunit of topoisomerase VI (Top6A). Guided by the crystal structure of Top6A, we engineered the *rec12* locus to encode Rec12 proteins each with a single amino acid substitution in a conserved residue. Of 21 substitutions, 10 significantly reduced or abolished meiotic DSBs, gene conversion, crossover recombination and the faithful segregation of chromosomes. Critical residues map within the metal ion-binding pocket toprim (E179A, D229A, D231A), catalytic region 5Y-CAP (R94A, D95A, Y98F) and the DNA-binding interface (K201A, G202E, R209A, K242A). A subset of substitutions reduced DSBs but maintained crossovers, demonstrating crossover homeostasis. Furthermore, a strong separation of function mutation (R304A) suggests that the crossover/non-crossover decision is established early by a protein–protein interaction surface of Rec12. Fission yeast has multiple crossovers per bivalent, and chromosome segregation was robust above a threshold of about one crossover per bivalent, below which non-disjunction occurred. These results support structural and functional conservation among Rec12/Spo11/Top6A family members for the catalysis of DSBs, and they reveal how Rec12 regulates other features of meiotic chromosome dynamics.

## INTRODUCTION

Meiosis couples DNA replication, the pairing of homologous chromosomes, and two rounds of chromosome

segregation to produce haploid meiotic products. Homologous recombination is induced to high levels in between DNA replication and the first, reductional division in which homologs migrate to opposite poles. Crossover recombination structures (chiasmata) function together with distal sister chromatid cohesion to align paired homologs (bivalents) on the metaphase plate of meiosis I in opposition to spindle tension (1). With few exceptions this process is crucial for a proper reductional division, because in the absence of chiasmata homologs segregate aberrantly and often to the same pole (non-disjunction). This leads to the production of aneuploid meiotic products that are typically inviable or, in some cases, contribute to the production of viable but aneuploid offspring (e.g. Down's syndrome) (2).

Meiotically induced, dsDNA breaks (DSBs) initiate recombination between homologous chromosomes (3). Meiosis-specific DSBs have been demonstrated directly in three highly diverged eukaryotes, the budding yeast *Saccharomyces cerevisiae* (4,5), the fission yeast *Schizosaccharomyces pombe* (6,7) and the ciliated protozoan *Tetrahymena thermophila* (8). Their presence in other eukaryotes, including mammals, has been inferred indirectly from PCR-based assays (9), from immunofluorescence localization of proteins that assemble at or near to the positions of DNA damage (10–12), and from the fragmentation of chromosomes in meiotic mutants (13,14). Such findings suggest that the initiation of meiotic recombination from DSBs is broadly conserved, which is not mutually exclusive with the hypothesis that some fraction of meiotic recombination is initiated by ssDNA nicks or gaps (15–21).

Proteins required for DSB-initiated meiotic recombination are, at least in part, also conserved. For example, budding yeast and fission yeast each require at least 10 different proteins for the formation of DSBs [(3,22,23) and references therein]. Some of these proteins have no reported orthologs in the other yeast; and some proteins are orthologous, but are required for DSB formation only in one of the two yeasts (e.g. Rad50 is required only in

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budding yeast, Rec8 is required only in fission yeast). Nevertheless, a subset of the proteins are conserved both at the amino acid sequence level and in their requirement for the formation of meiotic DSBs (Spo11/Rec12, Ski8/Rec14, Rec114/Rec7, Mei4/Rec24). And although overall sequence homology is often low (and hence can escape detection), functional orthologs of the yeast recombination proteins can be found even in mammals [for a recent example, see (24)]. Among conserved proteins known to be required for the formation of meiotic DSBs, Spo11 (Rec12) is of particular interest, because it is almost certainly the catalytic subunit of a protein complex that introduces recombination-initiating DSBs.

The Spo11 (Rec12) protein is ubiquitous in eukaryotes (25). Most eukaryotes have only one *SPO11* (*rec12*<sup>+</sup>) gene, while the remainder encode multiple paralogs among which one is dedicated to meiotic recombination (25–27). Spo11 family proteins share sequence homology with the catalytic subunit (Top6A) of type VI topoisomerases in Archaea (25,28–33). Type VI topoisomerases are members of the topoisomerase II superfamily. They function as A<sub>2</sub>B<sub>2</sub> heterotetramers in which an active site tyrosine in each A subunit catalyzes DNA cleavage via a transesterification reaction with one strand of the DNA backbone (34). Coordinate nicking of two strands produces a DSB in which each 5'-end is held by a covalent phosphotyrosine linkage to a Top6A protomer (protein–DNA cleavage complex). ATPase-dependent functions of the B subunits contribute to conformational changes involved in DNA strand passage (34). Following strand passage, the covalent protein–DNA cleavage complex undergoes a second pair of transesterification reactions to reseal the DSB and to release the topoisomerase.

Most eukaryotes lack a detectable ortholog of Top6B and exceptions (e.g. in *Arabidopsis* sp.) are thought to be the result of horizontal gene transfer (34). It therefore seems unlikely that Spo11 family proteins are, by and large, topoisomerases. Nevertheless, Spo11 is thought to cleave DNA in a fashion akin to that of Top6A based upon multiple criteria. First, in both budding yeast and fission yeast the Spo11/Rec12 protein becomes covalently linked to meiotic DSBs (29,35). Second, a strictly conserved, putative active site tyrosine of Spo11/Rec12 is essential for the formation of meiotic DSBs and for meiotic recombination (28,29,33). Third, the crystal structure of homodimeric Top6A from *Methanococcus jannaschii* has been solved and within this structure are a putative DNA-binding pocket, a conserved structural domain involved in metal ion-binding (toprim), and signature residues of the catalytic domains from the topoisomerase II superfamily (5Y-CAP) (36). Specific mutations in budding yeast Spo11 (28,37), in Spo11-1 of *Arabidopsis thaliana* (38), and in fission yeast Rec12 (33,39) map to structural motifs of Top6A and compromise meiotic recombination. It is therefore thought that Spo11/Rec12 proteins function, like Top6A, as a homodimer (within the context of a larger protein complex) in which each Spo11/Rec12 protomer cleaves one strand of the DNA by a transesterification reaction to produce DSBs. Subsequently, through a mechanism

distinct from that of topoisomerases, the protein–DNA cleavage complexes are released by endonucleolytic nicking of the DNA strands to which Spo11/Rec12 is bound covalently (40,41). This leaves behind 3'-protruding, ssDNA tails that are further processed, invade and base pair with a homologous chromosome template, and prime subsequent steps of the meiotic recombination pathway.

Structure-function analysis of Spo11/Rec12 proteins has been complicated by two factors. First, it has proven difficult to isolate soluble protein for biochemical studies *in vitro*. In two cases where soluble protein was obtained, they lacked catalytic activity (20,38). Second, it has proven difficult to dissect the functions of mutant Spo11 proteins *in vivo*. In some organisms, such as *Coprinus cinereus* and mice, *spo11* mutant cells undergo apoptosis during or shortly after meiotic prophase (42–44). In other organisms, such as budding yeast, essentially all meiotic products of *spo11* mutants are inviable (37,45), presumably due to a high frequency of aneuploidy. In contrast, mutants of fission yeast lacking meiotic recombination and recombination-dependent (chiasmatic) chromosome segregation progress through meiosis and produce a high frequency of viable meiotic products (23,33,46,47). With only three pairs of chromosomes, random assortment of homologs often produces meiotic products that receive by chance at least one copy of each chromosome, and spore viability is further improved by a backup system for achiasmatic (distributive) chromosome segregation (33,47).

