Distinct histone modifications define initiation and repair of meiotic recombination in the mouse

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Little is known about the factors determining the location and activity of the rapidly evolving meiotic crossover hotspots that shape genome diversity. Here, we show that several histone modifications are enriched at the active mouse *Psmb9* hotspot, and we distinguish those marks that precede from those that follow hotspot recombinational activity. H3K4Me3, H3K4Me2 and H3K9Ac are specifically enriched in the chromatids that carry an active initiation site, and in the absence of DNA double-strand breaks (DSBs) in *Spo11*^{-/-} mice. We thus propose that these marks are part of the substrate for recombination initiation at the *Psmb9* hotspot. In contrast, hyperacetylation of H4 is increased as a consequence of DSB formation, as shown by its dependency on *Spo11* and by the enrichment detected on both recombining chromatids. In addition, the comparison with another hotspot, *Hlx1*, strongly suggests that H3K4Me3 and H4 hyperacetylation are common features of DSB formation and repair, respectively. Altogether, the chromatin signatures of the *Psmb9* and *Hlx1* hotspots provide a basis for understanding the distribution of meiotic recombination.

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Introduction

During meiosis, reciprocal homologous recombination events or crossovers (COs) constitute physical connections required for the proper segregation of homologous chromosomes at the first meiotic division (Petronczki *et al*, 2003). In addition, COs increase genome diversity by reshuffling alleles over generations. COs result from a complex process of DNA double-strand break (DSB) formation and repair (Hunter, 2007). The induced DSBs are repaired by homologous recombination using alternative pathways. The repair using the homologous chromatid as a template and associated with a reciprocal exchange leads to CO. Alternatively, the repair can lead to a non-reciprocal exchange of genetic information,

a gene conversion event, without CO (Baudat and de Massy, 2007b). The initiation step of meiotic recombination, DSB formation, is catalyzed by the evolutionarily conserved Spo11 protein (Keeney and Neale, 2006). Spo11 is homologous to the catalytic subunit of the TopoVI family of type II DNA topoisomerases (Bergerat *et al*, 1997). Primarily on the basis of yeast studies, DSB formation has been shown to require several other proteins, the roles of which remain to be determined. DSBs are not randomly distributed in the genome and occur preferentially in regions called hotspots (Petes, 2001). How DSB frequency and position are determined is not known. In *Saccharomyces cerevisiae*, most DSBs occur in intergenic regions adjacent to transcription promoters (Baudat and Nicolas, 1997; Gerton *et al*, 2000). DSB frequency varies greatly from one promoter region to another according to undefined rules. However, transcriptional activity is not required for DSB formation. In *Schizosaccharomyces pombe*, DSBs occur preferentially in large intergenic regions, and with a positive correlation with the presence of non-coding RNA loci (Cromie *et al*, 2007; Wahls *et al*, 2008). On the basis of DSB mapping, at the nucleotide resolution, in *S. cerevisiae*, Spo11 seems to have no or little DNA sequence specificity (Liu *et al*, 1995; de Massy *et al*, 1995; Diaz *et al*, 2002). Several analyses show that chromatin accessibility is one important feature of initiation sites in *S. cerevisiae* and *S. pombe* (Lichten, 2008) and DSBs occur preferentially in open chromatin (Ohta *et al*, 1994; Wu and Lichten, 1994). In addition, acetylations of histone H3 and H4 and trimethylation of H3 K4 have been, respectively, detected at DSB sites either by local analysis at individual sites in *S. pombe* (Yamada *et al*, 2004) or, more recently, by genome-wide analysis in *S. cerevisiae* (Borde *et al*, 2009). Several mutations in enzymes involved in histone post-translational modifications (HPTMs) have also been shown to lead to a decrease or increase of DSB activity, suggesting a role for histone modifications in initiation activity (Sollier *et al*, 2004; Yamashita *et al*, 2004; Mieczkowski *et al*, 2007; Hirota *et al*, 2008; Merker *et al*, 2008).

In mammals, DSBs have not been directly mapped, but the molecular analysis of recombinant molecules at several meiotic CO hotspots has shown that CO hotspots are preferred initiation sites (Buard and de Massy, 2007). High-resolution mapping of CO by direct analysis of recombinant molecules at individual hotspots and by genome-wide population diversity analysis shows several striking properties of hotspot distribution in the human genome that seem to be also found in mice. COs cluster within ~2 kb-wide regions and are spaced on average every 50–100 kb (Myers *et al*, 2005). In the human genome, around 25 000 CO hotspots have been mapped, with activities varying over three orders of magnitude. These hotspots are mostly located outside of genes and a substantial fraction has been found to contain a degenerated 13-mer motif (Myers *et al*, 2008). In addition to the potential role for a specific DNA sequence or a sequence family, chromatin features have been invoked to explain DNA

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sequence-independent variations in recombination rates, such as those observed between male and female meiosis for instance. The recent high-resolution mapping of male and female hotspots in mice shows indeed that several hotspots have different activities in male and female meiosis (Paigen *et al*, 2008).

