Meiotic double strand breaks (DSBs) occur at discrete regions in the genome coined hotspots. Precisely what directs site selection of these DSBs is hotly debated and in particular it is unclear which chromatin features, and regulatory factors are necessary for a genomic region to initiate and resolve DSBs as a crossover (CO) event. In human and mouse, one layer of hotspot selection control is a recognition sequence element present at these sites that is bound by the Prdm9 zinc-finger protein. Furthermore, an overall open chromatin structure is thought to be required to allow access of the recombination machinery, and this is often dictated by the packaging of DNA around nucleosomes.

We recently defined the nucleosome occupancy maps of four mouse recombination hotspots throughout meiosis. These analyses revealed no obvious dynamic changes in nucleosome occupancy, suggesting an intrinsic nature of recombinogenic sites, yet they also revealed that nucleosomes define zones of exclusion for CO resolution. Here, we discuss new evidence implicating nucleosome occupancy in recombinogenic repair and its potential roles in controlling chromatin structure at mouse meiotic hotspots.

**Nucleosome Mapping of Mouse Recombination Hotspots on Chromosome 19**

We have defined previously the hotspots HS9, HS22, HS59.4, HS61.1 and HS59.5 in this study on mouse chromosome 19. HS89 and HS822 are intra-chromosomal, whereas HS59.4, HS59.5 and HS61.1 are sub-telomeric. All these five hotspots have crossover (CO) rates ranging from 0.5 to 6.0 x 10^{-4} per meiosis, which is typical to about two-thirds of all mouse meiotic hotspots. The 1.5-kb core of hotspot HS9 lacks polymorphisms between the two strains, whereas the cores of HS22, HS59.4, HS59.5 and HS61.1 harbor numerous single nucleotide polymorphisms (SNP) and insertions/deletions (indel), with an overall sequence divergence of 7% (49 SNPs, 5 indels), 0.3% (3 SNPs, 1 indel), 1% (15 SNPs, no indels) and 2.7% (34 SNPs, 6 indels).

In our earlier report, we used an improved FACs methodology to obtain highly-enriched fractions having >95% purity for pre-leptotene, leptotene-zygotene and pachytene-diplotene cells. Using this strategy, we generated precise nucleosome occupancy maps at hotspots HS9, HS22, HS59.4, HS61.1 and HS59.5 in this study on mouse chromo

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**Organization and roles of nucleosomes at mouse meiotic recombination hotspots**

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active hotspots, i.e., HS22 and HS59.5 (with recombination rate of $2.17 \times 10^{-4}$ and $2.22 \times 10^{-4}$, respectively) in comparison to those having large open domains; e.g., the more active HS9 and HS61.1 hotspots with recombination rates of $4.77 \times 10^{-4}$ and $4.99 \times 10^{-4}$, respectively. A similar trend has been observed for S. cerevisiae hotspots, where in general more active and wide hotspots, such as YAT1, NAT1 and WHI5, have overall low nucleosome occupancy and disordered chromatin structure. In accord with these findings, there is a close correlation between the locale of DNase I hypersensitive (DHSS) regions and the distribution of recombinogenic hotspots in S. cerevisiae. Finally, overlapping the yeast DSB map (obtained by sequencing genomic Spo11-bound DNA fragments) with whole-genome nucleosome occupancy profiles, revealed that Spo11-associated hotspots lacked nucleosomes.

Collectively, these findings would suggest that DSB occur exclusively on non-nucleosomal DNA and that nucleosomes would also prevent access of Spo11. Thus, loose chromatin structure and the openness of a particular genomic region would be thought necessary for establishing a hotspot. However, genomic regions with low-nucleosome occupancy are not necessarily restricted to hotspots. For instance, areas of open chromatin have been observed in regions flanking mouse meiotic hotspots, both in the immediate vicinity of hotspot cores and even in recombinogenically cold flanking regions at -25 kb to +25 kb from the hotspot core. Similarly, not all nuclease hypersensitive sites are DSB hotspots in yeast and there is no correlation between the degree of nuclease hypersensitivity and the frequency of DSB formation. Therefore, there must be additional factors