We took advantage of the biology of fission yeast to determine the functional significance of 21 different amino acids within Rec12 that are hypothesized, based upon the crystal structure of Top6A and the biochemistry of topoisomerases, to be important functionally. We report, first, that the structure and function of Rec12 in the catalysis of DSBs is likely broadly conserved among Rec12/Spo11/Top6A family members. Second, essentially all meiotic recombination in fission yeast is attributable to Rec12-catalyzed DSBs. Third, although fission yeast has multiple crossovers per chromosome pair (on average approximately 15), a single crossover is necessary and sufficient for chiasmatic chromosome segregation. Fourth, fission yeast has a crossover homeostasis mechanism that helps to maintain the presence of crossovers required for chiasmatic segregation. Fifth, the crossover/non-crossover decision is likely established early by a protein–protein interaction surface of Rec12.

## MATERIALS AND METHODS

### Strains, media and genetic methods

The genotypes of *S. pombe* strains used are listed in Supplementary Table S1 and the sequences of oligonucleotide primers are listed in Supplementary Table S2. Culture media, culture conditions, genetic crosses and genotyping for auxotrophic or conditional markers were as described (48–51). PCR diagnostics were used to determine mating type (52) and to distinguish among various

*rad50* (6,7) and *rec12* alleles. Each newly constructed *rec12* allele harbors a translationally silent restriction site polymorphism, relative to wild-type *rec12*<sup>+</sup>. PCR products from the *rec12* locus (primers *rec12*-TOPO-FP, *rec12*-TOPO-RP) were digested with one of *Bst*UI, *Mae*I, *Pvu*II, *Stu*I, *Eco*RV, *Pst*I, *Apa*I, *Hae*III, or *Apa*LI; depending upon the allele being sought (see Supplementary Table S2 for allele-specific restriction site polymorphisms).

### Mutagenesis *in vitro*

A *Bam*HI-*Nde*I fragment of pUC19-*Rec12* (33) harboring the *rec12*<sup>+</sup> gene was subcloned between the *Bam*HI and *Nde*I sites of pUra4-Sph (53) to generate pUra4-*Rec12*. Site directed mutagenesis (GeneTailor, Invitrogen) was used to introduce mutations into the *rec12* coding region of pUra4-*Rec12*. Primers were designed such that each product would also contain a diagnostic change in a restriction endonuclease recognition site (Supplementary Table S2). Candidate mutant plasmids were first screened by restriction digestion, then for each positive candidate the entire *rec12* region was sequenced to confirm the desired substitutions and to eliminate clones with extraneous mutations introduced during PCR.

### Allele replacement *in vivo*

For pop-in, pop-out allele replacement (54,55), we linearized each different pUra4-*Rec12* plasmid with an enzyme that cuts within the portion of the plasmid derived from the endogenous *rec12* locus. Some plasmids were digested with *Nco*I (for plasmids encoding *Rec12*-R76A, D79A, E83A, R94A, D95A, Y97F, K282A, R283A, D284A, R304A, E305A). Others were cut with *Mfe*I (for plasmids encoding *Rec12*-E179A, K201A, R209A, K210A, K214A, D229A, D231A, K242A). Linearized plasmids were transformed (56) into strain WSP 0589 (*h*<sup>-</sup> *ura4-D18*) and uracil-prototrophic transformants were selected for on NBA minimal medium lacking uracil (57). Since pUra4-*Rec12* lacks an origin of replication for fission yeast, stable transformants arise by integration of the plasmid into the chromosome. In fission yeast, such integrations can occur by homologous recombination at the target locus or via non-homologous recombination elsewhere in the genome (51,58,59). Transformants were screened by PCR to identify those in which the plasmid had integrated by 'pop-in' homologous recombination at the *rec12* locus to generate a tandem, direct repeat of the *rec12* gene (with *ura4*<sup>+</sup> and plasmid DNA in the middle). Such direct repeats are inherently unstable in the absence of selection because recombination between the two tandem copies causes the plasmid to 'pop out' of the chromosome. Since the plasmid lacks an origin of replication for fission yeast, following excision it is lost during subsequent rounds of cell division. Excision events involving recombination to one side of the site-directed mutation leave the mutation in the chromosome, whereas excision events to the other side leave a wild-type gene in the chromosome. For each candidate, a Ura<sup>+</sup> colony (tandem integrant) was

inoculated into 10 ml of non-selective rich medium (YEL), was grown to mid/late log phase, was split 1:100 into YEL and grown again to mid/late log phase, and then serial dilutions of the culture were plated on YEA containing 1 mg/ml of 5-fluoroorotic acid (5-FOA). The 5-FOA kills cells expressing *ura4*<sup>+</sup> (57), and hence selects for growth of cells that had lost the *ura4*<sup>+</sup> gene (pop-out) or suffered a mutation that inactivates *ura4*<sup>+</sup>. Diagnostic PCR and RFLP mapping was used to identify the pop-out class and to determine which candidate colonies had left the desired mutations in the genome. Subsequently, for clones that passed this test we sequenced the endogenous *rec12* locus (spanning the entire region of homology originally present in pUra4-*Rec12*) to confirm the presence of the desired mutations and to eliminate clones that harbored artifactual mutations.

### Analysis of meiotically induced DSBs

The induction of synchronous meiosis in *pat1-114* strains was as described (60–62). Meiotically-induced, *Rec12*-dependent DSBs were detected by Southern blotting of pulse field gels (6,7). Cells from synchronous meiotic cultures were washed three times in wash buffer (50 mM EDTA, 10 mM Tris-HCl, pH 7.6) and were re-suspended at  $3 \times 10^9$  cells per milliliter in cell wall digestion buffer (50 mM EDTA, pH 8.0) containing 2 mg/ml of Yeast Lytic Enzyme (MP Biochemicals Inc.). The cell suspensions were immediately mixed with an equal volume of cell wall digestion buffer containing 2% of dissolved agarose (pulse field grade, Bio-Rad Laboratories) at 45°C. Samples were placed in gel plug molds, then were incubated for 15 min each at 22 and 4°C. Solidified plugs were soaked in 50 vol of cell lysis buffer (500 mM EDTA, 10 mM Tris-HCl, 1% 2-mercaptoethanol, pH 7.6) for 3 h at 37°C. Plugs were transferred to 3 vol of protease buffer (100 mM EDTA, 10 mM Tris-HCl, 20 mM NaCl, 1% sarkosyl) containing 0.1 mg/ml of Proteinase K and were incubated for 16–24 h at 37°C. Plugs were then washed three times in 50 vol of 50 mM EDTA, pH 8.0 at 22°C for 15 min each. They were stored in 50 mM EDTA at 4°C (stable for months). For restriction digestion, plugs were soaked sequentially at 4°C in 50 vol of 10 mM Tris, pH 7.6 (three times,  $\geq 2$  h each) and in 10 vol of 1 $\times$  restriction endonuclease buffer (two times,  $\geq 8$  h each). Each plug (~100  $\mu$ l) was incubated for 8 h at 37°C in 300  $\mu$ l of 1 $\times$  restriction endonuclease buffer containing 50 units of *Pme*I (New England Biolabs). Plugs were cast in gels containing 0.5 $\times$  TBE and 1% pulse field certified agarose (Bio-Rad Laboratories). Gels were run in a Bio-Rad CHEF-DR II pulse field system for 16 h at 14°C, 6 V/cm, 3 s switch time. DNA molecules were transferred to Brightstar Plus membranes (Applied Biosystems) by inverse capillary blotting. These were probed using a radioactively labeled (Rediprime II DNA-labeling system, GE Healthcare) PCR product (primers *mbs1*-FP, *mbs1*-RP) using standard protocols (Hybond-N manual, Amersham). Methods for quantitative measurements are described in the text.

