Crossovers ensure the accurate segregation of homologous chromosomes from one another during meiosis. Here, we describe the identity and function of the *Drosophila melanogaster* gene recombination defective (*rec*), which is required for most meiotic crossing over. We show that *rec* encodes a member of the mini-chromosome maintenance (MCM) protein family. Six MCM proteins (MCM2–7) are essential for DNA replication and are found in all eukaryotes. *REC* is the *Drosophila* ortholog of the recently identified seventh member of this family, MCM8. Our phylogenetic analysis reveals the existence of yet another family member, MCM9, and shows that MCM8 and MCM9 arose early in eukaryotic evolution, though one or both have been lost in multiple eukaryotic lineages. *Drosophila* has lost MCM9 but retained MCM8, represented by *REC*. We used genetic and molecular methods to study the function of *REC* in meiotic recombination. Epistasis experiments suggest that *REC* acts after the Rad51 ortholog SPN-A but before the endonuclease MEI-9. Although crossovers are reduced by 95% in *rec* mutants, the frequency of noncrossover gene conversion is significantly increased. Interestingly, gene conversion tracts in *rec* mutants are about half the length of tracts in wild-type flies. To account for these phenotypes, we propose that *REC* facilitates repair synthesis during meiotic recombination. In the absence of *REC*, synthesis does not proceed far enough to allow formation of an intermediate that can give rise to crossovers, and recombination proceeds via synthesis-dependent strand annealing to generate only noncrossover products.

**Introduction**

Faithful segregation of homologous chromosomes in meiosis requires crossovers, which, in concert with sister chromatid cohesion, form the chiasmata that hold and orient homologs on the meiotic spindle. Crossovers are distributed nonrandomly between chromosomes, along each chromosome arm, and relative to one another, indicating that meiotic recombination is tightly regulated. One aspect of this regulation is the process that determines whether a recombination event becomes a crossover or a noncrossover.

Models of meiotic recombination must account for the production of both crossovers and noncrossovers. Current models are derived from the double-strand break (DSB) repair model of Szostak et al. [1]. In this model, recombination is initiated with a DSB on one chromatid (Figure 1A, parts a–f). Resection of the 5’ ends leaves 3’ single-stranded overhangs. One of these overhanging ends invades a homologous, non-sister duplex and primes repair DNA synthesis. The strand displaced by the migrating synthesis bubble is captured by the other 3’ overhang, which primes synthesis using the displaced strand as a template. Ligation of the newly synthesized ends to the resected 5’ ends generates an intermediate with two Holliday junctions. This double Holliday junction (DHJ) intermediate is resolved by an unknown endonuclease to form either crossover or noncrossover products.

Recent data from yeast has resulted in modification of this model. Allers and Lichten [2] physically monitored formation of recombination intermediates and products in *Saccharomyces cerevisiae* using an ectopic recombination system and found that noncrossover products appear before DHJ intermediates. They proposed that noncrossovers arise not through a DHJ intermediate, but through synthesis-dependent strand annealing (SDSA). In SDSA, the nascent strand dissociates from the template and anneals to the other resected end (Figure 1A, part g). Trimming of any overhangs and filling in of any gaps, followed by ligation, results in noncrossover products. Subsequent genetic tests of this model in *S. cerevisiae* are consistent with most noncrossovers coming from SDSA, while the remainder are derived from a DHJ intermediate [3].

These models also take into account the occurrence of gene conversion—nonreciprocal transfer of information from one duplex to another—that can be associated with both crossovers and noncrossovers. Figure 1B illustrates possible origins of gene conversion during SDSA. Heteroduplex DNA (hDNA), in which the two strands are derived from different parental molecules, is produced by both invasion of a single-stranded overhang into a homologous template and annealing.
Synopsis

Most of our cells have two copies of each chromosome. For sexual reproduction, these must separate from one another to produce sperm or eggs with one copy of each chromosome. This occurs during meiosis, when chromosomes pair and exchange DNA segments. This exchange—meiotic recombination—creates physical linkages between chromosome pairs and is also a source of genetic diversity. To learn more about the process of meiotic recombination, the authors characterized the gene recombination defective (rec) from the fruit fly Drosophila melanogaster. Molecular analysis revealed that rec is related to a large family of genes found in all animals, plants, and protists. These genes are thought to be important in DNA replication, but rec appears to have a novel function. The authors found that mutants lacking rec are unable to copy enough DNA during meiotic recombination to form linkages between chromosomes. This results in chromosomes segregating randomly during meiosis, so that most eggs have an incorrect number or composition of chromosomes.

Recombination is initiated with a DSB (a), that is resected to generate 3’ single-stranded ends (b). A 3’ end invades the homologous chromosome (c) and primes repair DNA synthesis (d). If repair synthesis proceeds far enough, second-end capture can occur (e). After repair synthesis from the second end, ligation of newly synthesized 3’ ends to resected 5’ ends results in a DHJ intermediate (f). Resolution of the DHJ can generate a CO or a NCO. In SDSA, the nascent strand dissociates after repair synthesis and anneals to the second broken end (g). Gap filling and ligation generates an NCO. Note that NCO products can come from either a DHJ intermediate or SDSA, or both.

(A) Model for the formation of crossovers (CO) and noncrossovers (NCO). Recombination is initiated with a DSB (a), that is resected to generate 3’ single-stranded ends (b). A 3’ end invades the homologous chromosome (c) and primes repair DNA synthesis (d). If repair synthesis proceeds far enough, second-end capture can occur (e). After repair synthesis from the second end, ligation of newly synthesized 3’ ends to resected 5’ ends results in a DHJ intermediate (f). Resolution of the DHJ can generate a CO or a NCO. In SDSA, the nascent strand dissociates after repair synthesis and anneals to the second broken end (g). Gap filling and ligation generates an NCO. Note that NCO products can come from either a DHJ intermediate or SDSA, or both.

