Mouse tetrad analysis provides insights into recombination mechanisms and hotspot evolutionary dynamics

Francesca Cole1,2,6, Frédéric Baudat3,6, Corinne Grey3, Scott Keeney4,5, Bernard de Massy3 & Maria Jasin1

The ability to examine all chromatids from a single meiosis in yeast tetrads has been indispensable for defining the mechanisms of homologous recombination initiated by DNA double-strand breaks (DSBs). Using a broadly applicable strategy for the analysis of chromatids from a single meiosis at two recombination hotspots in mouse oocytes and spermatocytes, we demonstrate here the unidirectional transfer of information—gene conversion—in both crossovers and noncrossovers. Whereas gene conversion in crossovers is associated with reciprocal exchange, the unbroken chromatid is not altered in noncrossover gene conversion events, providing strong evidence that noncrossovers arise from a distinct pathway. Gene conversion frequently spares the binding site of the hotspot-specifying protein PRDM9, with the result that erosion of the hotspot is slowed. Thus, mouse tetrad analysis demonstrates how unique aspects of mammalian recombination mechanisms shape hotspot evolutionary dynamics.

Sexual reproduction requires the formation of haploid gametes from diploid precursors through meiosis, which comprises two divisions following a single round of genome duplication. During the first meiotic prophase, recombination establishes physical connections between homologous chromosomes (homologs), essential for proper chromosome segregation1–3. Recombination is best understood in yeast, in part because all four chromatids from a single meiosis—a tetrad—can be recovered4. Tetrad analysis demonstrated that recombination can occur with an exchange of chromatid arms, as a crossover, or without an exchange, as a noncrossover5. Importantly, both crossovers and noncrossovers are often associated with gene conversion, the non-reciprocal transfer of information from a donor chromatid to a recipient. A model to account for this transmission, confirmed later by molecular approaches, holds that recombination initiated by DSBs leads to gene conversion at the DSB site using information from the uncut donor chromatid6. This model posited the formation of a double–Holliday junction intermediate that is resolved as a crossover or a noncrossover, such that either resolution type can lead to conversion of markers on the donor. Work in budding yeast supports this model for crossovers but demonstrated that most noncrossovers arise by pathways that do not involve the resolution of a double Holliday junction or alteration of the donor chromatid7–11.

In mammals, crossovers are detected by genetic mapping in pedigrees and by sperm and oocyte typing, and they can be inferred from population diversity analysis12–17. Events involving the transfer of short patches of genetic information attributed to noncrossovers have also been detected by sperm and oocyte typing18–22. Gene conversion has been inferred in mammals but not formally proven because, unlike in fungi, only single chromatids could be analyzed18,21,23. Although many aspects of meiotic recombination in mammals are likely conserved with yeast13, mammals differ in key features. For example, the ratio of noncrossovers to crossovers seems to be much higher in mammals, and inferred gene conversion tracts are shorter.

In mammals as in other organisms, recombination is initiated by DSBs generated by the Spo11 transerase24,25. DSBs occur most often at preferred sites, termed DSB hotspots, which are presumed to be recombination hotspots26. In mammals, unlike in yeast and other organisms, hotspot location is governed largely by PRDM9, a meiosis-specific histone H3 methyltransferase with a DNA binding specificity determined by a tandem array of C2H2 zinc fingers27–30. PRDM9 binding sites occur within hotspots28,31,32, suggesting that these binding sites are likely to undergo gene conversion during repair. This property raises a conundrum about PRDM9 binding site maintenance and, thus, about hotspot evolutionary dynamics: because the direction of gene conversion is biased (the cut chromosome copies the uncut donor), PRDM9 binding sites are predicted to be rapidly lost in the absence of additional constraints, as seen for the PRDM9 motif during human evolution29.

To understand these mechanistic and evolutionary aspects of mammalian recombination, we directly interrogated the structures of recombinant molecules by developing strategies to analyze all

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four chromatids of a single meiosis in the mouse, Mus musculus. We performed this equivalent of fungal tetrad analysis in both oocytes and spermatocytes because of fundamental differences in female and male meioses. Two recombination hotspots were analyzed, Psmb9 (ref. 23) and A3 (ref. 18), which are representative of the 50 or so mammalian hotspots described thus far for the width and distribution of exchanges12,14. These analyses demonstrate the occurrence of gene conversion, either associated with reciprocal exchange (crossover) or not (noncrossover). Notably, noncrossovers occur without modification of the donor chromatin. Many gene conversions do not include the PRDM9 binding site, providing a mechanism for lengthening of the evolutionary lifespan of hotspots.