**Frequency analyses of meiotic recombination and diploid meiotic products**

Frequencies of intragenic recombination between *ade6* heteroalleles were determined from the relative plating efficiencies of spores on minimal medium containing or lacking adenine, as described (49). Methods to determine the frequencies of diploid spore colonies and of intergenic recombination between *ade6* and *arg1* were as described (50,63). Diploid spore colonies, which could contain complementing markers, were excluded from recombinant frequency determinations. The frequencies of reciprocal, intergenic recombination between *ade6* and *arg1* were converted into genetic map distances (cM, the percent frequency of crossing over) using the function of Haldane (64).

**Statistical methods**

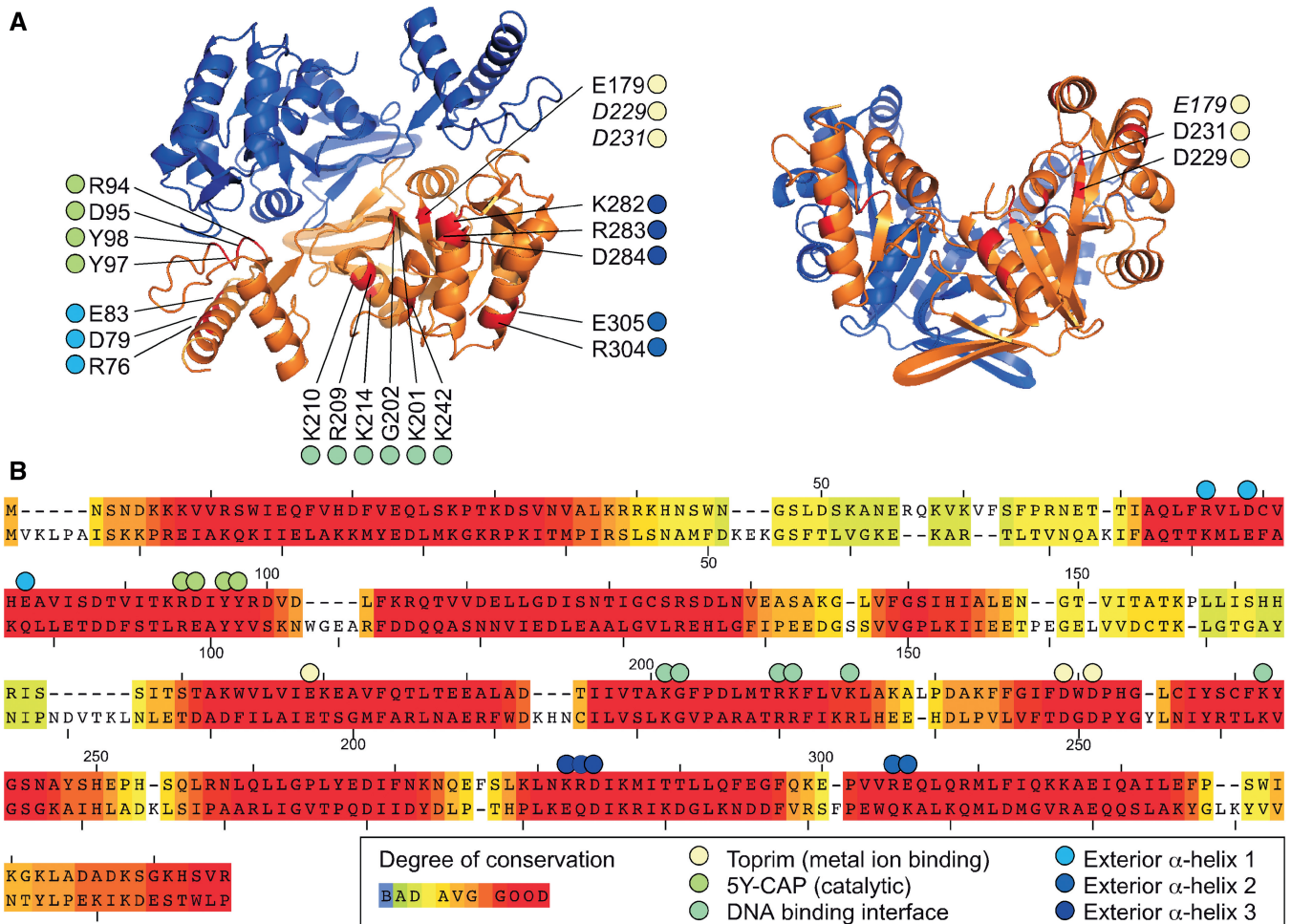
Each experiment was conducted three or more independent times. Mean and SD values were calculated using

Excel (Microsoft). The 95% confidence interval (mean  $\pm 2 \times$  larger SD) was used to determine whether mean values were significantly different from one another.

**RESULTS**

**Identification of amino acid targets in Rec12 based upon the crystal structure of Top6A**

Rec12 and other eukaryotic Spo11 proteins share ~30% sequence homology with Top6A of *M. jannaschii*, permitting one to model the positions of conserved and conservative amino acids within the crystal structure of Top6A (Figure 1) (33,36–39). We identified six regions of Rec12 protein that were of interest. The first region is the putative DNA-binding interface. Five basic residues of Rec12 (K201, R209, K210, K214, K242) are positioned such that they might make hydrogen bonding contacts with the phosphate backbone of DNA, and a sixth (G202) is positioned in the base of the cleft (39).



**Figure 1.** Amino acids of Rec12 targeted for replacement. (A) Crystal structure of Top6A homodimer from *M. jannaschii* (36). One subunit is colored blue and the other orange. Superimposed on the orange subunit are the locations of amino acids of Rec12 that were targeted for replacement (red). These are numbered according to their positions within Rec12; corresponding domain assignments are indicated (grouped and color coded, see key below). The positions of amino acids not visible (italics) in the top view (left) are shown in the front view (right). Also visible in the front view is the DNA-binding cleft. (B) Sequence alignment of Rec12 (top) and Top6A (bottom). Diagram indicates the position of each amino acid substitution, relative to degree of local sequence conservation and to map location within the crystal structure.

The second region maps to a domain that is conserved structurally between type II topoisomerases and primases (toprim) (65). Three amino acids of Rec12 (E179, D229, D231) are broadly conserved in toprim domains and these three residues coordinate metal ions in the crystal structures of Top6A and primase (36,66). The third region of Rec12 corresponds to the catalytic (5Y-CAP) domain that is conserved structurally between topoisomerase II proteins and catabolite activator proteins (34,67). Four amino acids of Rec12 (R94, D95, Y97, Y98) are positioned near the active site in the crystal structure and hypothetically contribute to catalysis (28,33,34,36,37). The tyrosine at position 98 is strictly conserved, is essential for meiotic recombination in all cases tested, and is thought to form the covalent phosphotyrosine linkage to meiotic DSBs. Position 97 is conservative, being tyrosine in the majority of Rec12/Spo11 proteins and phenylalanine in many others. Given its close proximity to active site tyrosine 98, tyrosine 97 might also carry out a transesterification/DNA cleavage reaction. Three additional regions of Rec12 were selected because they might participate in protein-protein interactions. Since hydrogen bonding often contributes to such interactions, we sought regions that map to an exterior surface, that have charge clusters, that have exposed side chains which could participate in hydrogen bonding, and that fall within a reasonably well conserved window of nearby sequence. Region four maps to an exterior  $\alpha$ -helix (R76, D79, E83); region five maps to an exterior  $\alpha$ -helix (K282, R283, D284); and region six maps to another exterior  $\alpha$ -helix (R304, E305).