(B) Sources of gene conversion. hDNA is first generated during strand invasion (a, dotted box); mismatch repair may replace the invading sequence with the sequence of the template (b). After synthesis (c) and dissociation, annealing of the nascent strand to the other end of the break also generates hDNA (d), which can also be acted on by mismatch repair (e). The final NCO product in this example (f) has a region of gene conversion derived from two rounds of mismatch repair, but one round is sufficient to generate a gene conversion. Also, mismatch repair can act on the DHJ intermediate (A) or the products of DHJ resolution, and thus gene conversion can also be associated with crossing over.

DOI: 10.1371/journal.pgen.0010040.g001

Figure 1. Meiotic Recombination Models

(B) Sources of gene conversion. hDNA is first generated during strand invasion (a, dotted box); mismatch repair may replace the invading sequence with the sequence of the template (b). After synthesis (c) and dissociation, annealing of the nascent strand to the other end of the break also generates hDNA (d), which can also be acted on by mismatch repair (e). The final NCO product in this example (f) has a region of gene conversion derived from two rounds of mismatch repair, but one round is sufficient to generate a gene conversion. Also, mismatch repair can act on the DHJ intermediate (A) or the products of DHJ resolution, and thus gene conversion can also be associated with crossing over.

DOI: 10.1371/journal.pgen.0010040.g001

To gain insight into the function of the REC protein in meiotic recombination, we identified rec molecularly and found that it encodes the Drosophila ortholog of MCM8. The eukaryotic mini-chromosome maintenance (MCM) family of proteins contains six members (MCM2–7) that form a heterohexameric helicase required for replication [19]. Though MCM2–7 are essential in all eukaryotes, rec mutants are viable, and we have been unable to find any function for REC outside of meiosis. To explore the defect in meiotic recombination further, we analyzed the distribution of crossing over in rec mutants and found that residual crossovers are distributed abnormally. This finding, coupled with epistasis analysis, suggests that REC might act at an intermediate step in recombination. Further insight into the function of REC comes from our finding that the frequency of noncrossovers is substantially increased in rec mutants, and that these noncrossover events have significantly shorter gene conversion tracts than those of wild-type females. Based on these phenotypes and the structural similarity between REC and MCM proteins, we propose that REC facilitates processive repair DNA synthesis, and is a prerequisite for formation of the DHJ intermediate during meiotic recombination. In the absence of REC, recombination proceeds through SDSA to generate noncrossovers.
Results
Molecular Identification of rec

Grell's early work on the null alleles of rec found that homozygous mutant females produce progeny with high levels of chromosome nondisjunction [18]. Elevated levels of nondisjunction are indicative of, among other things, defects in homolog synopsis or meiotic recombination. Grell found that although synaptonemal complex formation was normal in rec mutant females, the frequency of crossing over among progeny recovered was 3.6% of wild-type levels. This crossover reduction indicates that the product of rec is involved in the meiotic recombination pathway and is required to generate most crossovers. To understand the function of REC in generating crossovers, we first sought to identify the gene molecularly.

Matsubayashi and Yamamoto [20] used deletion and restriction mapping to place rec in a region on the right arm of Chromosome 3 between the genes c(3)G and spn-E. Subsequently, c(3)G and spn-E were molecularly identified and the region was sequenced, revealing nine predicted genes in the region to which rec was mapped. We identified CG31293 as a candidate for rec because it was the only gene in the region that encodes a protein whose proposed function is associated with DNA metabolism. CG31293 has two exons separated by a 6-kilobase intron. We sequenced CG31293 in rec and rec2 mutant flies and found mutations in both (Figure 2). Sequencing CG31293 in rec homozygotes revealed a C→T transition at position 889, which changes a glutamine codon to a stop codon. If translated, this mutation would result in a truncation after 295 of 885 amino acids. The rec2 allele has an A→T transversion that disrupts the CG31293 splice acceptor site.

To generate additional alleles of rec, we screened the progeny of females with an EMS-mutagenized Chromosome 3 in trans to rec2 for high levels of X chromosome nondisjunction (see Materials and Methods for details). Out of 1,238 lines screened, we obtained two alleles of rec, both of which have mutations in CG31293. The rec1 allele has a C→T transition at nucleotide 2251 that generated a nonsense codon near the end of the first exon; rec2 mutants could potentially produce a protein of the same approximate size as the rec2 mutant. The rec1 allele has a C→T transition at nucleotide 142 that generated a nonsense codon predicted to truncate the protein after only 47 residues. Based on the sequence of these four alleles, we conclude that rec corresponds to CG31293 and that all four alleles are likely to be null.

During the course of this study, Matsubayashi and Yamamoto, using a different strategy also concluded that rec corresponds to CG31293 [21]. Their sequence analysis of rec4 and rec5 corresponds with our findings. They also sequenced the temperature-sensitive allele rec5 and found a C→T transition at position 1379, which causes the substitution of a nonconserved serine for a phenylalanine at residue 455.

MCM8 and MCM9 Arose Early in Eukaryotic Evolution

CG31293 encodes an 885-amino acid protein that is homologous to the MCM family of proteins found throughout eukaryotes and archaea [19]. Archaeal species each have a single MCM that forms a homohexamer believed to be the replicative helicase. Eukaryotes have six related MCM proteins, MCM2–7, which constitute a hexameric helicase involved in replication, recombination, and transcription [22]. REC is the Drosophila ortholog of MCM8, a seventh eukaryotic member of this family that was identified recently [21,23–26]. MCM8 has been reported to be present in vertebrates and Drosophila, but not in fungi or nematodes, and thus the origin of this family member is unclear.