RESULTS

Direct evidence for meiotic gene conversion in mice

Meiotic recombination at the Psmb9 hotspot was previously detected using allele-specific PCR of DNA from pooled ovaries23. We adapted this analysis to single oocytes to examine all four chromatids of a given meiosis—a tetrad. As detailed below, the success rate for recovering recombinant recombinant molecules when present was very high (100% in most experiments), indicating that we indeed usually did succeed in analyzing all four chromatids.

Microdissected dictyate oocytes from 25- to 30-d-old B10 × R209 F1 hybrid mice were individually lysed, and the hotspot region was amplified using non-allele-specific primers (universal PCR, Fig. 1a). Crossover and parental chromatids were distinguished by TaqI-BstXI digestion of the PCR product. For example, for the oocyte shown in Figure 1b, four chromatids—two crossover and two parental—were identified. By this approach, 4 of 119 oocytes (3.4%) showed crossover chromatids, translating to a 1.7% per-gamete frequency similar to previous estimates from pooled ovaries (Table 1)23. When allele-specific PCR was used to amplify DNA from the universal PCR and crossover breakpoints were mapped (Fig. 1c), all four oocytes exhibited reciprocal exchange associated with gene conversion, that is, showing a 3:1 ratio for one or more polymorphisms between the breakpoints (Fig. 1c). Three crossovers involved conversion to the B10 genotype, indicating a DSB on an R209 chromatid, and one involved conversion to the R209 genotype, indicating a DSB on a B10 chromatid (Fig. 1e).

For some hotspots, preferential recombination initiation on one homolog has been proposed because crossover breakpoints cluster asymmetrically depending on which crossover chromatid is amplified23. In a few cases, such as with Psmb9, this property is directly correlated with PRDM9 binding and enrichment of trimethylation of histone H3 at lysine 4 (H3K4me3) on the homolog with predicted high initiation activity12,24,25. Tetrad experiments were thus performed at Psmb9 with a second F1 hybrid, B10.A × SGR, in which recombination is thought to initiate preferentially on the B10.A chromosome23. Moreover, the polymorphism density in this hybrid is higher in the center of Psmb9, allowing finer-scale recombination maps.

Six oocytes of 205 (2.9%) displayed crossovers, all of which were reciprocal (Fig. 1f), for a 1.5% per-gamete frequency (Table 1). All six displayed gene conversion to the SGR genotype, providing direct evidence for preferential initiation on the B10.A chromosome and substantiating the model that asymmetric crossover breakpoint distributions result from biased initiation. Overall, analysis in both hybrids provides formal proof for gene conversion and for the generation of reciprocal crossover molecules within a single meiotic cell.

The mean gene conversion tract length for B10.A × SGR hybrids was 446 bp (Fig. 1f), similar to the inferred length from the asymmetric crossover breakpoint distribution23. Because tetrad analysis captures both recombinant chromatids, gene conversion tract length could also be determined from the B10 × R209 hybrid that does not show biased initiation. The mean tract length for this hybrid was 566 bp (Fig. 1e), similar to that for the B10.A × SGR hybrid. Thus, mean tract lengths from hotspots with unbiased initiation are similar to those with biased initiation.

Noncrossovers identified by oocyte tetrad analysis

Noncrossovers are predicted to be the major outcome of mammalian meiotic recombination and to arise by a distinct mechanism from that of crossovers13. To identify noncrossovers, PCR was performed on DNA from the universal PCR of individual oocytes using allele-specific primers directed to specific polymorphisms (indicated by asterisks in Fig. 1d). DNA sequencing then verified the presence and extent of the noncrossover.

In the B10 × R209 hybrid, three polymorphisms were tested (Fig. 1g). Seven oocytes had detectable noncrossovers (5.9%), all of which converted only the BsrFI polymorphism in the hotspot center. Four noncrossovers were derived from initiation events on the B10 chromosome, and three were derived from initiation events on the R209 chromosome. The noncrossover frequency was 1.5% per gamete, similar to that for pooled ovaries (Table 1)23.