We used site-directed mutagenesis *in vitro*, followed by allele replacement *in vivo*, to modify the endogenous *rec12* locus such that it expressed mutant proteins from the normal *rec12* promoter and 3'-regulatory regions. A total of 21 single amino acid substitutions were analyzed, 19 of which were created *de novo* and two of which we described previously (33,39). With three exceptions, we replaced charged amino acids (Lys, Arg, Asp, Glu) with a non-polar, aliphatic amino acid (Ala). In two instances an aromatic side chain with a hydroxyl group (Tyr) was replaced by one lacking the hydroxyl group (Phe). And in one case a non-polar, aliphatic amino acid (Gly) was replaced by a charged amino acid (Glu) (39). Each substitution was chosen to alter hydrogen bonding potential or a specific functional group (-OH of Tyr), although one of the two substitutions reported previously (G202E) might also affect a bend between a  $\beta$ -sheet and  $\alpha$ -helix (39).

#### Specific amino acids in five of six domains targeted are required for meiotic DSBs

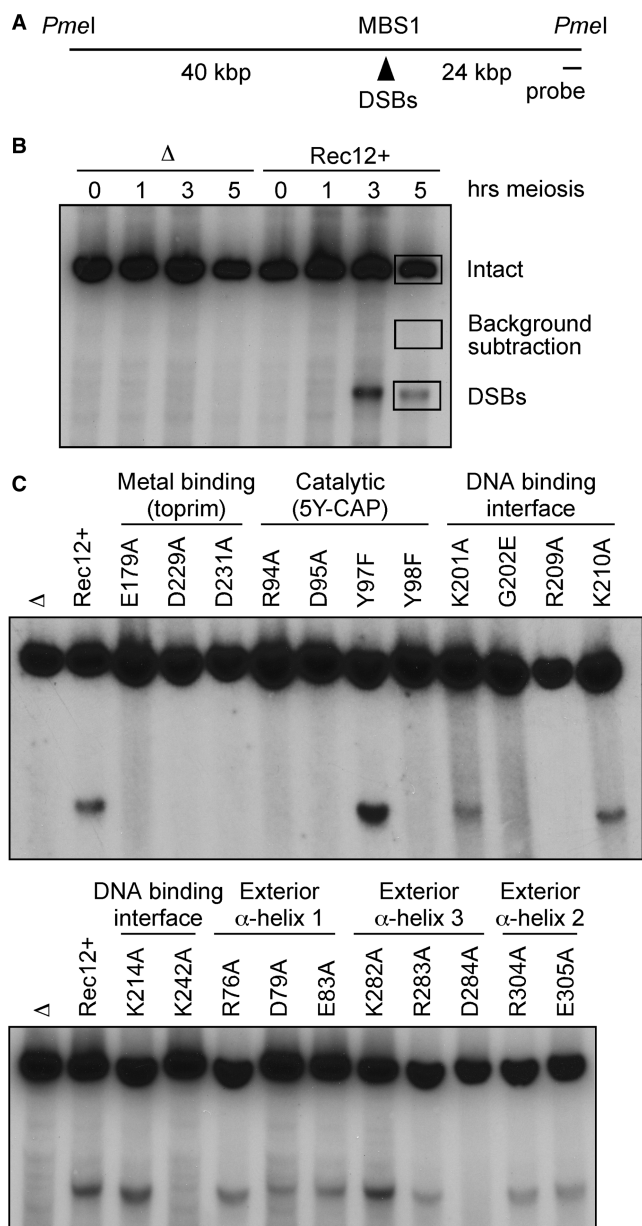
To determine the proficiencies with which Rec12 protein variants catalyze the formation of meiotic DSBs, we took advantage of the fact that DSBs accumulate unrepaired in *rad50S* cells (15). In fission yeast the *rad50S* mutation does not affect the distribution of Rec12-catalyzed DSBs, but rather it blocks subsequent processing of otherwise transient, covalent Rec12-DNA cleavage complexes (18,35).

Therefore, the frequency of meiotic DSBs that accumulates in *rad50S* cells provides a fairly accurate measure of the total frequency of DSBs introduced during meiosis.

We first analyzed time points of synchronous meiosis in cells expressing and lacking Rec12 and we probed for DSBs at the well characterized site *mbs1* (7,15). Because meiotic DSBs in fission yeast are clustered in widely separated peaks, and because large DNA fragments are susceptible to shearing forces, we employed pulse-field gel electrophoresis of DNA samples prepared after embedding cells in agarose plugs. Our Southern blot results for the positive and negative controls (Figure 2A and B) are consistent with those reported previously (7,15). First, in every sample there was a very low, uniform level of Rec12-independent DNA cleavage. Second, no meiotically-induced DNA cleavage was detected in cells lacking Rec12. Third, in cells expressing wild-type Rec12, DSBs were strongly induced during meiosis and these DSBs mapped to the position of *mbs1*. We used phosphorimage analysis of data from multiple experiments, with background subtraction from a relatively 'light' portion of each lane (e.g. Figure 2B boxes), to determine the frequencies of DSBs. The highest signal intensity detected in the negative control at any time point (0, 1, 3, 5 h) and in the positive control at early time points (0, 1 h) was  $0.41\% \pm 0.25$  (range of 0.25–0.41%). In contrast, at later time points the positive control yielded signal intensities that were higher and equivalent for the 3 h ( $7.1\% \pm 2.5$ ) and 5 h ( $6.7\% \pm 1.0$ ) time points, as expected for the accumulation of unrepaired DSBs in the *rad50S* genetic background. We therefore used the 5 h time point of meiosis to analyze DSBs in cells expressing Rec12 protein variants.

The 21 different amino acid substitutions were targeted to six different predicted functional regions of Rec12 (Figure 1). Mutations in five of the six regions significantly reduced the frequency of meiotic DSBs (Figure 2C, Table 1). Some substitutions reduced, but did not eliminate, DSBs. These mapped to the putative DNA-binding interface (K201A, K210A) and to exterior  $\alpha$ -helix 1 (D79A, E83A). Other substitutions eliminated detectable DSBs. These included all three substitutions targeted to the toprim (metal ion binding) signature (E179A, D229A, D231A), three of four targeted to the 5Y-CAP (catalytic) domain (R94A, D95A, Y98F), three of six within the DNA interface (G202E, R209A, K242A), and one of three substitutions targeted to exterior  $\alpha$ -helix 3 (D284A). The effects were specific to the aforementioned residues, because amino acid substitutions located elsewhere in Rec12 did not significantly alter the frequency of meiotic DSBs (Table 1).

Notably, there are two domains essential for DNA cleavage by all members of the type II topoisomerase superfamily (toprim and 5Y-CAP) (34), and six of seven substitutions in the corresponding positions of Rec12 gave a null phenotype for DSBs (Table 1). The other, conservative substitution (Y97F) affects a residue that is generally conservative in Rec12/Spo11 proteins and that has not been implicated in DNA cleavage by any topoisomerase. Also notable is that five of six substitutions in the



**Figure 2.** Effects of Rec12 amino-acid substitutions upon DSB formation. (A) Pulse field gel electrophoresis and Southern blotting were used to analyze DSBs (arrowhead) at major break site 1 (mbs1). (B) DSB formation at mbs1 requires meiosis and Rec12. Samples were from the indicated time points of synchronous meiotic cultures. Images are scans of X-ray films from autoradiography. For quantitative measurements, signal intensities within the indicated areas (boxes) of each lane were determined by phosphorimage analysis of the hybridized Southern blot membrane. The background was subtracted prior to calculating the frequency of DSBs. (C) Analysis of mutants. Each experiment included positive (Rec12<sup>+</sup>) and negative (Δ) controls. The frequencies of DSBs at 5 h (mean ± SD of data from multiple experiments) are provided in Table 1.

putative DNA-binding interface reduced (two cases) or abolished (three cases) DSBs. The other substitution (K214A) is positioned on the ‘wrong side’ of an  $\alpha$ -helix in the DNA-binding cleft for its side chain to participate in hydrogen bonding with the phosphate backbone of DNA (Figure 1). And with regard to the five of five, correctly

positioned DNA cleft substitutions that compromise catalysis, three similar substitutions in Spo11-1 of *A. thaliana* were recently shown to reduce DNA binding *in vitro* (38). In summary, these results provide compelling evidence for conservation in the structure and function of Rec12, relative to Top6A and other members of the topoisomerase II superfamily, and they provide insight into molecular mechanisms by which Rec12 catalyzes the formation of DSBs.