To learn more about the MCM family of proteins, we analyzed MCMs from a number of diverse eukaryotes. On the basis of molecular and ultrastructural analysis, eukaryotes can be divided into eight major phylogenetic groups [27]. Previously, MCMs have been analyzed from only two of these groups: plants, represented by Arabidopsis thaliana, and opisthokonts, which include fungi and animals. In addition, Gozuacik et al. [26] noted the existence of an MCM8 ortholog in the discicristate Leishmania major, suggesting a broad phylogenetic distribution of this family member among eukaryotes. To expand this analysis, we identified all of the MCMs from species for which complete or nearly complete genome sequence is available, including at least one from each of the eight eukaryotic phylogenetic groups (Table S1). We conducted alignments on the predicted protein sequence of each conserved MCM domain and used these alignments to construct phylogenetic trees (see Materials and Methods for details). Eukaryotic MCM domain proteins cluster into eight subfamilies comprising the six replicative MCMs, MCM8, and a novel group that we refer to as MCM9 (Figure 2).

Classification in these eight subfamilies was the same with two different algorithms for tree construction (maximum likelihood and neighbor joining), although the relationship of each group to one another and group members to each other varies with different methods of tree construction.

As expected, the six replicative MCMs are present in all eukaryotes, indicating that these six were present in the ancestral eukaryote. MCM8 and MCM9 are also widely distributed among eukaryotes, found in five of the six phylogenetic groups we examined, suggesting that the MCM8 and MCM9 subfamilies also arose early in eukaryotic evolution. Because both are absent from the excavaete Giardia lamblia, which is perhaps the most deeply branching species surveyed, we cannot determine whether these family members originated after a split between excavates and other eukaryotes, or whether they were lost in the lineage giving rise to Giardia. MCM8 and MCM9 are also absent from the nematode Caenorhabditis elegans and from all available fungal genomes, with the exception of the microsporidium Encephalitozoon...
Thus, MCM8 and MCM9 do appear to have been lost multiple times during the eukaryotic radiation. Interestingly, our survey suggests that in most eukaryotic genomes MCM8 and MCM9 are either both present or both lacking. This raises the possibility that these two family members function together.

Mammalian MCM9 is unique in that it lacks the carboxy-terminal half of the conserved MCM domain, including the Walker B box (Figure S1). This is unlikely to be an error in annotation, since all mammalian MCM9 sequences available have a similar structure, and several full-length cDNA sequences encoding the truncated human protein are present in the database. Although mammalian MCM9 would lack ATPase activity on its own, it is possible that this protein retains some other function.

Three features of the phylogenetic tree in Figure 3 indicate that *Drosophila* REC has diverged from other MCM8 proteins. First, REC never clusters adjacent to human MCM8, despite the fact that this is the most closely related of the species shown (compare clustering of replicative MCMs). Second,
Drosophila melanogaster is the only species surveyed for this figure that has MCM8 but lacks MCM9. This is true for other Drosophila species, including D. pseudoobscura and D. viridis, which diverged from D. melanogaster approximately 25 and 40 million years ago, respectively (Figure S2). However, another arthropod, the mosquito Anopheles gambiae, has retained both MCM8 and MCM9.

The third notable feature of REC is that it has a longer branch length than other MCMs, indicating that it has accumulated more substitutions than its orthologs. Alignment of MCMs from several diverse eukaryotes and several Drosophila species (Figure S2) shows some significant differences between REC and other MCMs. Replicative MCMs have consensus sequences for the Walker A and B boxes, which are involved in nucleotide binding and hydrolysis [28], and differ from those of other ATPases. The A box consensus sequence of GxxGxGKT is GDP[GS][SA]KS (x represents any residue; residues in brackets are alternative possibilities at a given site) in replicative MCMs. Although other MCM8 and MCM9 sequences match the replicative MCM consensus, REC has a sequence closer to the canonical ATPase consensus: GDP[Gx]x[S]xKS. Replicative MCMs also have a highly conserved Walker B signature of IDEFDKM. Even within the deeply branching protists, the only substitutions are replacement of the initial isoleucine with a different bulky aliphatic residue (L, V, or M), replacement of the central phenylalanine with a different hydrophobic residue (leucine in several G. lamblia MCMs), or replacement of the second aspartic acid residue with glutamic acid. The corresponding sequence of REC, however, is LDDVDKL, which has five substitutions in the seven-residue sequence. However, each substitution represents a conservative change, suggesting that ATPase function may have been conserved. We also note that the DK residues in this motif are not conserved in the MCM9 subfamily. A third highly conserved motif found in MCM proteins is the arginine finger found downstream of the Walker B box. The consensus for this motif is UUSRFDUU, where U is a bulky aliphatic residue (I, L, V, or M). This motif is found in all eukaryotic and archaean MCMs surveyed except Arabidopsis MCM9 and Drosophila REC. In REC, this sequence is LLRFHHLV, and thus the absolutely conserved SR and D residues are missing.

In spite of the important sequence differences between REC and other MCMs, it is clear that REC is a member of the MCM family. When REC is used as a query in BLAST searches, all MCM proteins are returned with a high score, while other sequences do not receive significant scores. In addition, searches for conserved domains identify the MCM domain with high confidence. The divergence of Drosophila REC from other MCMs may be related to the absence of MCM9 in Drosophila, perhaps by allowing REC to assume functions of both proteins. Alternatively, REC may have acquired a novel function that does not require MCM9.