In the B10.A × SGR hybrid, seven polymorphisms were queried (Fig. 1h). Five oocytes had detectable noncrossovers (2.4%), all incorporating polymorphisms within the central ~200 bp of the hotspot. The per-gamete noncrossover frequency was 0.6%, similar to that for pooled ovaries (Table 1 and Supplementary Table 1). As with oocytes, noncrossovers from pooled ovaries or sperm typing were also concentrated near the hotspot center (Supplementary Fig. 1 and Supplementary Table 1). Noncrossovers incorporating more than one polymorphism (co-conversion events) were also detected at similar proportions in oocytes (2 of 5 noncrossovers) and pooled ovaries (21 of 42), although, interestingly, sperm typing showed fewer co-conversion events (16 of 62; P = 0.0134, Fisher’s exact test, two-tailed). Overall, taking into account the distance to the adjacent polymorphisms, the mean noncrossover gene conversion tract length was 86 bp (minimum of 23 bp; maximum of 148 bp) in pooled ovaries and 68 bp (minimum of 15 bp; maximum of 124 bp) in sperm (Supplementary Fig. 1). All noncrossovers from tetrads were conversions to the SGR genotype on a B10.A chromosome (Fig. 1i), implying preferential initiation on the B10.A chromosome as observed with pooled ovaries (Supplementary Table 1). Notably, the donor chromosome was unaffected in all noncrossovers, excluding the possibility that they arose from two nearby crossovers.

Unidirectional transfer of genetic information in noncrossovers

Tetrad analysis was also applied to spermatocytes. Flow cytometry was used to isolate late prophase I primary spermatocytes25, which have completed meiotic recombination15 (Fig. 2a). To maximize noncrossover recovery, we analyzed the A3 hotspot, which has a high ratio of detectable noncrossovers to crossovers (~10:1)18. Fine-scale recombination analysis is possible because of the high density and relatively even distribution of polymorphisms, mostly SNPs. In total, 22 polymorphisms were queried in A/J × DBA/2 F1 hybrids, averaging ~100 bp apart and with a higher density at the hotspot center. In this hybrid, A3 was inferred to have preferential initiation on the DBA/2 chromosome18.

Pools of spermatocytes were analyzed rather than single cells to increase the recovery of recombination events. Pools were small, such that most recombinants detected would be derived from a single spermatocyte. Sorted spermatocytes were lysed in 522 pools of ~20 cells each, and the A3 hotspot was amplified by universal PCR followed
by nested PCRs with allele-specific forward primers and universal reverse primers (Fig. 2b, left). Amplified DNA was blotted onto replicate filters and probed with allele-specific oligonucleotides to identify and map recombinants (Fig. 2c)36. From the ~10,440 analyzed spermatocytes, 111 noncrossovers converted a DBA/2J chromosome segment to the A/J genotype, in comparison to 20 conversions in the opposite orientation (Fig. 2d). Thus, the noncrossover frequency was 1.25% per meiosis (0.31% per gamete), with most initiation on the DBA/2J chromosome, similar to findings in pooled sperm18. Even with the closely spaced polymorphisms, noncrossover gene conversion tracts most often included only a single polymorphism and averaged 86 ± 49 bp in length. Whereas noncrossovers clustered in the central 200 bp of A3, a substantial fraction was distributed ~1 kb to either side (Fig. 2d,e and Supplementary Fig. 2). All five noncrossovers identified by tetrad analysis involved conversion of Psmb9 hotspot sequences on the B10.A chromosome to those of the SGR chromosome. The noncrossover frequency was 2.4% (95% CI = 0.9–5.9%) per meiosis. Asterisks indicate the polymorphisms queried for noncrossovers, as in g.
chromosome. A nearly identical frequency (0.29% per gamete; Table 1) and similar distribution (Supplementary Fig. 2) of noncrossovers were obtained.

Overall, a single noncrossover was detected in each of 118 pools, whereas multiple noncrossovers on the DBA/2J chromosome were detected in 10 pools, close to the number predicted assuming that recombinant spermatocytes had a Poisson distribution among pools (12.5). Only 3 pools showed noncrossovers on both the DBA/2J and A/J chromosomes, matching expectation for noncrossovers from independent spermatocytes (3.9). These results indicate that gene conversion on the A/J chromosome results from infrequent recombination initiation on that chromosome not from initiation on DBA/2J.

Thus, the large number of analyzed spermatocytes and high polymorphism density provided compelling evidence that noncrossovers arise from the unidirectional transfer of genetic information.

**Crossover gene conversion tracts often encompass the hotspot center**

Crossovers at A3 were detected in spermatocytes but in much smaller numbers than noncrossovers: 2 reciprocal crossovers in the same experiments as noncrossovers (Fig. 2b, left) and 13 more from larger pools of spermatocytes (90 pools of ~100 spermatocytes) using 2 allele-specific primers (Fig. 2b, right), 10 of which were reciprocal. Consistent with preferential initiation on the DBA/2J chromosome, twice as many crossovers showed gene conversion to the A/J genotype (Fig. 3a). Recovery of three crossovers without the reciprocal product might reflect less efficient amplification from larger pools (thus, a 77% success rate for both products). This 0.08% crossover frequency per meiosis translates to a rate of 0.04% per gamete, similar to that determined by sperm typing (Table 1). The net noncrossover-to-crossover ratio at A3 was therefore ~15:1 (noncrossover, 1.25%; crossover, 0.08%).