We sought to directly investigate whether HPTMs participate in defining recombination hotspots in mammals by taking advantage of the features and extensive characterization of the mouse hotspot *Psmb9* (Shiroishi *et al*, 1991; Guillon and de Massy, 2002; Guillon *et al*, 2005; Baudat and de Massy, 2007a). Recombination events occur at a frequency 2000-fold higher than the genome average at this hotspot and spread over 1.5 kb with the highest density in a central 210-bp region. Depending on the presence of a region of chromosome 17 from the *M. m. molossinus wm7* haplotype, hybrid mice can have active initiation at *Psmb9* on both, only one or none of the homologues (Baudat and de Massy, 2007a). We first determined the level of enrichment of several HPTMs at *Psmb9* and in flanking regions in strains carrying active or inactive *Psmb9* hotspot in meiotic and somatic cells. We then determined whether the enriched HPTMs could be a cause or a consequence of recombination activity using two complementary approaches. First, taking advantage of single nucleotide polymorphisms (SNPs), we were able to determine whether in hybrids, in which only one homologue initiates, the HPTMs are enriched on both or only one homologue. Second, we tested the enrichment of HPTMs in *Spo11*^{-/-} mice deficient for the initiation of meiotic recombination (Baudat *et al*, 2000; Romanienko and Camerini-Otero, 2000; Mahadevaiah *et al*, 2001). We thus show that H3K4Me3 is specifically enriched in the chromatid that carries an active initiation site and independently from DSB formation by Spo11. Instead, H4 hyperacetylation is a chromatin modification that appears after DSB formation, as shown by its Spo11 dependence. Comparison between *Psmb9* and *Hlx1*, a hotspot on chromosome 1, shows distinct chromatin features and two common properties: the enrichment of H3K4Me3 and of H4 hyperacetylation. Interestingly, no transcript could be detected in the *Psmb9* region and the low level of transcript detected at *Hlx1* does not vary with H3K4Me3 levels. Therefore, the H3K4Me3 enrichment we detected at hotspots does not correlate with detectable transcriptional activity. Altogether, these histone modifications do highlight new features of the chromatin in intergenic regions of the mouse genome and provide a basis for understanding the distribution of meiotic recombination in eukaryotes.

Results

Distinct HPTMs at active or inactive *Psmb9* hotspot

HPTMs were measured at *Psmb9* and in flanking regions in strains carrying recombinationally active (R209) and inactive (B6) *Psmb9* alleles, in meiotic and somatic cells. Native chromatin from elutriated pachytene spermatocytes or from liver cells was immunoprecipitated with antibodies raised against a series of HPTMs. Immunoprecipitated DNA was quantified using real-time PCR (see Materials and methods). We observed significant enrichments for a set of HPTMs in pachytene spermatocytes from a strain carrying an active *Psmb9* hotspot (R209) relative to the recombinationally inactive B6 strain (Figure 1). The enrichment was restricted

to the 1.5-kb hotspot region for H3K4Me3, H3K4Me2, H3K9Ac, hyperacetylated H4 and H3K27Me1 (Figure 2). For all five modifications, the enrichment was statistically significant (Supplementary Figure S1). In contrast, there was no difference for these modifications in somatic cells (liver) between the two strains. Conversely, H3K27Me3 and H3K9Me3 were significantly enriched in B6 relative to R209 in spermatocytes (Figure 2 and Supplementary Figure S1). No difference for total histone H3 could be detected between the two strains within the hotspot region, suggesting a similar density of nucleosomes. In addition, micrococcal nuclease digests of chromatin prepared from a 1:1 mix of spermatocytes from the two strains did not show any difference in sensitivity between the B6 and R209 alleles (Supplementary Figure S2), consistent with a previous analysis of DNase I hypersensitivity at this hotspot (Mizuno *et al*, 1996).

Strains R209 and B6 not only differ in the activity of the *Psmb9* hotspot but also in DNA sequence all along the most proximal 33-Mb region of mouse chromosome 17, corresponding to the *wm7* haplotype, and including SNPs within the hotspot region. We have recently isolated the congenic strain RB2, which possesses a shorter fragment of the *wm7* haplotype than R209, located at least 10-Mb away from the hotspot and still conferring high recombination activity on the *Psmb9* hotspot (Grey *et al*, 2009). The RB2 × B10 hybrid has, therefore, a local DNA sequence identical to B6 at and around *Psmb9* and a hotspot predicted as active as in R209 (Figure 1). We observed a similar HPTM pattern, both qualitatively and quantitatively, in RB2 × B10 hybrid mice and in R209 mice (Supplementary Figure S3), showing that