Though REC has sequence homology to replicative MCMs, rec mutants do not have phenotypes consistent with an essential role in replication. The genes encoding Drosophila MCM2–7 are essential [29–33], but rec mutants are viable and do not exhibit any of the gross developmental defects observed in the replicative MCM mutants. However, rec is expressed in somatic tissues—CDNs have been identified in libraries made from embryos, adult head, and cell lines [34]—suggesting a function outside of meiosis. Since many of the genes required for meiotic recombination are also required for mitotic repair of DNA damage [35], we hypothesized that REC may be involved in repair of DNA damage in somatic cells. To test this hypothesis, we measured the sensitivity of rec mutants to agents that cause DSBs (gamma irradiation) and block replication (methyl methanesulfonate [36] and nitrogen mustard), following previously published methods [37,38]. We found that rec mutants are not hypersensitive to either of these agents when compared to their heterozygous siblings (unpublished data). We conclude that REC is not essential for repair of DSBs or blocked replication forks in mitotically dividing cells. Matsubayashi and Yamamoto also found that rec mutants are not hypersensitive to X-rays or methyl methanesulfonate [21].

**REC Functions in an Intermediate Step of Meiotic Recombination**

The only known function for REC is in generating meiotic crossovers. As a putative replicative helicase, there are many steps in recombination where REC might act to promote crossing over, including pre-meiotic S phase, resection, strand invasion, repair synthesis, and resolution. Because rec mutants have normal synaptonemal complex formation [18], it is unlikely that REC is required to complete pre-meiotic S phase. To determine where REC acts in subsequent steps of meiotic recombination, we first placed rec genetically in the Drosophila recombination pathway relative to previously characterized genes. To accomplish this, we conducted epistasis studies and analyzed the distribution of residual crossovers in rec mutants. In Drosophila, null mutations in genes whose products are required to generate meiosis-specific DSBs (mei-W68, mei-P22) abolish essentially all crossovers [4]. Because null rec mutants have residual crossovers, we conclude that REC is not involved in DSB formation like MEI-W68 and MEI-P22. After DSB formation, broken ends are resected and Rad51 homologs catalyze strand invasion. Females mutant for spn-A, which encodes the Drosophila ortholog of Rad51, are sterile and lay eggs with developmental patterning defects [9]. These phenotypes are due to an activated DNA damage-dependent cell-cycle checkpoint caused by persistent unrepaired meiotic DSBs, which disrupts communication between the oocyte and the somatic follicle cells that pattern the eggshell [10]. The proteins required for resection are not known, but we expect Drosophila resection mutants to have a phenotype similar to spn-A mutants because they would also be unable to repair meiotic DSBs. Because rec mutants are not sterile and have normal eggshell patterning, it is unlikely that REC is required for resection or strand invasion. To determine whether REC functions before or after strand invasion, we generated rec spn-A double mutants. The phenotype of the double mutant females was similar to that of spn-A single mutants, including sterility and patterning defects (unpublished data). Though this does not exclude the possibility that REC could be acting in an accessory role at either resection or strand invasion, it suggests that REC acts after strand invasion.

Further insight into the function of REC can be gained by examining the distribution of residual crossovers in rec mutants. In wild-type females, most crossovers are located in the middle of a chromosome arm and very few occur near centromeres or telomeres. In most recombination mutants that do not completely abolish crossing over, the reduction in
Crossing over is polar, with a stronger reduction in medial intervals and less reduction in intervals proximal to the centromere [39,40]. Null mutations of mei-218 and hypomorphic alleles of genes required for DSB formation and strand invasion have this phenotype [41]. The only known recombination mutants that do not have a polar reduction are mei-9, which encodes the Drosophila ortholog of the endonuclease XPF, and mus312, which encodes a protein required for the meiotic function of MEI-9 [13,39]. MEI-9 and MUS312 are believed to act together in the final steps of the crossover pathway, perhaps in resolution of DHJ intermediates. Resolution of Holliday junctions in Escherichia coli requires the branch migration activity of the helicase RuvB (reviewed in [42]), and in mammalian cells Holliday junction branch-migration activity co-purifies with resolvase [43]. If REC has helicase activity that is required for resolution, we would expect rec mutants to exhibit a uniform reduction in crossing over, as in mei-9 mutants.

To examine crossover distribution in rec mutants, we measured the frequency of recombination on Chromosome 2 using intervals flanked by visible markers that span the entire left arm and centric heterochromatin. We calculated the ratio between the reduction in crossover frequency in the centromere-proximal interval (pr-cn) and the reduction across the entire arm (al-cn) (Table 1). In mei-9 mutants this ratio was 1.1, showing that the reduction in crossing over is the same in the centromere-proximal interval as in other regions of the chromosome. In mei-218 mutants, however, this ratio was 4.0, due to a more modest reduction in the interval proximal to the centromere. When we analyzed the frequency and distribution of crossovers in rec mutants, we found a more modest reduction in crossing over in the centromere-proximal interval compared to the total crossover reduction (pr-cn to al-cn ratio of 3.3). Based on this analysis, we conclude that REC does not act with MEI-9 at the resolution step of meiotic recombination.

Because mei-9 and rec have different distributions of residual crossovers, we were able to perform epistasis analysis between these two genes. As in the rec single mutant, the reduction in crossing over is polar in the double mutant, with a pr-cn to al-cn ratio of 6.2. Although this ratio is actually higher than that of the rec single mutant, it is unclear whether this difference is functionally significant or is merely an anomaly caused by genetic background effects or relatively low sample size. (Due to the difficulty of the genetic manipulations involved in generating and assaying the double mutant, we were not able to generate as large a sample size for this genotype as for the single mutants; for example, we recovered only a single crossover within the b-pr region, resulting in an apparently more severe decrease in this region than in other regions, though recovery of one additional crossover would have made this region appear more like the al-dp region.) Regardless of the reason for the differences between the double mutant and the rec single mutant, it is clear that the double mutant exhibits a polar phenotype, confirming that REC does not function at the same time as MEI-9 but most likely acts at an intermediate step in the recombination pathway.