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**Figure 1.** Nucleosome profiles at HS22 and HS59.5 meiotic hotspots in various wild-type mouse strains. Nucleosome occupancy maps are obtained by real-time PCR. Profiles are normalized to the highest peaks, with the vertical scale being the fold enrichment between the mono-nucleosome MNase-digested DNA fraction and undigested genomic DNA, calculated using the Equation 2. The x-axis represents the location across the analyzed locus. Nucleosome occupancy maps at various meiotic stages were identical for each of the backgrounds and the final averaged profile of at least three experiments is shown. Nucleosomes positions at hotspots regions are indicated by black circles and central nucleosomes at the hotspot cores (blue bars) are shown in red. Hotspot cores are outlined within central recombinogenic regions in the hotspots, and are shown in more detail in CO profiles in reference 2 and in Figure 3. “Hot” and “cold” HS22 and HS59.5 regions in various mouse strains are marked with flame and ice cube images when known, respectively. Additional nucleosome profiles in various meiotic stages and in somatic primary thymic cells at HS9, HS22, HS59.4 and HS61.1 have been published in reference 2. ND, no data; MNase, micrococcal nuclease.
that establish a particular genomic region as a recombination hotspot. 

Nucleosome profiles at mouse meiotic hotspots appear to be pre-established early on at the spermatogonia stage, well before the initiation of meiosis, and remain nearly identical throughout meiosis.2 Remarkably, nearly identical nucleosome landscapes are also observed in somatic thymic cells, supporting the notion that no significant chromatin remodeling occurs at the hotspots during meiosis.2 Similar findings have been described in yeast, where whole-genome nucleosome occupancy maps also showed no differences in pre-miotic and early meiotic profiles.3 This agrees with an earlier yeast study, showing that DNase I hypersensitive regions that overlap with DSB sites, are identical in mitotic and meiotic cells and that this pattern does not change before, during, or following DSB formation in meiotic cells.9 Overall, these findings indicate that the sites of stably bound nucleosomes, one of the criteria for sites of opportunity for Spo11 cleavage at the leptotene stage, are pre-established in mice and appears to be conserved with what is observed in yeast. In addition, nucleosome profiles appear to be identical for a hotspot locus that is either active or inactive in different type mouse strains. Specifically, similar nucleosome profiles were observed for HS22 locus in C57Bl/6J and B6/DBA strains, where this hotspot is active, and in DBA/2J and CAST/DBA hybrids where this hotspot is inactive3 (Fig. 1). Similarly, nucleosome profiles at the HS59.5 hotspot were identical across strains and hybrids irrespective of their active or inactive recombination status (Fig. 1). Thus, the absence of recombination activity in a particular strain background cannot be simply attributed to addition of stable nucleosomes or to alterations in the patterns of stable nucleosomes.
Figure 3. Nucleosome positioning within the hotspot cores is correlated with crossover repulsion zones. A representative example is shown for HS59.5. B6-to-DBa (black) and DBa-to-B6 (gray) profiles are shown as a mirror representation. Recombination rate and the number of molecules analyzed to obtain the CO profile are indicated. Nucleosomes are indicated by black circles, central ones are shown with a red vertical line and are drawn to scale. Prdm9b putative binding sites are indicated with a red vertical line for Prdm9b-C57Bl/6J allele. Sequence alignment is provided in Table 1. Additional CO profiles (and nucleosome positioning) at HS22, HS59.4 and HS61.1 cores have been published in reference 2.

We predict that higher order chromatin structure, of which nucleosome patterning is a critical component, should still play a significant role in establishing the suitable environment for the Spo11 complex to bind a given genomic region. Nucleosome occupancy pattern may affect the distribution of chromatin loops on the axis during synaptonemal complex formation and thereby influence the positioning of favorable sites for DSB initiation (Fig. 2). In support of this notion, in yeast, DSBs are known to be depleted in cohesin-enriched regions found at the bases of chromatin loops.11 Alternatively, the initiation of DSBs may be regulated by epigenetic means, for example by histone modifications and/or DNA methylation, that may cause changes in chromatin conformation via interaction with chromatin remodeling machinery, rendering DNA loops more or less accessible for the Spo11 complex (Fig. 2).12,13,18

Multiple reports studying different organisms suggest that active histone modifications, such as acetylation, methylation and ubiquitination may position meiotic recombination (Fig. 2).17,18 In contrast, repressive histone modification marks are depleted in mouse meiotic hotspots (Fig. 2).12,13,17

Other modes for control of hotspots have also been suggested, for example remote cis-acting DNA motifs affecting the initiation and activity of hotspots.15,19 Further, in S. pombe meiotic recombination hotspots preferentially occur in loci that express the prl class of long polyadenylated noncoding RNAs (ncRNAs),20 where activation of the hotspot site by ncRNAs is thought to provoke chromatin remodeling. Alternatively, ncRNA-DNA hybrids (R-loops) have been proposed to trigger events that open chromatin for the Spo11 complex.20

Taking all of these potential mechanisms into account, it is likely that DSB initiation and hotspot activity are controlled by several different extrinsic and intrinsic cues, and that at least some of these are also dependent upon a particular genomic background. However, one commonality is the need to form an open chromatin conformation that is conducive to DSB initiation.