Figure 1 Mouse strains with the *Psmb9* initiation site of meiotic recombination active on both, only one or none of the two homologous chromosomes. The *Psmb9* hotspot is on mouse chromosome 17. The *wm7* haplotype, drawn in orange (or dark grey), is required for *Psmb9* activity. Fragments from B10 or B6 origin are in black and that from the A strain is in blue (or light grey). The initiation activity at *Psmb9* on each homologue is indicated (+ or -). CO frequencies (percentage of recombinant molecules per total sperm DNA) in B10.AxSGR and R209xB10 are from the study by Baudat and de Massy (2007a). In R209, B6 and RB2 × B10 frequencies are in parentheses as they are predicted based on genetic analyses that showed that the presence of the R209 or RB2 chromosome (Grey *et al*, 2009) was sufficient to induce *Psmb9* initiation activity on both homologues (Shiroishi *et al*, 1991; Baudat and de Massy, 2007a). A full-colour version of this figure is available at *The EMBO Journal* Online.

the local DNA sequence is not the determinant for the HPTM pattern at the hotspot.

Distinction between HPTMs that precede from those that follow DSB recombinational repair

Our observations raised the question whether the different levels of histone modifications were a cause or a consequence of recombination activity. If some HPTMs contribute to determine initiation site activity, two predictions can be made: (1) in hybrids in which initiation takes place on only one homologue, these HPTMs should be specifically enriched on the chromatid that initiates; (2) these HPTMs should be present at similar levels in *Spo11*^{-/-} and *Spo11*^{+/-} meiotic cells. To address the first issue, we took advantage of the B10.AxSGR hybrid, in which initiation takes place only on the B10.A chromosome (Figure 1). To determine on which homologue the HPTMs occur, we added an allele-specific quantification step to native ChIP and Q-PCR. Strikingly, within the central region of the hotspot, the enrichment was significantly biased towards the actively initiating B10.A allele for H3K4Me3, H3K4Me2, H3K9Ac and H3K27Me1, and towards the inactive SGR allele for H3K27Me3 (Figure 3). In contrast, there was no bias for hyperacetylated H4. The bound chromatin for sequences outside and on either side of the hotspot showed no bias towards one allele versus the other, neither did unbound chromatin within the central region of the hotspot (Supplementary Figure S4). These data show that the combination of HPTMs found enriched at the active hotspot is preferentially associated with the allele initiating meiotic recombination, except for hyperacetylated H4. Second, the analysis of chromatin from testes of 15-day-old *Spo11*^{+/-} and *Spo11*^{-/-} mice showed that two modifications enriched at the centre of the hotspot, H3K4Me3 and H3K9Ac, are enriched to similar levels in the presence or absence of DSB (Figure 4). This strongly suggests that these modifications are part of the substrate for recombination initiation at *Psm9*. This interpretation implies that these modifications are maintained at least from the time of initiation (leptonema) to the pachytene stage. We further directly confirmed the presence of H3K4Me3 at the active hotspot during early stages of meiotic prophase I, in pre-puberal mice testes (Supplementary Figure S5) containing spermatocytes almost exclusively at leptonema and zygonema stages (Supplementary Table S3). Whether H3K4Me2 also marks the initiation site could not be definitively answered given that the enrichment was slightly lower in *Spo11*^{-/-} compared with *Spo11*^{+/-}, with a difference at the limit of significance ($P=0.06$). Interestingly, a significantly lower level of H4

Figure 2 Enrichment for specific histone modifications at the active *Psm9* hotspot. A 4.8 kb region covering the *Psm9* hotspot, defined by high CO rates (cM per kb) is shown together with nine sequence tagged sites (STSs; red bars). Normalized ratios of immunoprecipitated fractions of chromatin between R209 (*Psm9* active) and B6 (*Psm9* inactive) for each STS are shown for elutriated spermatocytes (dark) and for liver cells (light) for different histone post-translational modifications. For H3K9Me3, H4K20Me3 and H3, ratios are from spermatocytes only. The data plotted result from several independent experiments; the experimental values and statistical analysis are shown in Supplementary Figure S1. A full-colour version of this figure is available at *The EMBO Journal* Online.

Figure 3 Enrichment for specific histone modifications on the initiating chromatid. The enrichments of histone modifications along the hotspot region in B10.AxSGR relative to B6 are shown as in Figure 2, here as black curves. On the right Y-axis, percentage of B10.A allele detected in the immunoprecipitated fractions. Values are shown for each histone modification and for each of four STSs, as vertical bars together with s.d. Student's *t*-test was used to test the null hypothesis according to which the percentages of B10.A allele in immunoprecipitated fractions do not differ between the 5' flanking STS (STS 1) and the two STSs inside the hotspot region (STS 8 and 11, treated as a group). A full-colour version of this figure is available at *The EMBO Journal Online*.

hyperacetylation was detected in *Spo11*^{-/-} as compared with *Spo11*^{+/-} mice, suggesting that this modification is enriched upon DSB repair, consistent with the allele-specific analysis described above showing equal levels of H4 acetylation on both chromatids. Other modifications, such as H3K27Me1