### Table 1. Crossing Over on the Second Chromosome in Recombination Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>al – dp*</th>
<th>dp – b</th>
<th>b – pr</th>
<th>pr – cn</th>
<th>Total al-cn</th>
<th>Ratio of pr-cn/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1,601</td>
<td>11 (100)</td>
<td>24 (100)</td>
<td>6 (100)</td>
<td>2 (100)</td>
<td>43 (100)</td>
<td>1.0</td>
</tr>
<tr>
<td>mei-9</td>
<td>2,352</td>
<td>0.55 (5.0)</td>
<td>2.2 (9.2)</td>
<td>0.34 (5.7)</td>
<td>0.17 (8.5)</td>
<td>3.3 (7.7)</td>
<td>1.1</td>
</tr>
<tr>
<td>mei-218</td>
<td>2,547</td>
<td>0.55 (5.0)</td>
<td>1.7 (6.9)</td>
<td>0.71 (12)</td>
<td>0.63 (32)</td>
<td>3.5 (81)</td>
<td>4.0</td>
</tr>
<tr>
<td>rec&lt;sup&gt;1&lt;/sup&gt;/rec&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2,300</td>
<td>0.48 (4.4)</td>
<td>0.70 (2.9)</td>
<td>0.23 (3.8)</td>
<td>0.26 (13)</td>
<td>1.7 (40)</td>
<td>3.3</td>
</tr>
<tr>
<td>mei-9&lt;sup&gt;2&lt;/sup&gt;; rec&lt;sup&gt;1&lt;/sup&gt;/rec&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1,357</td>
<td>0.29 (2.6)</td>
<td>1.3 (5.2)</td>
<td>0.07 (1.2)</td>
<td>0.66 (33)</td>
<td>2.3 (53)</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Recombination frequency is expressed as map units across the intervals shown. Numbers in parentheses denote the percentage of wild-type recombination frequency.

** DOI:** 10.1371/journal.pgen.0010040.t001

**Rec Mutants Have Increased Rates of Noncrossover Recombination but Shorter Gene Conversion Tracts**

Our data place REC in an intermediate step of the recombination pathway, sometime after strand invasion. These steps include repair DNA synthesis, capture of the second resected end, and ligation to form a DHJ intermediate. Because REC is homologous to MCM replication proteins, we hypothesized that REC acts during repair DNA synthesis. Repair synthesis is necessary for formation of both crossovers and noncrossover gene conversions. If REC is essential for all repair synthesis during meiosis, then rec mutants should exhibit a reduction in the frequency of noncrossovers similar to the reduction observed in crossovers. Alternatively, if REC merely facilitates repair synthesis, then there may be no reduction in the frequency of noncrossovers in rec mutants, but gene conversion tracts should be shorter than in wild-type.

To determine the frequency of noncrossovers and the length of conversion tracts, we recovered noncrossover gene conversions. Because there are no hotspots for recombination in Drosophila, we used a system originally developed by Chovnick and colleagues to select for recombination events that occur at the rosy (ry) locus [44,45]. The ry gene encodes xanthine dehydrogenase, an enzyme that metabolizes purine and is required for normal eye pigmentation; ry mutant larvae die when the food is supplemented with purine. Females trans-heterozygous for ry<sup>321</sup> and ry<sup>606</sup>, missense mutations separated by 3.8 kilobases, are crossed to males that are homozygous for a deletion of ry (Figure 4). The progeny are treated with purine so that only rare ry<sup>1</sup> recombinants and mosaics that have both ry<sup>1</sup> and ry mutant tissue (products of PMS) survive. Visible markers flanking ry (kar and cv-c) were used to distinguish crossovers from noncrossover recombination events.
We screened more than 2.3 million progeny of wild-type females and more than 0.7 million progeny of rec mutant females (Table 2). As expected, the frequency of crossing over in rec mutants was less than 10% of the wild-type frequency. In contrast, noncrossover gene conversions were recovered almost twice as frequently in rec mutants as in wild-type. Increased recovery of noncrossovers in rec mutants could reflect an actual increase in the frequency of events, or it could be caused by an increase in gene conversion tract length. An increase in tract length increases the frequency of recovery of recombination events because selecting for rec recombinants enriches for longer conversion tracts. Since there are no recombination hotspots in Drosophila, recombination could be initiated at any site within or near ry [46]. Therefore, the longer a conversion tract is, the greater the probability that it crosses either the ry606 or ry531 mutant site. (There is also a selection against extremely long tracts, probability that it crosses either the nation could be initiated at any site within or near ry531 7.3-kilobase region that includes the morphisms, or small insertion/deletion heterologies, within a because the events recovered. We were able to map gene conversion tracts lengths in the mutants, we measured gene conversion tract lengths in the recovered more noncrossover gene conversions in REC facilitates repair synthesis, and to determine why we can conclude that REC is not essential for noncrossovers, we can treat larvae with purine, recover ry+ recombinants Figure 4. Intragenic Recombination at rosy Cross scheme used to recover recombination events within the ry gene. Brackets indicate that females were either trans-heterozygous for different rec alleles or were completely wild-type at rec. The three types of ry- progeny recovered after purine selection are indicated. DOI: 10.1371/journal.pgen.0010040.g004