The mean crossover gene conversion tract length at A3 was 626 ± 319 bp, similar to that at Psmb9 but substantially longer than for noncrossovers at A3 (86 ± 49 bp). Unlike in noncrossovers, most crossover gene conversion tracts overlapped each other and the hotspots center, such that five polymorphisms spanning ~200 bp of the center were converted in 75% of crossovers (Fig. 3a). Of note, however, three crossovers had short, off-center gene conversion tracts (Fig. 3a), which could not have been inferred from sperm typing (Supplementary Fig. 3a–c).

We determined A/J transmission relative to DBA/2J transmission at each polymorphism (Fig. 3b). Gene conversion, primarily from noncrossovers, resulted in transmission distortion (deviation from the mendelian ratio) in favor of A/J sequences for all polymorphisms, especially those in the central ~200 bp, agreeing with sperm typing18. The polymorphism that showed the greatest distortion was 8.2-fold more likely to convert to the A/J genotype, resulting in A/J genotype transmission to 50.04% of gametes. Both crossover and noncrossover gene conversion events contributed to transmission distortion of this polymorphism: 5 of 7 crossovers (71%) and 17 of 19 noncrossovers (89%) involved conversion to the A/J genotype. However, because noncrossovers greatly outnumbered crossovers, the net impact of noncrossovers was greater.

**PRDM9 binds to the center of the A3 hotspot**

PRDM9 specifies the location of recombination hotspots in mice and humans. DBA/2J and A/J mice express PRDM9b from *M. musculus domesticus*27,30. We assayed PRDM9b binding to A3 sequences in vitro to determine whether preferential recombination initiation on the DBA/2J chromosome was associated with higher binding affinity. *M. musculus molossinus* PRDM9wm7 (which has different DNA contact residues27) was used as a control.

Overlapping fragments spanning ~1.7 kb of the A3 alleles from DBA/2J and A/J were used as probes in southwestern analysis (Fig. 3c). PRDM9b showed substantial binding to a DBA/2J fragment from the hotspot center (probe 5) but not to other DBA/2J fragments. PRDM9b also bound the A/J fragment from the hotspot center but much less efficiently (Fig. 3c), consistent with less frequent DSB formation on the A/J chromosome. Weak binding was also detected for probe 3 from both DBA/2J and A/J. The relevance of this binding is uncertain, however, as the frequency of recombination in this region of the hotspot was considerably higher on the DBA/2J chromosome than on the A/J chromosome (Fig. 2e). PRDM9wm7 also bound weakly to probe 3 from A/J but did not bind any of the DBA/2J fragments (Supplementary Fig. 3d) confirming the specificity of PRDM9b binding.

To further localize binding, we tested a 57-bp fragment (Fig. 3b) spanning sequences unique to probe 5. This fragment maps to the hotspot center and contains a partial match to the site predicted from PRDM9b zinc-finger composition and a nearly exact match to the consensus sequence from whole-genome DSB analysis (Fig. 3b)28. Accordingly, PRDM9b bound to both the DBA/2J and A/J probes but with ~8-fold better binding to DBA/2J (Fig. 3d).

The C57BL/6J A3 allele has substantially more activity than the A/J allele, although not as much as the DBA/2J allele18. Likewise, the corresponding 57-bp fragment from C57BL/6J (Fig. 3b) had an intermediate level of PRDM9b binding (Fig. 3d). The predicted PRDM9 binding site contains two sequence differences between these strains: a C/T base substitution and a 2-bp insertion/deletion (indicated by red arrowheads in Fig. 3b). The hierarchy of PRDM9b binding (DBA/2J > C57BL/6J >> A/J) suggests that the 2-bp deletion common to C57BL/6J and A/J reduces binding efficiency by about half and the A/J-specific base substitution reduces it even further. Thus, despite the long PRDM9 recognition site owing to its zinc-finger array, one or a few residues can significantly affect PRDM9 binding, reinforcing the idea that PRDM9 binding efficiency modulates recombination hotspot activity31,32.