Crossover Repulsion Zones

The detailed crossover (CO) profiles obtained for the highly polymorphic HS22, HS59.4, HS61.1 hotspots24 and in this study for the HS59.5 locus (Fig. 3), have revealed domains that are resistant to CO resolution. These regions, termed as CO repulsion zones, have been observed in many mammalian hotspots.25,26,27,28

They appeared to be rather randomly distributed across hotspot regions, where they can either occur at the center of the hotspot core (e.g., in HS22, HS59.4 and HS59.5).27 (Fig. 3) or at the 3' boundary of the core (e.g., HS61.1).27 Strikingly, the superposition of nucleosome occupancy maps of these four loci with the CO profiles established that in all four hotspots nucleosomes are localized exactly at the sites that are resistant to CO resolution. Detection of nucleosomes at the sites of CO repulsion zones may shed new insights in the mechanism of homologous recombination.

In an earlier study,27 we proposed a CO repulsion zone model, in which stable nucleosomes may generate constraints to D-loop extension and second end capture. This model favors the notion that the distribution of nucleosomes directs the selection of the preferential binding sites by the Spo11 complex to open chromatin regions. There are several predictions of this model. First, the local nucleosome patterns in the vicinity of a hotspot will control strand invasion and direct D-loop formation by regulating the accessibility and positioning of the nucleosome-free region on the donor homologous chromosome. Second, nucleosome positioning and possibly certain histone modifications would control hotspot activity by affecting the distribution of CO and NCO. In support of this model, recent analysis of CO profiles and the distribution of histone modification marks at Prdm9 and H1ef super-hotspots show an enrichment of selectively modified histones, such as H3K4Me3 and hyperacetylated H4 at their cores, in the regions corresponding to the highest CO rates.24

Instructive Role of DNA Motifs in Hotspot Formation

Multiple DNA regulatory sites of meiotic recombination have been identified in yeast. These sites, coined n-hotspots, mostly have preferential DNA sequences for binding various transcription factors that also may promote recombination.27,28,29 Examples of these sequences are CRE-like M26 DNA site (5'-ATG ACT G-3'), 5'-CCA AT-3' and 5'-CCC CGC A-3' motifs, which are binding sites for Art1-Pdr1, CCAAT-binding transcription factors, and Scr1, Ruv1, Hsl1 and Rut2 zinc-finger transcription factors respectively.15,27,28 However, other meiotic yeast hotspot regions, the so-called B-hotspots, are not associated with transcription

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factor binding. These sites rather contain intrinsically open nucleosome-excluding DNA sequences located in intergenic genomic regions. For example, the common DNA repeat (CCGG)n motif (where n = 12, 24 or 48), which excludes nucleosomes, can either augment (i.e., the 12-mer) or repress (i.e., the 48-mer) recombination.4,5

In humans, several short DNA motifs were identified that are enriched in hotspots.4,6,7 In particular, the 7-mer motif CCT CCC T and the 9-mer motif CCC CAC CCC are each found in about 10% of human hotspots. The recent breakthrough for the recombination field was the identification of a degenerate 13-mer sequence motif CCN CCN TNN CCN C that is present at the core of approximately 40% linkage disequilibrium (LD)-defined hotspots in humans.8 These findings suggested the existence of a DNA binding factor that could direct the DSB initiation machinery to specific sites. Further, genetic studies in mice identified a region that appeared to encode a major trans-acting regulator in mice identified a region that appeared to encode a major trans-acting regulator in mice.41 Finally, it has been established that this motif is present in active recombinogenic regions.5