Previous work to determine the mean length of conversion tracts at ry has utilized models that take into account the selection for longer tract lengths [48,49]; we employed a similar strategy. We sequenced the region flanking the selected site of conversion (ry531 or ry606) in 29 wild-type and 18 rec noncrossover gene conversion events and determined which nonselectable polymorphisms had also been converted (co-convertants: Figure 5B and C). To calculate the mean conversion tract length for each genotype, we derived a maximum likelihood model that incorporates standard errors, allowing us to compare the mean tract lengths of wild-type and rec mutants with 95% confidence intervals (see Materials and Methods). Conversion tract lengths generated by rec mutants are shorter than those from wild-type, with mean tract lengths of 250 basepairs (bp) and 441 bp, respectively. Although the 95% confidence intervals (160–340 bp and 323–558 bp, respectively) overlap slightly, the difference in tract lengths between rec and wild-type is statistically significant (p = 0.01). The increased recovery of noncrossover gene conversions from rec mutants therefore reflects an actual increase in the frequency of events and is not the result of longer conversion tracts. Indeed, because the conversion tracts are significantly shorter in rec mutants, the frequency of events recovered actually underestimates the true increase in noncrossover gene conversions.

Gene conversion tracts are the product of hDNA formation and repair. To determine whether the shorter tract length in rec mutants is due to a defect in the repair of hDNA, we used two different methods to determine whether PMS, a readout for hDNA repair, occurs in wild-type or rec mutants. Since the ry gene product is non–cell autonomous, any ry+ recombinant could be mosaic, containing genetic information for both ry+ and ry in different cells. To test for germ-line mosaicism, recombinant progeny were mated to ry flies; germ-line mosaics produce both ry and ry+ progeny. To test for somatic mosaicism, PCR was performed using primers specific for the ry606 and ry531 sites. Mosaicism was not detected in any of the 29 noncrossovers recovered from wild-type females or the 18 noncrossovers from rec mutants. Since mosaicism has been detected using both methods in noncrossover events from mei-9 mutants (SJR and JS, unpublished data), we conclude that PMS does not occur at an appreciable frequency in the absence of REC.

Discussion

Understanding how crossovers form is crucial to understanding the mechanisms eukaryotes use to faithfully pass half of their genetic information to the next generation. In
Drosophila, many components of the meiotic recombination pathway have been identified, but a complete picture of the process has yet to emerge. In this paper, we molecularly and genetically characterized an important participant in this pathway—REC, the Drosophila homolog of MCM8—giving us new insight into requirements for crossover formation.

Our data support a model in which REC acts at an intermediate step of meiotic recombination. REC is not required for pre-meiotic S phase because homologous chromosomes in rec mutant females form normal synaptonemal complex, indicative of complete replication of genomic DNA [18]. Our finding that rec mutant females have about twice the normal number of noncrossover gene conversions indicates that initiation of recombination is not impaired in rec mutants; rather, very few DSBs are repaired as crossovers. Our data suggest that REC functions after strand invasion, since females mutant for both rec and spn-A, which encodes the Rad51 ortholog, phenocopy spn-A single mutants. Based on the distribution of residual crossovers in rec mutants and in mei-9; rec double mutants, it is likely that REC does not function with MEI-9 at resolution but acts at some previous step.

Normally, some recombination events become crossovers and some become noncrossovers. An increase in noncrossovers would occur if the crossover pathway were blocked so that most or all events followed the noncrossover pathway. In the ry intragenic recombination assay, noncrossover gene conversions are recovered only if they span a mutant site and convert that site to the wild-type sequence. In contrast, a crossover can be recovered if it occurs anywhere between the two mutations, as long as it generates a wild-type chromosome. Based on conversion tract lengths and the distance between the two mutations, we expect that many of the crossovers we recover would not be detected if they instead became noncrossovers, because they would not contain a conversion tract long enough to span a mutant site. The increase in noncrossovers that we observed in rec mutants, therefore, appears to be more than expected from this simple interpretation. A possible explanation for the increased frequency of noncrossovers in rec mutants comes from a hypothesis proposed by Bhagat et al. [41], who suggested that crossover distribution is disrupted as the result of a feedback mechanism that ensures one crossover per chromosome. The proposed feedback mechanism senses some intermediate in the crossover pathway (e.g., the DHJ structure). In mutants in which this intermediate does not form, a signal causes the cell to initiate additional recombination events to ensure that a crossover is obtained. These initiations may occur outside the normal constraints, leading to a disruption of the normal distribution and an apparent polar reduction in crossing over. According to this model, rec mutants are impaired in...
formation of some crucial intermediate leading to crossovers. As a result, more recombination events are initiated, but most of these still become noncrossovers. Thus, the frequency of noncrossovers is elevated, and the crossovers that are produced do not follow the normal distribution.

The defect in rec mutants is not limited to an increased production of noncrossovers at the apparent expense of crossovers. We also found that noncrossover gene conversion tract length is significantly reduced in rec mutants. This could result from a defect in generating hDNA or a defect in repairing hDNA. Defects in repair of hDNA result in PMS of markers within the heteroduplex tract. We did not detect PMS in any of the events from wild-type or rec mutant females. Thus, rec mutants are not defective in repair of hDNA; rather, formation of hDNA may be compromised.