### Recombination rate is proportional to frequency of DSBs

Meiotic DSBs (and perhaps other lesions such as ssDNA nicks) initiate recombination that produces, ultimately, either non-crossover or crossover recombinant products. We therefore measured intragenic recombination at *ade6* and intergenic recombination between *ade6* and *arg1*. In the former, *ade6*<sup>+</sup> recombinants are due to gene conversion events, a subpopulation of which is accompanied by reciprocal exchange (68–71). In the latter, essentially all recombinants are due to reciprocal exchange (crossing over), because the frequency of exchanges between two distant markers is far higher than the frequency with which individual markers undergo conversion (72,73). As positive and negative controls, we measured recombination in cells expressing and lacking (null mutant) Rec12. All ( $\geq 99.5\%$ ) of recombination required the presence of Rec12 protein (Table 1), as reported previously (33).

As expected, each of the 14 single amino acid substitutions in Rec12 that significantly reduced the formation of meiotic DSBs also significantly reduced the frequencies of intragenic recombination, or intergenic recombination, or both (Table 1). Linear regression analysis of each data set revealed a positive correlation between the recombinant frequencies and the frequencies of DSBs ( $R^2 = 0.70$ ,  $0.70$ ) (Figure 3). In each linear regression the ordinal intercept has a slight, positive value ( $\leq 5\%$  of wild-type recombinant frequencies for intragenic recombination,  $\leq 10\%$  for intergenic recombination). This might reflect a fraction of Rec12-dependent recombination initiating from lesions other than DSBs (e.g. from ssDNA nicks). Alternatively, and seemingly more likely, it might reflect the fact that the method used to detect DSBs (Southern blotting) has a minimum detection limit above zero. In either case, the results from titrating the frequency of Rec12-dependent DSBs nicely fill the void between ‘all or none’ phenotypes reported previously, suggesting that the majority of meiotic recombination in fission yeast ( $\geq 90$ – $95\%$ ) is initiated from Rec12-catalyzed DSBs.

### Rec12-catalyzed DSBs are not sufficient for Rec12-dependent recombination

In several cases the frequency of meiotic DSBs was not significantly different from that in wild-type cells, and yet the frequency of intragenic recombination was significantly reduced (Y97F, K214A, R76A, K282A, R304A, E305A) (Table 1). This indicates that Rec12 is required for a step of recombination following the catalysis of DSBs.

**Table 1.** Effects of Rec12 amino acid substitutions upon meiotic DSBs, two types of recombination and recombination-dependent chromosome segregation

Protein or putative domain	Rec12 protein expressed	Frequencies <sup>a</sup>						
		Meiotic DSBs		Intergenic recombination		Intragenic recombination		Diploid meiotic products (%)
		(%)	(ratio)	(cM)	(ratio)	( $\times 10^4$ )	(ratio)	
Positive control	Rec12 <sup>+</sup>	4.97 ± 1.01	1.00	72 ± 1.4	1.00	30 ± 2.3	1.00	0.51 ± 0.56
Negative control	None ( $\Delta$ )	0.24 ± 0.18	0.05	≤0.32	<0.01	0.025 ± 0.010	<0.01	19 ± 1.4
Metal ion binding (toprim)	Rec12-E179A	0.23 ± 0.19	0.05	≤0.24	<0.01	0.015 ± 0.0055	<0.01	21 ± 1.1
Metal ion binding (toprim)	Rec12-D229A	0.19 ± 0.14	0.04	≤0.29	<0.01	0.021 ± 0.017	<0.01	17 ± 1.8
Metal ion binding (toprim)	Rec12-D231A	0.18 ± 0.18	0.04	≤ 0.20	<0.01	0.021 ± 0.0044	<0.01	26 ± 3.0
Catalytic (5Y-CAP)	Rec12-R94A	0.29 ± 0.16	0.06	≤ 0.45	<0.01	0.016 ± 0.014	<0.01	16 ± 0.88
Catalytic (5Y-CAP)	Rec12-D95A	0.19 ± 0.12	0.04	≤ 0.27	<0.01	0.013 ± 0.0055	<0.01	16 ± 4.9
Catalytic (5Y-CAP)	Rec12-Y97F	6.37 ± 3.00	1.28	68 ± 13	0.94	18 ± 2.1	0.60	≤0.32
Catalytic (5Y-CAP)	Rec12-Y98F	0.12 ± 0.23	0.02	≤0.20	<0.01	0.012 ± 0.0060	<0.01	12 ± 1.0
DNA-binding interface	Rec12-K201A	1.53 ± 0.21	0.31	17 ± 2.0	0.24	4.4 ± 1.4	0.15	1.1 ± 0.65
DNA-binding interface	Rec12-G202E	0.33 ± 0.24	0.07	≤0.20	<0.01	0.073 ± 0.024	<0.01	11 ± 2.1
DNA-binding interface	Rec12-R209A	0.13 ± 0.13	0.03	4.1 ± 1.0	0.06	0.93 ± 0.15	0.03	6.2 ± 0.65
DNA-binding interface	Rec12-K210A	2.67 ± 0.26	0.54	70 ± 14	0.97	14 ± 1.3	0.47	≤0.23
DNA-binding interface	Rec12-K214A	4.90 ± 1.06	0.99	72 ± 6.4	1.00	19 ± 1.9	0.63	≤0.13
DNA-binding interface	Rec12-R242A	0.30 ± 0.24	0.06	21 ± 5.1	0.29	3.4 ± 0.67	0.11	3.2 ± 1.4
Exterior $\alpha$ -helix 1	Rec12-R76A	4.33 ± 0.70	0.87	49 ± 3.4	0.68	20 ± 2.8	0.67	≤0.13
Exterior $\alpha$ -helix 1	Rec12-D79A	1.63 ± 0.58	0.33	87 ± 31	1.21	23 ± 0.55	0.77	≤0.080
Exterior $\alpha$ -helix 1	Rec12-E83A	2.74 ± 0.93	0.55	69 ± 23	0.96	20 ± 2.7	0.67	0.15 ± 0.3
Exterior $\alpha$ -helix 2	Rec12-R304A	3.50 ± 0.97	0.70	59 ± 10	0.82	7.6 ± 1.2	0.25	0.21 ± 0.20
Exterior $\alpha$ -helix 2	Rec12-E305A	3.90 ± 0.85	0.78	54 ± 15	0.75	18 ± 1.8	0.60	≤0.21
Exterior $\alpha$ -helix 3	Rec12-K282A	5.42 ± 0.82	1.09	66 ± 16	0.92	22 ± 2.1	0.73	≤0.26
Exterior $\alpha$ -helix 3	Rec12-R283A	2.97 ± 0.78	0.60	49 ± 12	0.68	26 ± 1.9	0.87	0.27 ± 0.54
Exterior $\alpha$ -helix 3	Rec12-D284A	0.29 ± 0.17	0.06	14 ± 6.2	0.19	2.3 ± 0.44	0.08	3.2 ± 1.5

<sup>a</sup>Data are mean ± SD from three or more independent experiments.