We investigated how recombination affected the transmission of the PRDM9 binding site, considering transmission from either crossover or noncrossover gene conversion of the polymorphisms inferred to have the greatest impact on PRDM9 binding.
Figure 2. Mouse spermatocyte tetrads demonstrate that noncrossovers result from the unidirectional transfer of information. (a) Single-cell suspensions from the testes of adult F1 hybrid mice were stained with Hoechst 33342 and propidium iodide. Cells with the highest blue and red fluorescence intensities were sorted using the indicated gate (oval). In three independent experiments, all sorted cells were primary spermatocytes, with ~98% in diplonema or MLH1-positive pachynema, on the basis of staining for the axial element marker SYCP3 and the crossover marker MLH1 (refs. 37,58,59), as shown in the representative images chosen from 188 analyzed spermatocytes. Scale bar, 10 µm. (b) A3 hotspot amplification strategy for spermatocytes. Left, to amplify both noncrossovers and crossovers, cells were plated in pools of ~20 cells per well, and DNA was universally amplified across the A3 hotspot on chromosome 1. The amplified DNA was used to seed two separate PCRs using an allele-specific forward primer and a universal reverse primer. Right, to amplify only crossovers, cells were plated in larger pools of ~100 cells per well for universal amplification. Universally amplified DNA was used to seed two separate PCRs using primer sets to detect both recombinant chromatids. (c) Representative crossover and noncrossover recombinants from spermatocyte analysis. Replicate blots were generated from PCRs in the A/J-to-universal (U) orientation (top) or the DBA/2J-to-universal orientation (bottom) and probed with allele-specific oligonucleotides to genotype polymorphisms across the A3 hotspot. The genotype of a representative crossover with the length of the gene conversion tract is shown between the blots and that of a representative noncrossover is shown below the blots. Dot blot legend: solid colored circles or squares, genotype determined by blotting; dashed colored circles or squares, inferred genotype; black squares in the upper right corner, loading control of amplified DBA/2J or A/J DNA; black rectangles in the lower left corner, dilutions of the loading control; #, well containing noncrossovers on both the A/J and DBA/2J chromosomes. (d) Spermatocyte noncrossovers at the A3 hotspot. Because recombination initiates preferentially on the DBA/2J chromosome (red), the majority of noncrossovers involve conversion of DBA/2J polymorphisms to the A/J genotype (blue). The noncrossover frequency was 1.25% (95% CI = 1.0–1.5%) per meiosis. A schematic of the central polymorphisms is shown at the top. Red arrowheads indicate polymorphisms implicated in differential PRDM9 binding between A/J and DBA/2J; yellow shading indicates the predicted PRDM9 binding site (Fig. 3b). Indel 3 is located within a direct repeat such that only the A/J polymorphism can be genotyped; <4% of noncrossovers on the DBA/2J chromosome were converted only at this polymorphism. An asterisk indicates the noncrossover event highlighted in c. (e) Total Poisson-adjusted noncrossover frequencies on the DBA/2J (top) and A/J (bottom) chromosomes. Noncrossover frequencies at each tested polymorphism are normalized for co-conversion events. Ticks in the center represent the 22 polymorphisms tested.
The C/T transition and the 2-bp insertion-deletion were much more likely to convert to the A/J sequences, resulting in clear transmigration distortion (Fig. 3b). However, 80% of noncrossovers converted polymorphisms flanking the center of A3 without converting those affecting PRDM9 binding. Thus, most recombination events are predicted not to affect A3 hotspot activity.

**DISCUSSION**

In this study, we sought to test long-standing assumptions about the mechanism of meiotic recombination in mammals by developing mouse tetrad analysis, a strategy to assess multiple chromatids from a single meiosis at recombination hotspots. Our experiments demonstrated the non-mendelian transfer of information—gene conversion—during recombination. Crossing over in both oocytes and spermatocytes was associated with gene conversion. Noncrossovers were non-reciprocal exchanges of genetic information without observable modification of the donor locus. Notably, gene conversion frequently occurred away from the PRDM9 binding site, with implications for understanding recombination mechanisms and hotspot evolution.

Gene conversion tracts were observed in each of 22 crossovers recovered from mouse tetrads. The strong association of gene conversion with crossovers and the relatively uniform conversion tract lengths (average of 566 ± 277 bp) together suggest that most crossovers arise from a common mechanism. Consistent with this idea, ~90% of crossovers in mouse are dependent on the MLH1 protein37,38. Gene conversion associated with crossing over is compatible with double-Holliday junction resolution, as predicted by the original DSB repair model from yeast6. However, in mammals, the two Holliday double–Holliday junction resolution, as predicted by the original DSB repair model from yeast6. However, in mammals, the two Holliday complexes might be closer at the time of resolution given that the hotspot (ref. 32).
density-dependent effects might exist and cause variations, which could be uncovered by genome-wide deep sequencing\(^{15}\).