Figure 4 Enrichment for histone modifications in *Spo11*^{-/-} mice. Bound fractions, normalized to *Psm9*-STS 1 (the 5' most flanking STS), of chromatin of whole testes cells from R209xB10 *Spo11*^{+/-} (red) and R209xB10 *Spo11*^{-/-} (green) pre-puberal littermates are shown for nine sequence tagged sites along the *Psm9* hotspot region. Mann-Whitney statistical test was used to determine the statistical significance of the difference between bound fractions of *Spo11*^{+/-} and *Spo11*^{-/-} mice for STSs included within the CO hotspot region (STSs 6, 7, 8, 11 and 13). A full-colour version of this figure is available at *The EMBO Journal Online*.

and H3K27Me3, show little variation along the hotspot region, possibly as a result of the heterogeneity of the cell population in these analyses. Global analysis of

the different HPTMs in meiotic prophase nuclei by immunostaining showed highly dynamic processes that probably reflect different DNA-related events, including in particular transcription and recombination (Supplementary Figure S6). This analysis essentially indicates that H3K4Me3 and H3K9Ac are present at the stage of DSB formation (leptonema), even in the absence of SPO11, and that H4 hyperacetylation is detected at all stages during meiotic prophase, including the stage of DSB repair (zygonema and pachynema).

H3K4Me3 and hyperacetylated H4 enrichment at the *Hlx1* hotspot

To test the generality of our finding at the *Psmb9* hotspot, we monitored HPTMs at the *Hlx1* hotspot on chromosome 1, which is specifically active in hybrids between B6 and Cast/Eij (Paigen *et al*, 2008; Parvanov *et al*, 2009). We observed a 2.5% sperm CO rate at *Hlx1* in an R209 × Cast/Eij hybrid consistent with the CO pedigree analysis in B6 × Cast/Eij by Paigen *et al*. (Supplementary Figure S7). Similar to *Psmb9*, specific enrichment of H3K4Me3 and hyperacetylated H4 was observed at the active *Hlx1* hotspot (Figure 5 and Supplementary Figure S8). Interestingly, H3K9Ac was not enriched and H3K27Me1 was even depleted at the active *Hlx1* hotspot, suggesting distinct features between hotspots. The functional role for H3K4Me3 with respect to initiation activity at *Hlx1* was further supported by the enrichment of this mark in *Spo11*^{-/-} mice, at a level similar to that observed in *Spo11*^{+/-} littermates (Supplementary Figure S9).

Absence of correlation between recombination and transcription activities

Several histone modifications (H3K4Me3, H3K4Me2, H3K9Ac and H4 hyperacetylation) found enriched at the active *Psmb9* hotspot are known for their association with transcription and are found at promoters (Barski *et al*, 2007; Kouzarides, 2007; Li *et al*, 2007; Mikkelsen *et al*, 2007). This raises the question whether transcription occurs at the *Psmb9* hotspot. We first verified that in spermatocytes, promoters from expressed or repressed genes carry the expected HPTMs described in somatic cells. We therefore analysed the enrichment of HPTMs at the well-characterized promoter of *Sycp1* (Sage *et al*, 1999), a meiosis-specific gene expressed during meiotic prophase and at the promoter of *Nestin*, a neural marker gene (Burgold *et al*, 2008). Consistent with previous analysis of histone modifications, H3K4Me3, H3K4Me2, H3K9Ac and hyperacetylated H4 were present at the *Sycp1* promoter and H3K27Me3 was enriched at the *Nestin* promoter. All modifications were detected at levels higher than those detected at the centre of the *Psmb9* hotspot (Supplementary Figure S10). We then directly monitored transcription activity in spermatocytes at the *Psmb9* and *Hlx1* hotspots either when active or when inactive. No transcript was detected along the *Psmb9* region both by northern blot (Figure 6) and by random primed RT-qPCR (data not shown). A weak transcriptional activity was detected at the *Hlx1* hotspot by northern blot and at equal levels in strains in which *Hlx1* was active or not, as determined by RT-qPCR (around 1% of the actin level, Supplementary Figure S11), and therefore showed no correlation with the enrichment of H3K4Me3 described in Figure 5.

Figure 5 Enrichment for specific histone modifications at the active *Hlx1* hotspot. A 8 kb region covering the *Hlx1* hotspot, defined by high CO rates (cM per kb) is shown together with seven sequence tagged sites (STSs; red bars). Normalized ratios of immunoprecipitated fractions of H3K4Me3 chromatin between R209 × Cast (*Hlx1* active) and B6 (*Hlx1* inactive), and of H4Ac5 chromatin between R209 (*Hlx1* active) and B6 are shown for each STS. Normalized ratios of immunoprecipitated fractions for each STS are shown. The data plotted result from several independent experiments; the experimental values statistical analysis are shown in Supplementary Figure S8. A full-colour version of this figure is available at *The EMBO Journal* Online.