In S. cerevisiae, the hotspot activity associated with the HIS4 gene was previously shown to require the Baslp transcription factor.8 Nonetheless, a genome-wide analysis of Baslp transcription factor binding sites and their recombinogenic activity has demonstrated that Baslp stimulated recombination only in 16% of these sites. Finally, if instructive, the 13-mer sequence motif CCN CCN TNN CCN C, which is present in 40% of human meiotic hotspots, can only promote recombination for 10% of its locations in the genome.8

In mice, two different 36- and 33-mer motifs have been predicted to be recognized by two distinct Prdm9 proteins for b and c alleles in M. m. domesticus and M. m. molossinus strains.8 We have screened HS61.1, HS9 and HS59.5 sequences for the presence of Prdm9 b allele motif and HS22 sequence for the presence of Prdm9 c allele motif and HS59.5 allele motifs (Table 1). For HS22 and HS59.5 loci, sequences matching from 6 to 10 out of 15 highest score bases of Prdm9 predicted recognition motifs were identified (Table 1 and Fig. 1). Notably, screening of the HS22 hotspot core DNA sequences in C57Bl/6J strain background in which this hotspot is active and in CAST/EiJ background where it is “cold” shows that sequences with high-score matches to the recognition motifs for Prdm9b and Prdm9awc proteins are present in both (up to 9 out of 15 matches for C57Bl/6J and up to 9 out of 14 matches for CAST/EiJ background, Table 1). Thus, other levels of control besides the presence of corresponding Prdm9 protein binding site must be operational in these different backgrounds to turn the HS22 region to a hotspot. 

These findings are consistent with data obtained for MSTN1a and MSTN1b human hotspots, which showed that major variation in their hotspot activity and the presence/absence of a hotspot can occur without any change in local DNA sequence.24 Finally, computational approaches to predict Prdm9 binding sequences are not always being confirmed by actual biochemical binding studies with hotspot sequences.25 In this study, 26 sequences for Prdm9b and Prdm9awc binding were generated using C4H zinc finger prediction program and tested in vitro for three mouse hotspots, G7c, Het6 and Pdm9. However, only one of them could bind Pdm9b using a southwestern assay.26 This failure to adequately determine Pdm9 binding sites indicates that the other factors besides DNA sequence might regulate its binding to a certain region, and/or that Pdm9 binding alone is not sufficient.

Future Directions: Is the Chromatin Structure of Hotspots Controlled by Labile Histone Variants?

Open chromatin regions are necessary but not sufficient for conferring DSBs. Very little is known about how open chromatin domains at hotspots are formed and what regulatory factors control chromatin architecture. Several recent studies have established that “nucleosome-free
regions’ are in fact not really free but are rather occupied by labile H3.3/H2A.Z nucleosomes.46-49 Unlike most of “stable” nucleosomes, which are deposited on DNA during replication phase (S-phase), H2A.Z and H3.3 histone variants are highly mobile,20-22 thus they must replace resident histones. For example, histone H3.3 slowly replaces H3 after differentiating cells have exited the cell cycle23 and also during spermatogenesis before DNA becomes repackaged with canonical nucleosomes.24,25 H2A.Z nucleosomes containing both H3.3 and H2A.Z histone variants are unusually unstable at the conditions normally used for nucleosome preparations.26-30 Intriguingly, a recent genome-wide study of H3.3/H2A.Z distribution around transcription regulatory regions in human cells showed that H3.3/H2A.Z nucleosome core particles mark “nucleosome-free” regions of active promoters, enhancers and insula-
tor regions.31 In addition, double-variate nucleosomes correspond to sites of tissue-specific Dnase I hypersensitivity.32 Accessing a site occupied by a nucleo-some for DNA binding, transcription, replication or repair requires nucleosome removal or sliding, and/or unraveling of the supercoiled DNA double helix. H3.3/H2A.Z nucleosomes could be produced and destabilized as part of this dynamic process.33 It has been proposed that they may serve as “place holders,” preventing the region from being covered with stable canonical nucleosomes and nonspecific factors.34 Alternatively, these labile nucleo-somes may be displaced by transcrip-tion factors.35 We speculate that H3.3/H2A.Z nucleosomes may be present in the “nucleosome-free” regions of potential meiotic recombination hotspots and mark accessible sites of DSB opportunity. Once a Spo11 protein complex is anchored to the labile nucleosome sites, they are get-
ting replaced with recombination protein machinery stimulating initiation of homologous recombination (Fig. 2). Certainly this is a hypothesis that needs to be further investigated in order to uncover the fundamental steps leading to DSB initiation.

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Nucleus 249
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