Double–Holliday junction resolution can, in principle, also give rise to noncrossovers\(^6\). However, this model predicts the presence of heteroduplex DNA on both the recipient and donor chromatids such that the donor can become modified. The absence of donor modification in the $>140$ noncrossovers we examined is more consistent with an alternative pathway(s) involving the unidirectional transfer of information from the donor to the recipient, for example, synthesis-dependent strand annealing. Dissolution of a double Holliday junction by branch migration can also lead to noncrossover formation without donor modification\(^5\). In yeast, most noncrossovers are thought to derive from either of these latter mechanisms rather than from double–Holliday junction resolution\(^7,10\).

This study found short noncrossover gene conversion tract lengths (94±6 bp), matching previous reports in mouse and human\(^{18,21–23,43}\). As with crossovers, noncrossover gene conversion tract lengths are much shorter than in yeast (220-fold shorter)\(^{10,39,46}\), suggesting that there are critical differences in either the proteins involved or the chromatin organization of the recombining region, which could affect DNA end processing, heteroduplex formation and extension, or mismatch correction. Mechanistic differences between mammals and yeast might reflect differences in the biological processes in which meiotic recombination participates. For example, strand invasion involves a search for sequence homology and is thought to contribute to the stabilization of interactions between homologs\(^{47}\). Limiting the extent of repair synthesis in mammals in comparison to yeast until multiple interhomolog recombination interactions occur along the chromosome might reduce opportunities to involve repetitive, non-allelic DNA, in turn reducing the potential for ectopic exchange. Moreover, shorter strand extension could produce a structure favorable for strand displacement and therefore participate in controlling the noncrossover pathway.

An unanticipated and new finding that affects the understanding of meiotic DSB repair mechanisms is the broad distribution of gene conversion at the A3 hotspot despite highly localized PRDM9 binding. From single-stranded DNA mapping at resected DSBs, it has been proposed that DSBs arise most frequently near PRDM9 binding sites\(^{28,48}\). This hypothesis is supported by the mapping of meiotic DSBs by SPO11-oligonucleotide sequencing (ref. 49 and J. Lange, M.J. and S.K., unpublished data): At the A3 hotspot in C57BL/6 mice, sixfold more DSBs (SPO11 oligonucleotides) occurred in the central 200 bp in comparison to the flanking 1.8 kb (900 bp on each side; J. Lange, M.J. and S.K., unpublished data). By contrast, only half of the detected noncrossovers mapped to this central region (57 of 111 noncrossovers). Thus, it seems likely that gene conversion distribution reflects a feature(s) of the recombination mechanism rather than DSB distribution alone.

Crossover gene conversion tracts include central polymorphisms more often than noncrossovers; however, some crossover gene conversion tracts do not (25–50%), which would not have been evident by single-chromatid analysis (Supplementary Fig. 3a–c). Hotspots with biased initiation show crossover breakpoint asymmetry, such that most breakpoints from one orientation are offset by those of the other orientation\(^{53}\). The subset of breakpoints from both orientations that overlap could have been attributed to rare initiation on the other chromosome. Instead, our results show these to be crossovers (and the intervening gene conversion tract) offset to one side of the hotspot center. These tracts are typically shorter, suggesting that they have other mechanistic differences as well.

In principle, gene conversion at the hotspot center could be reduced relative to DSB frequency by mismatch repair of heteroduplex intermediates biased toward restoration of parental sequences at the initial site of strand invasion\(^{10,50}\). Alternatively, parental sequences could be restored by initial strand invasion into the sister chromatid followed by polymerization\(^1\) (Fig. 4a). Ejection of the newly synthesized DNA from the sister chromatid would free it to switch templates and prime further DNA synthesis from the homolog, leading to gene conversion at a distance from the DSB site. In support of this model, intermediates involving invasion into the sister chromatid have been detected physically in budding yeast\(^{51,52}\), as have interstrsir repair events\(^{53}\). Our data suggest that these template switches might be prevalent in mouse, and one could speculate that sister chromatid invasion might be favored in situations where heterologies occur near DSB sites, as in A3. A variation of this model is that both ends initiate strand invasion, one into the sister and the other into the homolog\(^1,10\), such that asymmetry in intermediates leads to off-center gene conversion. Such multitemplate engagement might enhance the efficiency of recombination\(^4\), as well as reducing gene conversion at the hotspot center.

Gene conversion presents a dilemma for understanding how hotspot locations might be maintained in organisms in which the recombination landscape is determined by the sequence-specific DNA-binding protein PRDM9. In individuals heterozygous for hotspot alleles that display differential PRDM9 binding, gene conversion will tend to favor the transmission of the hotspot-disrupting polymorphisms to offspring, leading to hotspot erosion over time. The fact that hotspots exist despite this notion that they will rapidly extinguish themselves has been termed the ‘hotspot paradox’ (ref. 54). Indeed, signatures
of human PRDM9 motif decay are readily apparent in the human genome in comparison to the chimpanzee genome. Further, some human recombination hotspots show transmission distortion of hotspot-disrupting polymorphisms that has been modeled to lead to rapid hotspot extinction. Yet, hotspots are long-lived enough to cause linkage disequilibrium, implying that they can be maintained over tens of thousands of generations.