Discussion

We show here that a set of histone modifications mark the activity of mouse meiotic recombination hotspots, and we further tease apart marks that are associated with the initiation of recombination from post-repair modifications using two complementary approaches. With respect to DSB repair, acetylation of H4 seems to result from meiotic DSB formation at the *Psmb9* hotspot and is also associated with *Hlx1* hotspot activity, an observation consistent with H4 acetylation by the Tip60/NuA4 protein as a response to phosphorylation of H2AX and accompanying DSB repair in somatic cells (van Attikum and Gasser, 2005; Altaf *et al*, 2007). This modification is the first identified chromatin signature of recombinational repair at mammalian hotspots. We note that, albeit highly statistically significant, the level of enrichment of H4 acetylation is low when the hotspots are active, which could be because of the relatively low frequency of DSB repair events at the hotspots tested. With respect to initiation, our results suggest that a specific chromatin organization contributes to the definition of initiation sites for meiotic recombination in mice: From the analysis of the two hotspots, *Psmb9* and *Hlx1*, we have identified one common feature associated with initiation, the enrichment of H3K4Me3 at active hotspots. This modification is also enriched in *Spo11*^{-/-} mice, as expected for a mark that contributes to hotspot activity. This interpretation is strongly supported by the specific enrichment of H3K4Me3 on the initiating chromatid in heterozygous hybrids in which only one chromatid initiates. This modification is the first identified chromatin

Figure 6 Absence of detectable transcription activity at the *Psmb9* hotspot. Northern blot of total RNA (18 µg per lane) prepared from elutriated testes cells of RJ2 and of B10. RJ2 has a shorter *wnt7* fragment as compared with RB2 but confers the same CO activity at *Psmb9* and *Hlx1* (Grey *et al*, 2009). The probes used were PCR products from *Psmb9* 6F-13R, which includes the hotspot centre, and from *Psmb9* 15F-18R, which lies outside the hotspot region and includes the most 3' exon (ex) of the *Psmb9* gene. A full-colour version of this figure is available at *The EMBO Journal* Online.

signature of initiation of recombination at hotspots in mammals. It is interesting to note that this modification is well known to be enriched at transcriptional promoters (Barski *et al*, 2007; Mikkelsen *et al*, 2007), and was recently found to also mark transcription start sites of long non-coding transcripts (Guttman *et al*, 2009). In the *Psmb9* region, we did not detect any transcript by northern blot hybridization and RT-PCR. At the *Hlx1* hotspot, a weak transcriptional activity is detected, which might be associated with the expression of the gene located next to the hotspot, encoding for a ribosomal protein. However, the level of transcription is not different in strains in which *Hlx1* hotspot is active or not, whereas the level of H3K4Me3 differs by a factor of 5. One cannot formally exclude that small-size, low-abundance or unstable RNAs have escaped our detection. In any case, given the human CO map, which shows that most hotspots do not localize near transcription promoters (Myers *et al*, 2005), one expects H3K4Me3 not to be sufficient to promote hotspot activity, and that other chromatin features are most probably involved in hotspot localization. Furthermore, the chromatin configuration might slightly differ between hotspots as suggested by

our comparative analysis between *Psmb9* and *Hlx1*, in which H3K27Me1 is enriched in the former only.

A large number of H3K4 methyltransferases containing a SET domain has been described in mammals (Ruthenburg *et al*, 2007; Shilatifard, 2008). One of them, *Meisetz* (*Prdm9*), is specifically expressed in meiotic cells and has an essential function during meiotic prophase (Hayashi *et al*, 2005). Interestingly, we observed strong variations of the global level of H3K4Me3 in prophase nuclei with a peak at the leptotema/zygotema stages (Supplementary Figure S6). In *Meisetz*^{-/-} spermatocytes, the level of H3K4Me3 is reduced compared with wild type, but is still higher than that in somatic mutant cells (Hayashi *et al*, 2005). In both male and female *Meisetz*^{-/-} mice, gametogenesis is disrupted at the pachytene stage associated with a defect in pairing and DSB repair. One interpretation for this is that these phenotypes are due to an altered expression of one or several genes (Hayashi and Matsui, 2006). No difference between wild type and *Meisetz*^{-/-} testis was detected at the level of initiation of recombination, as measured by γH2AX staining. However, given the residual H3K4Me3 and the limits of immunofluorescence, it is possible that changes in the level or localization of DSB events have been undetected by this analysis. The hypothesis that the enrichment of H3K4Me3 we detected at *Psmb9* and *Hlx1* results from *Prdm9* activity is fully consistent with the observation that the *Prdm9* gene lies within the recently described critical genomic region, defined as *Dsbc1*. This region has been shown to contain a locus involved in genome-wide control of the distribution of recombination and hotspot activity (Grey *et al*, 2009; Parvanov *et al*, 2009).

Interestingly, in *S. cerevisiae*, H3K4Me3 is enriched at DSB sites before DSB formation. An analysis of the *Set1* mutant, the only H3K4 methyltransferase in *S. cerevisiae*, shows a general decrease of DSB levels (Borde *et al*, 2009). These observations suggest that H3K4Me3 might be an evolutionarily conserved feature of meiotic initiation sites. The analysis of *set1Δ* yeast also shows a minority of sites in which DSB levels are not reduced, suggesting that DSB can occur in the absence of H3K4Me3. Either in yeast or mice, the question of what directs H3K4 methyltransferase activity to specific sites in meiotic cells remains to be answered, as well as the role of this modification in generating a substrate for the protein complex generating DSB.