### A subset of mutations differentially affect gene conversion and crossing over

Overall the rate of Rec12-dependent recombination is proportional to the frequency of Rec12-catalyzed DSBs (Figure 3), but there are differences in the outcome. Eight of the single amino acid substitutions significantly reduced intragenic recombination at *ade6* without significantly affecting intergenic recombination between *ade6* and *arg1* (Y97F, K210A, K214A, D79A, E83A, K282A, R304A, E305A) (Table 1). In contrast, there were no substitutions that significantly reduced intergenic recombination without also significantly reducing intragenic recombination. Other substitutions significantly reduced both types of recombination to intermediate levels, and in one of those cases the percent reductions were identical (R76A). Thus, the preferential reduction of intragenic recombination is specific to a subset of the amino acid substitutions that reduce recombination. A clear implication is that Rec12 protein itself is involved in determining whether recombination events are partitioned into crossover or non-crossover recombinant products.

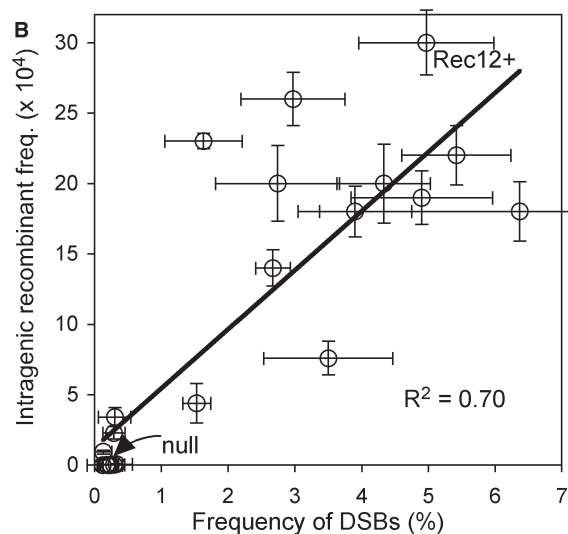
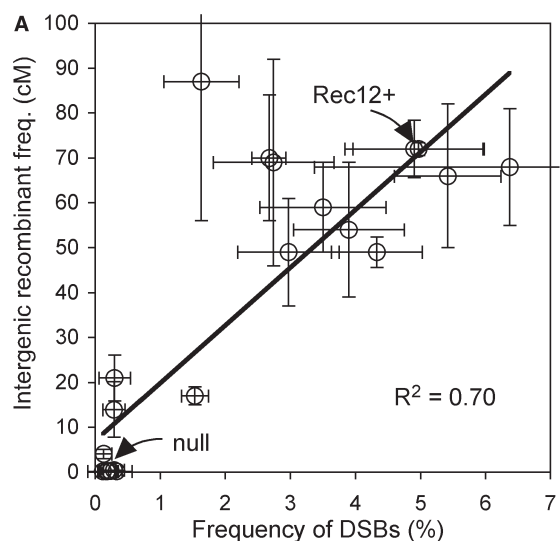
### All mutants hypomorphic for DSBs exhibit crossover homeostasis

An equally intriguing, and related finding is that three substitutions significantly reduced the frequency of DSBs without significantly reducing the frequency of crossover recombination (K210A, D79A, E83A) (Table 1). This property is shared by some hypomorphic mutants of

budding yeast in which Spo11 is crippled artificially by the presence of epitope tags (74). Such 'crossover homeostasis' helps to maintain the presence of crossover recombination events, presumably because they are required for chiasmatic chromosome segregation. When the overall frequency of recombination initiation (DSBs) falls, the cells can sense this reduction and take compensatory action.

A prediction is that when recombination intermediates are redirected preferentially towards crossover resolution, then this redirection/compensation should come at the expense of the non-crossover class. This prediction is met in fission yeast, because in each of the three mutants where DSBs were significantly reduced and crossovers were maintained between *ade6* and *arg1*, the intragenic (conversion) recombinant frequency at *ade6* was significantly reduced (K210A, D79A, E83A) (Table 1). For example, in K210A mutants DSBs were reduced (54% of wild-type levels), crossovers were maintained (97%), and conversions were reduced (47%). Similarly, when both types of recombination were significantly reduced, intragenic recombination was reduced to a greater extent (K201A).

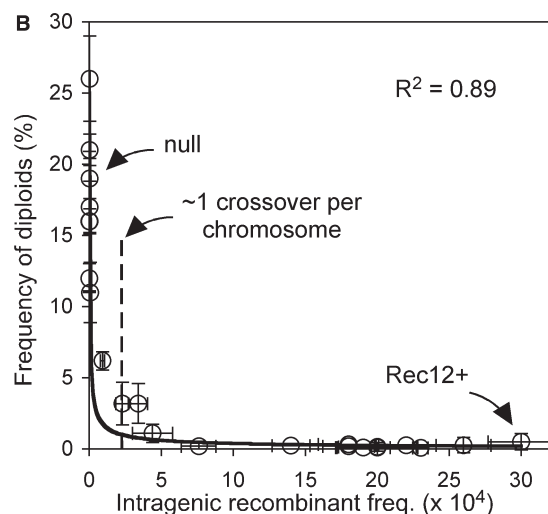
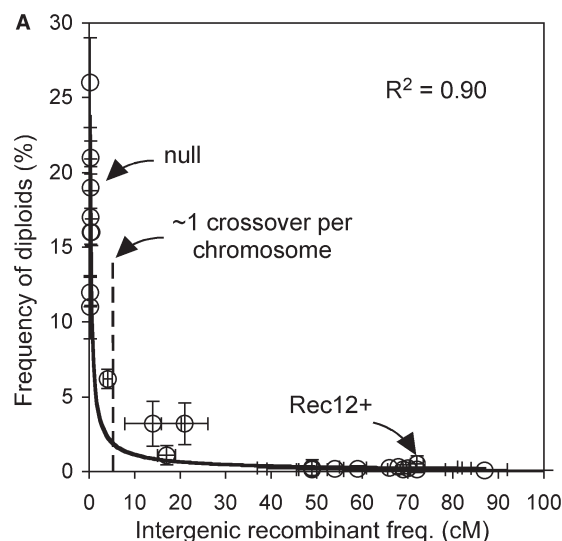
In total, each of the four hypomorphic mutants with significantly reduced DSBs exhibited crossover homeostasis. Many other amino substitutions within Rec12 significantly reduced DSBs, but gave a null phenotype for DSBs and for recombination. Those mutants are uninformative as to the presence or absence of crossover homeostasis.



**Figure 3.** Relationship between frequencies of Rec12-catalyzed DSBs and rates of meiotic recombination. The frequencies of intergenic (A) and intragenic (B) recombinants were plotted versus the frequencies of DSBs for each Rec12 protein variant and the negative control lacking Rec12. Data are mean (circles)  $\pm$  SD (thin lines). Results from linear regression analyses are also indicated (lines,  $R^2$  values).