Given that PRDM9 binds to hotspot centers, as shown here for A3 and previously for Psmb9 and other hotspots, how can hotspots persist over evolutionary time scales? Examining a large number of gene conversion events at A3, we found that polymorphisms favoring PRDM9 binding are frequently preserved in recombinant molecules. This would be predicted to promote hotspot longevity. Multiple factors contribute to the preservation of the PRDM9 binding site. Noncrossover gene conversion tracts are short and are distributed such that only a fraction include the hotspot-disrupting polymorphisms (20%). Although crossover gene conversion tracts are longer, they also do not always incorporate the hotspot-disrupting polymorphisms (75% incorporate them). Further, noncrossovers are much more frequent (by 10- to 20-fold) than crossovers, such that the greater likelihood of conversion at the PRDM9 site in crossovers is offset by the overall lower number of crossovers.

Thus, the polymorphisms inferred to make the A3 hotspot highly active would be expected to be much more slowly extinguished when considering transmission data from tetrad analysis than assuming that every recombination event results in conversion to the hotspot-disrupting allele, as is usually modeled. To evaluate the slowing of hotspot extinction, we performed Monte Carlo simulations using a Wright-Fisher population model of genetic drift (Fig. 4b). Assuming that every recombination event results in conversion to the hotspot-disrupting allele, the polymorphisms inferred to make the hotspot more active were extinguished in 1,160 generations on average (Fig. 4b, total). However, given that 80% of the gene conversions detected at A3 by tetrad analysis (crossovers and noncrossovers combined) preserved the PRDM9 binding site, extinction of either of the hotspot-active polymorphisms was predicted to take a much more substantial 4,176 generations (Fig. 4b, C and/or TT). Even a more modest frequency of PRDM9 binding site retention (in 40% of gene conversions) would substantially extend the time to extinction (3,665 generations). Not shown are similar simulations for the Psmb9 hotspot; however, recombination would lead to its extinction in 113 generations if the hotspot was extinguished in every event, whereas the observed retention of the critical PRDM9 binding polymorphism in a substantial fraction of gene conversions (6/11) predicts a much longer time to extinction (536 generations). Thus, gene conversion frequently spares the PRDM9 binding site with the result that the erosion of hotspots is slowed.

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AUTHOR CONTRIBUTIONS

F.C., F.B., S.K., B.d.M. and M.J. conceived the study, interpreted the data and wrote the manuscript. F.C. and F.B. performed the spermatocyte and oocyte (and associated) recombination experiments, respectively. F.B. and C.G. performed the southwestern blotting. F.C., M.J. and S.K. estimated the fixation rates.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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URLs. R version 2.15.3, http://cran.r-project.org/; R script for the transmission distortion simulation algorithm, http://cbio.mskcc.org/public/Cole_Mouse_Tetrads/. Confidence intervals (CIs) were calculated at http://vassarstats.net/.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.
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Isolation of 4C spermatocytes. Testes from 2- to 4-month-old A/J × DBA/2J F1 hybrids were decapsulated, and seminiferous tubules were incubated at 33 °C in Gey’s balanced salt solution (GBSS) with 0.5 mg/ml collagenase (Worthington, CL54) for 15 min, shaking at 500 r.p.m. The tubules were rinsed and treated with 0.5 mg/ml trypsin (Sigma, T9935) supplemented with 1 μg/ml DNase I (Sigma, DNEP) at 33 °C, shaking at 500 r.p.m. for 15 min. Trypsin was inactivated by the addition of 5% FCS. Cells were individualized by repeated pipetting and filtration through a 70-μm cell strainer (BD Falcon, 352330). Cells were washed several times in GBSS with 5% FCS and 1 μg/ml DNase I and were stained with 5 μg/ml Hoechst 33342 (resuspended in DMSO) for 2 h at 33 °C, shaking at 500 r.p.m. Just before fluorescence-activated cell sorting, cells were stained with 0.2 μg/ml propidium iodide and filtered through a 40-μm cell strainer. Cells were sorted on a MoFlo cytometer (Dako) with a 350-nm argon laser35. Late-stage primary spermatocytes exhibit the highest blue and red fluorescence intensities owing to their DNA content and chromatin structure63, respectively, allowing them to be readily sorted from cells that do not contain a full complement of chromatids, such as secondary spermatocytes and spermatids. Dead cells were gated by propidium iodide staining. Live cells with the highest blue and red fluorescence intensities were sorted, repeatedly washed with TBS and counted with a hemocytometer. Cells were diluted and plated at a density of 20 (noncrossover/crossover assays) or 100 (crossover assays) cells in 5 μl of TBS per well in 96-well plates and frozen at −80 °C for storage. A portion of the sorted cells was surface spread44 and stained45 with antibodies that recognize the axis component SYCP3 (1:500 dilution; Abcam, ab15093), the crossover marker MLH1 (1:75 dilution; BD Biosciences, 51-1327GR) and DAPI to allow staging and purity assessment. Splenic cells from the same mice were used as a somatic control. They were separated by macerating through a 70-μm cell strainer, repeatedly washed in GBSS with 5% FCS and 1 μg/ml DNase I, and stained with Hoechst and propidium iodide for cell sorting. Live splenic cells were plated at the same cell numbers as spermatocytes, and no recombinants were detected (16,200 tested cells or 64,800 molecules). Epididymides (sperrn) and liver (somatic control) from the same mice isolate were used for DNA extraction and recombination analysis36. No recombinants were detected in liver controls (14,400 molecules).