Whatever the mechanism, an epigenetic control of hotspot localization could provide a flexible regulation of meiotic recombination and contribute to the surprisingly marked variations of hotspot activities described, for instance, between individuals in the human population (Neumann and Jeffreys, 2006; Coop *et al*, 2008).

Materials and methods

Mouse strains

Nine mouse strains were used, including C57Bl/6JCr, C57Bl/10JCr (B6 and B10; purchased from Charles River laboratories, <http://www.criver.com>); B10.A (purchased from The Jackson Laboratory, www.jax.org); CAST/Eij (CAST); B10.A(R209) (abbreviated R209); B10.MOL-SGR (SGR); RB2 derived in our mouse facility by back-crossing B10 × R209 F1 with B10; RJ2 (carrying a recombinant chromosome 17 from RB2 × B10.A); and *Spo11*^{+/-} mice carrying the *Spo11* null allele *Spo11*^{tm1M} (Baudat *et al*, 2000). Sets of 8, 15 and 12 B6 male mice 35, 6 and 40 weeks old, respectively; 8, 11 and 10 R209 male mice 29, 40 and 33 weeks old, respectively; 10 and 9 B10A × SGR hybrid male mice 29 and 30 weeks old, respectively; 12

RB2 × B10 hybrid males 16–21 weeks old; 6 R209 × CAST hybrid males 10 weeks old; and R209xB10 *Spo11*^{−/−} (11 mice) and *Spo11*^{+/−} littermates (13 mice) 14–15 days old were used for independent spermatocytes chromatin preparation experiments. All experiments were carried out according to CNRS guidelines and approved by the regional ethics committee on live animals experimentation (project CE-LR-0812).

Enrichment for spermatocytes by centrifugal elutriation of testes cells

Mice were killed by CO₂ inhalation. Whole testes without their tunica albuginea were incubated in 20 ml TIM + 0.5 mg Liberase Blendzyme 3 (Roche Diagnostics, ref 11814176001) for 15 min at 33 °C with gentle shaking, then mixed by pipetting 20 times through the mouthpiece of a 10 ml plastic pipette, incubated again for 15 min at 33 °C before addition of 0.5 mg Liberase, and final incubation for 15 min at 33 °C. After homogenization by pipetting 100 times through the mouthpiece of a 10-ml plastic pipette, filtration twice through a 60-μm nylon mesh and centrifugation at 1500 r.p.m. for 10 min, an average of 7×10^7 cells per adult and of 2×10^6 cells per young mouse were obtained. Separation of testes cell populations from adults was carried out in TIM + 0.1% BSA by centrifugal elutriation with counter flow, using a JE-5.0 rotor and a 5-ml elutriation chamber (Beckman). A total of eighteen 45-ml fractions were collected during elutriation, at a constant centrifugation speed (1800 r.p.m.) and increasing flow rate from 15 to 35 ml/min (Supplementary Table S2). Cell fractions elutriated between 23 and 34 ml/min were subsequently pooled together, yielding on average 5×10^6 cells per male. Immunofluorescence analysis (see below) of spreads of elutriated cells allowed the estimation of the percentages of cells at different spermatogenic stages (Supplementary Table S3). Most elutriated cells were late pachytene and diplotene spermatocytes, which were, therefore, at a stage at which recombination is completed (Guillon *et al*, 2005).

Native chromatin immunoprecipitation

Antibodies purchased from Upstate were those against H3K9Me3 (no. 07-442, lot JBC1361819), H3K27Me3 (no. 07-449, lot DAM1387952), H4K20Me3 (no. 07-463, lot 31392), H3K27Me1 (no. 07-448, lot JBC1361682), H3K4Me2 (no. 07-030, lot 26335), H3K9Ac (no. 07-352, lot 31388), H4Ac5 (no. 06-946, lot 29860), and those purchased from Abcam and Upstate were against H3K4Me3 (no. ab8580 and 07-473 lot JBC1350098, respectively) and H3 (ab1791 and no. 06-755 lot 31949, respectively). Native chromatin was prepared from up to 6×10^7 elutriated cells or from whole testes cells obtained for young mice as described by Umlauf *et al* (2004), with minor modifications described in an updated protocol at www.methdb.de. A total of 6–12 μg of chromatin was immunoprecipitated using 5 μg of antibody. Numbers of replicates of chromatin preparations per mouse strain and of immunoprecipitations per preparation carried out are shown in Supplementary Table S4. A negative control was carried out in parallel for each chromatin preparation without adding any antibody. DNA extracted from chromatin fraction bound to antibody and from unbound fraction was re-suspended in 80 and 400 μl H₂O, respectively.