### Fidelity of chromosome segregation is a non-linear function of recombination rates

The *rec12Δ* mutants lack meiotic recombination and hence chiasmata, suffer non-disjunction of homologs in meiosis I, and produce a high frequency of diploid meiotic products diagnostic for the chromosome segregation errors (33,47). As expected, wild-type cells produced few diploid meiotic products, whereas cells lacking Rec12 produced them at a much higher frequency (Table 1). Remarkably, many of the mutants with significantly reduced recombination exhibited a wild-type phenotype for chromosome segregation. The data fit well to power functions ( $R^2 = 0.90, 0.89$ ), the shapes of which illustrate a key finding (Figure 4). Recombination rates can be titrated far below those in wild-type cells without



**Figure 4.** Non-linear dose response for recombination-dependent chromosome segregation. Non-disjunction of homologs in fission yeast meiosis produces a high frequency of diploid meiotic products (33,47). The frequencies of diploid spore colonies were plotted versus the frequencies of intergenic (A) and intragenic (B) recombination for each Rec12 protein variant and the negative control lacking Rec12. Data are mean (circles)  $\pm$  SD (thin lines) and are fit to power functions (curved lines,  $R^2$  values). There are approximately 44 crossovers per fission yeast genome per meiosis and those exchanges are distributed, without crossover interference, within each of the three chromosome pairs (bivalents) in proportion to chromosome size (72). Based upon the frequency of recombination in wild-type cells, one can extrapolate to the frequency of recombination (dotted line) that corresponds to approximately 3 crossovers per genome per meiosis (i.e. on average approximately 1 crossover per bivalent per meiosis).

deleterious effects upon segregation. However once recombination rates fall below a minimum threshold, chromosomes segregate aberrantly. Notably, the critical threshold for high fidelity chromosome segregation occurred at a recombinant frequency that corresponds to approximately 1 crossover per chromosome pair (bivalent) per meiosis (Figure 4) (72). We conclude that a single crossover per bivalent is necessary and sufficient to ensure high fidelity, chiasmatic segregation of



chromosomes in fission yeast. There is an ‘obligate cross-over’ (chiasma) with regard to faithful chromosome segregation. The introduction of multiple crossovers per chromosome pair, the preferential maintenance of chiasmata via crossover homeostasis (described above), and a backup distributive (achiasmatic) system (33,47) each help to ensure that meiotic products will be euploid.

## DISCUSSION

The biology of fission yeast, in particular its ability to produce viable meiotic products in the complete absence of meiotic recombination and recombination-dependent chromosome segregation (23,33,46,47), allowed us to determine the functional significance of 21 different amino acid residues of Rec12. Single amino-acid substitution, variant proteins were expressed from the endogenous *rec12* locus (normal promoter and 3'-regulatory regions) and were unencumbered by epitope tags which can affect function. Mutant phenotypes exhibited Mendelian inheritance, were linked genetically to the respective *rec12* mutations, and where tested were recapitulated following reconstruction of alleles. Therefore, the phenotypes are attributable to the single amino acid substitutions within Rec12 protein. The results provide insight into the biochemistry of Rec12, its functional conservation, multiple strategies employed for chromosome segregation, and regulation of the crossover/non-crossover pathway decision.

### Rec12 uses a conserved, topoisomerase-like mechanism to catalyze meiotic DSBs

Meiotic recombination has been characterized most extensively in two model organisms, budding yeast and fission yeast. While often lumped together as ‘yeast’, these organisms are highly diverged and they employ different strategies for many steps of recombination (e.g. they differ in the constellation of proteins required for the catalysis of DSBs). Nevertheless, a key, rate-limiting step of meiotic recombination—its initiation by Rec12/Spo11—is thought to be employed by all eukaryotes that undergo meiosis.

Our results (Table 1), coupled with those reported for the Spo11 protein of budding yeast (37) and Spo11-1 of *A. thaliana* (38), point to broad conservation of structure and function of Rec12/Spo11/Top6A proteins. Moreover, our findings strongly support the following inferences as to biochemical mechanisms by which Rec12 cleaves DNA.

(A) Rec12 functions as a homodimer in which each protomer contains one active site tyrosine. Rec12 contains two tyrosines in the active site region (Y97 and Y98), so hypothetically one Rec12 protomer could carry out two transesterification reactions (one for each DNA strand) to produce a DSB. However, one tyrosine is dispensable for the formation of DSBs (Y97) and the other is essential (Y98), so with only one requisite tyrosine in the active site region cleavage of both DNA strands requires more than a single protomer acting once.

Similarity to Top6A (conservation of key, functionally important residues) also supports action of Rec12 as a homodimer. We note that evidence for dimeric function of budding yeast Spo11 is also indirect (37), and that purified Rec12 and Spo11-1 of *A. thaliana* exist in solution as monomers and higher order complexes, respectively (20,38). Other proteins might coordinate homodimerization of Rec12 *in vivo* (20).

- (B) Rec12 has a DNA-binding cleft and a series of basic amino acid residues whose side chains are exposed on the surface of that cleft contribute to DNA binding via hydrogen bonding with the negatively charged phosphate backbone of DNA. Similarly, Spo11-1 of *A. thaliana* binds to DNA *in vitro* and substitutions at each of three arginine residues in the DNA cleft reduce DNA binding, whereas substitutions outside of the cleft do not (38). Direct interaction between Rec12 and DNA establishes the register in which DNA cleavage occurs.
- (C) Rec12 has a functional toprim domain which, like those of topoisomerase II superfamily proteins, coordinates the positioning of a metal ion cofactor that is essential for catalysis.
- (D) Rec12 has a functional 5Y-CAP domain, which, like those of topoisomerase II proteins, coordinates the transesterification reaction involving active site tyrosine at position 98. The DNA-binding interface positions the active site of each Rec12 protomer relative to their target DNA strands on opposite sides of the helix.
- (E) Exterior surfaces of Rec12 interact with other proteins required for recombination. One such interaction is with Rec14, which binds in the vicinity of Rec12-Q308/R309 (23). This interaction is conserved in the orthologous protein pair of budding yeast (Rec12/Spo11-Rec14/Ski8) (75,76). Another likely protein-protein interaction surface of Rec12, for which the binding partner is yet unidentified, helps to regulate the crossover/non-crossover pathway decision (see below).

### Rec12 is required for meiotic DSBs and for at least one step of recombination subsequent to formation of DSBs

The 21 targeted amino acid substitutions in Rec12, plus wild-type and null mutant, effectively titrated the frequency of meiotic DSBs over a  $\geq 25$ -fold range (there was a non-zero, minimum detection limit to the assay). As expected, substitutions that reduced the frequency of Rec12-catalyzed DSBs also reduced the rate of meiotic recombination (Table 1). Moreover, with exceptions discussed below, the overall rate of recombination is proportional to the frequency of meiotic DSBs (Figure 3). These linear dose response data fill the gap between ‘all or none’ phenotypes reported previously, showing nicely that Rec12-catalyzed DSBs are necessary for meiotic recombination.

While Rec12-catalyzed DSBs are necessary for recombination, they are unexpectedly not sufficient with regard to Rec12 protein itself. Six different amino acid

substitutions in Rec12 had no significant effect upon DSBs, and yet they significantly reduced recombinant frequencies (Table 1). Therefore Rec12 is required for a step of recombination subsequent to the catalysis of DSBs, which is consistent with 'late' functions of Rec12 protein reported previously (33,39). Rec12 might be required for a proximal pathway step such as the processing of covalent protein–DNA cleavage complexes, or it might function at some downstream step(s) of the pathway, or both. Additional evidence that Rec12 regulates recombination events downstream of DSBs is discussed below.