Amplification and detection of recombinants in isolated spermatocytes. Cells were lysed and extracted in 0.38% Igepal CA-630 (Sigma), 0.38% Tween-20 and 0.3 μg/μl proteinase K (Qiagen) at 55 °C for 2 h. Proteinase K was inactivated at 96 °C for 15 min before PCR. Extracted wells were diluted sixfold into PCR buffer containing the universal primers A3f600 and A3r6000 (5.4 kb; Supplementary Table 2c,d) that amplify across the entire A3 hotspot. PCR conditions were 95 °C for 1 min followed by 6 cycles at 96 °C for 30 s and 65 °C for 7 min and 19 cycles at 96 °C for 20 s, 58 °C for 30 s and 65 °C for 7 min. One-third of the reaction was treated with 7.0 U S1 nuclease (Invitrogen) in a volume of 50 μl. The PCR products were then digested with TaqI and BstXI, generating fragments of allele-specific length (B10, 2,099 bp; DBA, 2,062 bp). Recombinants were genotyped by dot blotting (Supplementary Table 2a,b) and several were confirmed by cloning and restriction digest and/or genotyping36. Spermatocytes were analyzed from three mice.

Southwestern blotting assays. Southwestern blotting assays were performed as described previously32, using full-length His-tagged mouse PRDM9m7 and PRDM9p. The 200- to 300-bp probes covering the A3 hotspot were generated by PCR amplification of genomic DNA from either strain (A/J or DBA/2), with XbaI or Nhel site-tailed primers (Supplementary Table 2f). PCR fragments were digested with XbaI or Nhel to generate a CTAG 5′ overhang at each end. The 57-bp probes containing the PRDM9-binding motif of the center of the A3 hotspot were generated by annealing complementary oligonucleotides, leaving a 3′-bp 5′ overhang with a G in the second position at each end (Supplementary Table 2g). DNA fragments were then labeled by end filling with [α-32P]dCTP as described previously32. His-tagged PRDM9 was detected with a primary monoclonal mouse antibody to polyhistidine (1:2,000 dilution; Sigma, H1029, clone HIS-1).
Transmission distortion calculations. To estimate the number of generations required for polymorphisms that are causative for transmission distortion to become fixed in a population, Monte Carlo simulations were performed with 1,000 samples using a Wright-Fisher model. For each generation starting from an initial population of 10,000 heterozygous individuals, 20,000 gametes were chosen at random, with alleles transmitted at the frequency experimentally determined by mouse tetrad analysis (A3: TT = 0.4998040, C = 0.4997082, both sites = 0.4995121; Psmb9: polymorphism 70 = 0.4939024) or at the frequency predicted from the model that all DSBs lead to loss of PRDM9 binding (total recombination for A3 = 0.4975647 and for Psmb9 = 0.4646341). Transmission frequencies were determined at A3 using the formula

\[
\text{% A/J transmission} = \frac{(N_{\text{cell}} \times 2) + (A_{\text{conv}} - D_{\text{conv}})}{N_{\text{cell}} \times 4} \times 100
\]

where \( N_{\text{cell}} \) is the number of cells tested and \( A_{\text{conv}} \) and \( D_{\text{conv}} \) are the number of conversions to the A/J and DBA2/J genotype at a particular polymorphism, respectively. The same approach was used to determine transmission frequencies at Psmb9. Each simulation ended when the allele was fixed or it reached 50,000 generations. Simulations were performed in R version 2.15.3.

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