CO PCR assay at hotspot Hlx1

Allele-specific primers were designed on the basis of SNPs between B6 and CAST sequences and those flanking the *Hlx1* hotspot (also called hotspot 186.3; Paigen *et al*, 2008). Sperm and tail DNAs were extracted from R209 × CAST males and digested using *XhoI*. Single CO molecules could be amplified only from sperm DNA in 10 μl reactions containing 50 pg DNA in two rounds of nested PCRs (primer sequences shown in Supplementary Table S5). Cycling conditions were 96 °C for 30 s, followed by *n* cycles of 96 °C for 10 s, A °C for 20 s, 68 °C for 3 min:30 s + 1 s per cycle using *n* and A °C as described in Supplementary Table S5.

CO breakpoints were mapped using RFLP (*HpyCH4IV*, *AvaII*, *MspI* and *EcoRI*) and further refined by sequence analyses within the 2.9-kb amplicon. Distributions of 40 and 33 CO breakpoints in CAST-B6 and in B6-CAST orientations, respectively, were shown to be significantly different by chi-square analysis, as previously shown for B6 × CAST hybrids (Paigen *et al*, 2008), which is strongly suggestive of a higher level of initiation on the B6 chromatids.

Primers, Q-PCR, correction and normalization

Primers were designed using Beacon designer or Primer3 programs for amplifying the central region of the *Sycp1* promoter, a region located 350 bp upstream of the TSS of the *Nestin* gene and overlapping the previously defined bivalent domain (Burgold *et al*, 2008), nine STSs along a 4.8-kb region spanning the hotspot *Psmb9* and flanking sequences (*Psmb9*-1–*Psmb9*-18) and seven STSs spreading over a 8-kb region of the *Hlx1* hotspot and flanking sequences (*Hlx1*-1.2–*Hlx1*-4). Primer sequences, working concentration in PCR reactions and annealing temperatures are given in Supplementary Table S6. Triplicates (for bound samples) and duplicates (for unbound samples) of 10-μl PCR reactions containing $1 \times$ LC480 SYBR Green mix (Roche) and pairs of primers were seeded using 2 μl ChIPped DNA each. Real-time amplification was carried out in an LC480 LightCycler (Roche) as follows: 95 °C for 5 min, 42 cycles of 95 °C for 10 s, A °C (see Supplementary Table S5) for 15 s and 72 °C for 15 s with 4.3, 2 and 4.3 °C/s ramping rates, respectively, and fluorescence acquisition during the extension step. Melting curves of the PCR product were obtained by carrying out a PCR as follows: 95 °C for 10 s, 70 °C for 15 s with 2 °C/s ramping rates and five fluorescence acquisitions per second up to 95 °C. Absolute quantification of input DNA in samples was carried out by amplifying a set of five-fold serial dilutions of a standard B6 genomic DNA, from 32 pg up to 20 ng, within each PCR microtitre plate.

The amount of non-specific bound DNA ('no antibody' control) ranged from 0.5 to 0.02% of the total input DNA (bound + unbound) between chromatin preparations. The average amount of DNA immunoprecipitated by each antibody amplified from the genomic regions tested exceeded over 10-fold the amount of non-specific bound DNA, except for H3, H3K9Me3 and H4K20Me3 antibodies, which bound on average only two- to fourfold over the background for the genomic regions tested (data not shown). The non-specific background signal was subtracted from the value of bound DNA for each antibody ($B_{corrected}$ or B_c = bound – background). The corrected bound fraction of total chromatin input ($B_c / (B_c + UB)$) amplified for each *Psmb9* STS was normalized to the corrected bound fraction of *Psmb9*-1, the most 5' flanking STS of the hotspot region, chosen as an arbitrary reference (Supplementary Figure S1). For a given tissue, HPTM and STS, the ratio between two mouse strains is the ratio between the average values of the normalized bound fractions (ratios plotted in Figures 2, 3 and 5 and Supplementary Figure S3). The enrichments relative to B6 are given in Supplementary Table S1.

Allele-specific quantification in immunoprecipitated DNA

Hybridization probes were designed for allele quantification, synthesized and purchased from TIB MOLBIOL. For each of four STSs, including two STSs outside the *Psmb9* hotspot (STS1 and 18) and two STSs located less than 200 bp away from the centre of the hotspot (STS8 and 11), one anchor and one sensor probe were designed, which annealed one base away from each other, the sensor probe lying on a SNP between the two alleles (Supplementary Table S7). A total of 10 μl PCR reactions seeded with 2 μl DNA and containing $1 \times$ Lightcycler480 Genotyping Master (Roche), a pair of PCR primers (see Supplementary Table S5) and 150 nM of each hybridization probe were amplified in duplicate using the same cycling conditions as above. Relative quantification of both alleles was carried out by measuring the peak areas of FRET signals emitted by hybridization probes annealed to PCR products during a 40–75 °C increasing temperature step. A standard set of SGR:B10A genomic DNA mixes (9:1, 8:2, ... 1:9) and B10A × SGR hybrid genomic DNA were included in amplification and the subsequent melting curve analysis. The equation giving the calculated proportion of both alleles as a function of the known proportion of both alleles was used for correcting the experimental values obtained. For each STS, the equations are