#### **A single crossover is sufficient to ensure chiasmatic chromosome segregation in fission yeast**

Crossover recombination structures (chiasmata) are required for the faithful segregation of homologs in the first (reductional) meiotic division (1). Fission yeast places approximately 44 crossovers into its genome (three chromosome pairs) in each meiosis (72). This begs the question of whether a single crossover is sufficient to direct chiasmatic segregation or whether multiple crossovers function together in the process. Our data on chromosome segregation in meioses where recombination rates were titrated over a  $\geq 100$ -fold range (Table 1) provide a clear answer. A single crossover per chromosome pair per meiosis is apparently necessary and sufficient to ensure high-fidelity chromosome segregation in fission yeast (Figure 4). A corollary of this conclusion is that the additional crossovers are not required for chiasmatic segregation, but are seeded by Rec12 to ensure a high probability that each chromosome pair receives at least one crossover. For example, the shortest chromosome (chr. III) receives approximately 11 crossovers per meiosis and, based upon the Poisson distribution, chromosome III pairs would lack crossovers only in 1 of approximately 50 000 wild-type meioses (72).

#### **Crossover homeostasis helps to maintain the presence of chiasmata**

While chiasmatic chromosome segregation in fission yeast is achieved by a seemingly stochastic process that is incredibly robust (above), this mechanism is demonstrably not random. We discovered that fission yeast also employs crossover homeostasis. Several amino acid substitutions within Rec12 (K210A, D79A, E83A) significantly reduced the frequency of meiotic DSBs without significantly reducing the frequency of crossover recombination events (Table 1). An additional prediction of crossover homeostasis was met, for each of those mutants maintained the crossovers between *ade6* and *arg1* at the expense of intragenic recombination events within *ade6*. Therefore fission yeast cells apparently have a mechanism with which to sense the frequency of DSBs (or DSB-dependent recombination intermediates), and in response to a reduced frequency of recombination initiation they preferentially redirect intermediates into the crossover pathway. Notably, the magnitude of crossover homeostasis in fission yeast is similar to that of budding yeast cells harboring mutated, epitope-crippled versions of Spo11 (74), the only other organism in which crossover

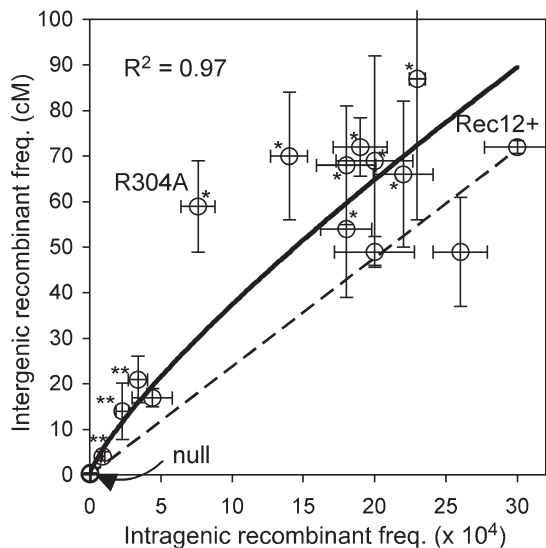
homeostasis has been reported. One presumes, given the high degree of divergence between budding yeast and fission yeast, that crossover homeostasis is both important and broadly conserved.

It has been proposed that crossover homeostasis in budding yeast is regulated by components of the synaptonemal complex (SC) and is related to crossover interference (74). This idea is at first approximation appealing, for both homeostasis and interference exert their effects upon crossovers. However, homeostasis regulates frequency and interference regulates distribution, so the two processes are at least partially distinct. Indeed, a subsequent study found that components of the SC and crossover interference pathway have only a nominal impact upon crossover homeostasis (77). Fission yeast lacks both SC and crossover interference (78), so those entities do not regulate its crossover homeostasis.

It was recently reported that differential choice of recombination partners, specifically the ratio of intersister (IS) to interhomolog (IH) recombination, helps to maintain crossovers in fission yeast (79). It seems likely that this process, called 'crossover invariance', is another manifestation of the crossover homeostasis reported here. Be that as it may, three factors suggest that regulation of the crossover/non-crossover decision by crossover invariance is exerted early. First, like crossover homeostasis, crossover invariance senses directly or indirectly the abundance of recombination-initiating DSBs. Second, it must act at or near the time when IS/IH interactions are partitioned, presumably by the time DSBs invade a homologous DNA molecule (sister or homolog). Third, it is regulated in part by DNA strand exchange proteins, which function shortly after the catalysis of DSBs.

#### **Early crossover/non-crossover decision regulated by an external surface of Rec12**

The partitioning of recombination intermediates into crossover and non-crossover outcomes occurs downstream of recombination initiation, but the decision can be made much earlier (80). Our results suggest that Rec12 protein helps, directly, to regulate the decision. The preferential reduction of intragenic (conversion) recombination occurs only for a subset of Rec12 protein variants that reduce recombination overall (Table 1), and hence can be ascribed to Rec12 protein itself. This is best illustrated by comparing the recombinant frequencies from the two classes (Figure 5). Eight amino acid substitutions significantly reduced intragenic recombination without significantly reducing intergenic recombination. An additional three substitutions significantly reduced both classes of recombination, but affected intragenic recombination to a greater extent ( $\geq 2$ -fold difference). Overall, the data fit a non-linear function ( $R^2 = 0.97$ ) with substantial preservation of crossovers when recombination rates are titrated over a broad range. And one substitution in particular, R304A, elicits a very strong separation of function phenotype (Figure 5). This substitution maps to an exposed face of an exterior  $\alpha$ -helix in the crystal structure of Top6A (Figure 1), suggesting strongly that a corresponding exterior surface of Rec12 helps to



**Figure 5.** Amino acid substitution-dependent, differential regulation of recombination outcome. The frequencies of intergenic recombination between *ade6* and *arg1* were plotted versus the frequencies of intragenic recombination within *ade6* for each Rec12 protein variant and the negative control lacking Rec12. Data are mean (circles)  $\pm$  SD (thin lines). Data points deviate from expected ratios (dotted line) and better fit a power function (curved line,  $R^2$ -value). Note that eight of 21 amino acid substitutions significantly reduce intragenic recombination without substantially affecting intergenic recombination (asterisk). Another three substitutions affect both types of recombination, but preferentially reduce intragenic recombination by a factor of  $\geq 2$  (double asterisk). The R304A substitution, which confers a strong separation of function for phenotypes, maps to a putative  $\alpha$ -helix on the exterior face of Rec12.

regulate the crossover/non-crossover decision. It presumably does so via interaction with another protein that docks to this surface. These hypotheses await further testing.

## CONCLUSIONS

We used structure-based, targeted mutagenesis to test the hypothesis that Rec12 protein catalyzes the formation of meiotic DSBs by a topoisomerase II-like mechanism. Single amino acid substitutions in five of six domains targeted, including those in each domain essential for the function of topoisomerase II superfamily proteins, coordinately reduced the formation of meiotic DSBs and meiotic recombination. We conclude that the structure and function of Rec12 in the catalysis of meiotic DSBs is broadly conserved among Rec12/Spo11/Top6A members of the topoisomerase II superfamily.

Our results also expand the repertoire of known mechanisms by which Rec12 protein ensures the faithful segregation of chromosomes in meiosis. First, although a single crossover is necessary and sufficient for chiasmatic segregation, Rec12 introduces multiple crossovers per chromosome pair (on average  $\sim 15$ ) to help maintain the presence of an 'obligate' crossover. Second, when the frequency of Rec12-catalyzed DSBs is reduced, the cells deploy crossover homeostasis to preferentially maintain the

crossovers. Third, this crossover/non-crossover pathway decision is regulated in part by Rec12 protein itself, most likely through a protein interaction surface that maps to Rec12-R304. Fourth, it has been shown previously that when achiasmatic chromosomes are detected, a backup (distributive) system helps to ensure that homologs go to opposite poles in meiosis I (33,47). This distributive system also seems to be regulated in part by Rec12 (33). These functions make sense, mechanistically, for Rec12 protein is well positioned to monitor the status of recombination initiation and, as required, to regulate its outcome.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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