The length of hDNA can be affected by the extent of strand invasion and the amount of repair synthesis. In S. cerevisiae, the Mer3 helicase has been shown in vitro to stimulate Rad51-mediated strand invasion [50]. As in rec mutants, mutations in the gene that encodes Mer3 cause a reduction in the frequency of crossovers and an increase in the frequency of noncrossovers [51]. However, in physical assays mer3 mutants are defective in the transition from DSB to strand invasion intermediate. Our data suggest that REC acts after strand invasion, so we do not favor the notion that REC performs a function similar to that of Mer3. Furthermore, based on the similarity of REC to replicative MCMs, we think it plausible that rec mutants have shorter conversion tracts because repair synthesis is diminished.

What is the relationship between reduced repair synthesis and decreased crossing over in rec mutants? In S. cerevisiae, crossovers are believed to arise through resolution of the DHJ intermediate. Although this process can also give rise to noncrossovers, most noncrossovers are thought to arise through SDSA [2]. There is evidence in S. cerevisiae that formation of a DHJ intermediate requires more repair synthesis than SDSA [3]. If this is also the case in Drosophila, then decreased repair synthesis would increase the probability that a meiotic DSB will be repaired through SDSA instead of the DHJ pathway.

We propose a model in which REC drives crossover formation by acting at the repair synthesis step of meiotic recombination (Figure 6). In the absence of REC, synthesis does not proceed far enough to allow second-end capture and formation of the DHJ intermediate, resulting in a deficit of crossovers. Noncrossovers may still be formed through SDSA. There are two versions of this model. First, REC may facilitate repair synthesis at all sites of meiotic recombination. In this version, noncrossovers may normally arise through the DHJ pathway or the SDSA pathway, but in rec mutants the SDSA pathway is favored; the decrease in gene conversion tract length in rec mutants reflects an overall decrease in repair synthesis. Alternatively, REC may facilitate synthesis only at those recombination sites designated to become DHJ intermediates (Figure 6B). In this version of the model, sites lacking REC in wild-type flies undergo SDSA. The decrease in mean tract length in rec mutants is due to loss of those noncrossovers that would have arisen via a DHJ intermediate.

Our data do not indicate whether noncrossovers in wild-type flies arise through SDSA, DHJ, or a combination of the two. In Drosophila, SDSA is a primary pathway for DSB repair in nonmeiotic cells [16,17]. It may be that SDSA is the...
Materials and Methods

Genetics. Flies were propagated at 25 °C on standard medium containing agar, cornmeal, dextrose, and brewer’s yeast. Information on loci mentioned, but not described here, is available on FlyBase (http://flybase.bio.indiana.edu/search/) [34].

Screen for new rec alleles. Two- to three-day-old y/ y ; kar ry506 cv-c females were starved for 1 h in an empty vial and transferred to a bottle containing 25 mM EMS in 1% sucrose. After eating EMS overnight, the males were transferred to a clean bottle for 1 d. Treated males were crossed to y ; MMRST6B Hu ry females (60 females and 30 males per bottle). Single males with a balanced mutagenized Chromosome 3 were crossed to y ; ru mus312 Z2035 st rec1 Ubc83-34/TM6B Hu ry females. Nonvigin females that were y ; ru mus312 Z2035 st rec1 Ubc83-34/ry kar cv-c were placed in a new vial with brothers. The progeny resulting for non-yellow females and yellow males which result from X chromosome nondisjunction. Stocks were made of all lines that exhibited nondisjunction, and new alleles of rec were confirmed by complementation analysis. Z2035 denotes an uncharacterized meiotic recombination mutation recovered in a screen of a collection of EMS-mutagenized chromosomes (SM and JS, unpublished data).

Phylogenetic analysis of MCM domain proteins. MCM proteins were identified by BLASTP and TBLASTN searches of the genome sequence databases at http://www.ncbi.nlm.nih.gov/BLAST1. Accession numbers of each protein are listed in Table S1. Initial alignments were done with ClustalX [52], using the default settings. Regions outside of the core MCM domain were removed, as were large insertions within the core. A de novo alignment was generated with these core sequences, using the BLOSUM scoring matrices. This alignment was used to generate phylogenetic trees using the neighbor-joining method implemented in ClustalX, with 10,000 boot-strap trials, and independently using the maximum likelihood method of Tree Puzzle [53].

Crossover frequency and distribution. Crossing over on Chromosome 2 was assayed by crossing all dp b pr Bl cv-c females in different genetic backgrounds to al dp b pr cv-c males. Recombination frequencies are expressed in map units; 1 map unit is equal to a recombination frequency of 1%.

Sequencing of rec mutants. Single homozygous mutant flies were homogenized in Squishing Buffer (10 mM Tris-HCl [pH 8.2], 1 mM EDTA, 25 mM NaCl, 1 mg/ml Proteinase K) incubated at 37 °C for 30 min and then 95 °C for 5 min. PCR was performed using gene-specific primers. Products were isolated by agarose gel electrophoresis, purified from a gel slice, and sequenced at the University of North Carolina Genome Analysis facility. Mutations were verified by sequencing the opposite strand from a independent amplification.

Intragenic recombination at the rosy locus. Females trans-heterozygous for ry806 and ry781 were crossed to yf/N; kar ry506 cv-c males (see Figure 4 for all crosses used). Crosses were set up in bottles containing alkaline medium using 50 females and 30 males (rec mutants) or 30 females and 10 males (wild-type). After 3 d, the adults were transferred to new bottles to generate the second brood, and the first brood bottles were treated with 0.75 ml of 0.2% purine. This was repeated to generate a third brood. One out of every 25 bottles was not treated with purine so that the total number of flies screened could be estimated.