$$\text{Psmb9-1: } y = -2.8113x^3 + 4.168x^2 - 0.3578x - 0.0037;$$

$$\text{Psmb9-8: } y = -0.9772x^3 + 1.3313x^2 + 0.6409x + 0.0089;$$

$$\text{Psmb9-11: } y = 1.8099x^3 - 2.5085x^2 + 1.6779x - 0.0043;$$

$$\text{Psmb9-18: } y = -2.1277x^3 + 2.8731x^2 + 0.2601x - 0.0106,$$

where *x* is the observed ratio between SGR allele peak area and total area (SGR + B10.A peaks) and *y* is the actual ratio of the two alleles in the DNA mix.

The experiment was repeated for all four STSs, with a second chromatin preparation of B10A × SGR elutriated spermatocytes for H3K4Me3, H3K9Ac, H3K4Me2 and H3K27Me1.

Immunostaining

Immunostaining of spermatocyte spreads was carried out as described by Moens *et al* (1997), using a milk-based blocking buffer (5% milk, 5% donkey serum in $1 \times$ PBS). The antibodies used were: guinea pig anti-SYCP3 at 500-fold dilution, mouse monoclonal anti-phospho-H2A.X (Upstate 05-636) at 625 000-fold dilution, rabbit polyclonal anti-H3K4Me2 at 1:5000, rabbit polyclonal anti-H3K4Me3 at 1:100, rabbit polyclonal anti-H3K9Ac at 1:750 and rabbit polyclonal anti-H3K27Me1 at 1:750 dilution. All incubations with primary antibodies were carried out overnight at room temperature. Secondary antibodies were goat anti-guinea pig Alexa Fluor 488 (Molecular probes), donkey Cy3-conjugated anti-mouse and Cy5-conjugated anti-rabbit antibodies. Incubations with secondary antibodies were carried out at 37°C for 90 min. Nuclei were stained with DAPI ($2\mu\text{g/ml}$) during the final washing step. Digital images were obtained by using a cooled CCD camera, Coolsnap HQ (Photometrics), coupled to a Leica DMRA2 microscope using the same exposure time for all acquisitions. Each colour signal was acquired as a black-and-white image using appropriate filter sets and was merged with Photoshop Imaging software using the same entry levels for each histone modification in order to be able to compare the staining intensity at different stages of meiosis.

RT-qPCR and Northern blot

A total of 2–4 μg total RNA per million cells was extracted using the Genelute mammalian total RNA mini prep kit (Sigma) from 2.5, 6, 5, 2.5, 20 and 25 million elutriated spermatocytes of R209, RB2 $\times$ B10, B10A $\times$ SGR, B6, RJ2 and B10 mice, respectively. After quality monitoring by gel electrophoresis, RNA preparations were treated with DNase (DNA-free kit, Ambion) and reverse transcribed (Superscript III reverse transcriptase, Invitrogen) by random priming (random 10-mer oligonucleotides, MWG) in the presence of ribonuclease inhibitor (RNase-OUT, Invitrogen).

Duplicates of $10\mu\text{l}$ PCR reactions, including each primer pair amplifying STS along the hotspot region (Psmb9-1–Psmb9-18; Supplementary Table S5), were seeded with 5 ng of RT products and amplified in Light-Cycler LC480 as described above. The amount of transcripts detected along the 4.8-kb hotspot region was compared with the amount of actin transcript using the $2^{-\Delta\text{C}_\text{T}}$ method and the absolute number of amplifiable molecules deduced from parallel amplification of diluted B6 genomic DNAs (16 and 80 pg).

Northern blot analysis was carried out using 18 μg total RNA from RJ2 and B10 elutriated testes cells and using PCR products labelled by random priming using α - ^{32}P -dCTP as probes. These PCR

products were from the *Psmb9* hotspot (6F–13R), the 3' last exon of the *Psmb9* gene (15F–18R), the *Hlx1* hotspot (5F–3R) and a 743-bp restriction fragment of a cloned *Spo11* cDNA used as a positive control.

Statistical analyses

The Mann–Whitney test was used to test the null hypothesis according to which the values of normalized, bound fractions obtained for STSs covering the hotspot region (*Psmb9* STSs 6–13; *Hlx1* STSs 5–3) and for different immunoprecipitation experiments (Supplementary Table S4) are not different between two mouse strains (B6 and R209 for *Psmb9*, B6 and R209 $\times$ CAST for *Hlx1*) or between *Spo11*^{+/−} and *Spo11*^{−/−} mice.

The Student's *t*-test was used to test the null hypothesis according to which the proportion of B10.A allele immunoprecipitated from the chromatin of B10.A $\times$ SGR spermatocytes was not different between *Psmb9* STSs included within the CO hotspot region (STSs 8 and 11) and *Psmb9* STS 1 (Figure 3).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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