For every ry progeny recovered, the type of recombinant was determined by examining the presence of flanking markers kar (0.3 map units to the right of ry) and cv-c (2.1 map units to the left of ry). Crossover progeny are wild-type for all three markers; a noncrossover gene conversion of ry781 produces a crosswise (cv-c) fly, and a noncrossover gene conversion of ry806 results in a karmoyins (kar-) eyed fly. Because of the proximity of these markers to one another, it is unlikely that individual ry progeny represent more than one recombination event in the region. Recombinant progeny were crossed to kar ry506 cv-c flies. After mating the recombinant fly, it was removed and homogenized in Squishing Buffer. To determine whether PMS occurred, allele-specific PCR was performed on the noncrossover gene conversion progeny using primers specific to either the ry781 or ry806 allele.

All experiments, the crossing to generate females trans-heterozygous for ry806 and ry781 were done at 25 °C. When adults began to emerge, they were kept at 18 °C overnight for virgin collection, and 25 °C during the day. For wild-type, crosses of these females to ry806 males were incubated at room temperature, typically 20-22 °C. We analyzed 22 conversion tracts from these crosses. Crosses of rec mutant females using to ry506 males were incubated at 25 °C. We expected that the difference in temperatures would not have a strong effect on meiotic recombination, since recombination occurs about 4 d prior to mature oocyte formation, which in most cases would be during virgin collection. Nonetheless, to ensure that the decreased tract length in rec mutants was not due to the different temperatures, we generated eight noncrossover gene conversions from wild-type females at 25 °C. These conversion tracts were in fact longer than those from the room temperature experiments (mean of 666 bp versus 374 bp). Because of this small sample size, however, we have based our analysis on all 29 wild-type tracts. We note that if tracts are truly longer at 25 °C, then the decreased tract length in rec mutants is even more severe than our analysis suggests.

Statistical analysis of gene conversion tract lengths. We assume that the number of co-conversions follow a binomial distribution with parameter $\phi$, where $k$ is the number of bp between the selected and nonselected sites. In this analysis, we consider 44 distinct values of $k$. The values of $k$, co-conversions, and total conversions for all the wild-type data are given in Table S3. Let $n_{k}$ be the number of co-conversions at the $k$th site, and let $n_{k}$ be the total number of conversions. The likelihood of the entire dataset is given by a product of binomial probabilities:

$$ L(\phi) = \prod_{k=1}^{44} \left( \binom{n_{k}}{k} \phi^{k}(1-\phi)^{n_{k}-k} \right) $$

The log of the likelihood in (1) is given by

$$ \log(L(\phi)) = \text{constant} + \sum_{k=1}^{44} n_{k} \log(\phi) + \sum_{k=1}^{44} (n_{k} - n_{k}) \log(1-\phi) $$

To find the maximum likelihood estimate of $\phi$, we take the derivative of (2) with respect to $\phi$ and set it equal to zero. We denote the maximum likelihood estimate of $\phi$ by $\hat{\phi}$. Using standard large sample theory arguments [54], it can be shown that $\hat{\phi}$ is approximately normally distributed with large sample variance approximately equal to

$$ \text{Var}(\hat{\phi}) = \frac{1}{\hat{\phi}(1-\hat{\phi})} $$

The formulae in (3) and (4) facilitate the computation of large sample confidence intervals for $\phi$, which take the form $\hat{\phi} \pm z_{0.05}/\sqrt{\text{Var}(\hat{\phi})}$, where $\text{SE}(\hat{\phi}) = \sqrt{\text{Var}(\hat{\phi})}$ is the standard error of the estimate, and $z_{0.05}$ is the 95% confidence interval. For example, for a 95% confidence interval, $z_{0.05} = 1.65$ (see Table S3) and assume that the tract length distribution follows a geometric distribution with parameter $\phi$, and hence the mean tract length $m = \frac{1}{\phi}$ and the expected selected tract length $m = \frac{n_{k}}{\phi}$ (Table S3). Using standard large sample theory along with the delta method [54], it can be shown that $\hat{m}$ is approximately normally distributed with approximate large sample variance

$$ \text{Var}(\hat{m}) = \text{Var}(\hat{\phi}) \left( \frac{1}{(1-\hat{\phi})^{2}} \right) $$

and thus the $(1 - z) \times 100$% confidence interval for $m$ is given by $\hat{m} \pm z_{0.05}/\sqrt{\text{Var}(\hat{m})}$, where $(\hat{m}) = \sqrt{\text{Var}(\hat{m})}$.

Using the above results, we can formally test for differences between the mean tract lengths between any two datasets $i$ and $j$ using a $Z$-statistic, which takes the form

$$ Z = \frac{\hat{m}_{i} - \hat{m}_{j}}{\sqrt{\text{Var}(\hat{m}_{i}) + \text{Var}(\hat{m}_{j})}} $$

All computations of $\phi$ were done in XLIPSTAT [55] using double precision. The maximum likelihood estimate was computed in XLIPSTAT using the Nelder-Mead algorithm, which converged within 500 iterations for all datasets using a tolerance level of 10^{-10}.

Supporting Information

Figure S1. Alignment of the Most Highly Conserved Region of MCM Proteins

This alignment shows the central region from all MCMs from Homo Drosophila MCM8 in Meiotic Recombination
Figure S2. Phylogenetic Analysis of Eukaryotic MCM Family Proteins
The tree shown was generated by the neighbor-joining method of ClustalW, using the alignment of the most highly conserved region of the MCM core domain shown in Figure S1 (correcting for multiple substitutions but including positions with gaps/unscored). Not all the branches are shown. The positions of the Walker A and B boxes and the arginine finger (RF) are indicated.

Found at DOI: 10.1371/journal.pgen.0010400.sg001 (43 KB PDF).

Table S1. Sequences Used for Phylogenetic Analysis
Found at DOI: 10.1371/journal.pgen.0010400.st001 (40 KB DOC).

References


