HELLS and PRDM9 form a pioneer complex to open chromatin at meiotic recombination hot spots

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Chromatin barriers prevent spurious interactions between regulatory elements and DNA-binding proteins. One such barrier, whose mechanism for overcoming is poorly understood, is access to recombination hot spots during meiosis. Here we show that the chromatin remodeler HELLS and DNA-binding protein PRDM9 function together to open chromatin at hot spots and provide access for the DNA double-strand break (DSB) machinery. Recombination hot spots are decorated by a unique combination of histone modifications not found at other regulatory elements. HELLS is recruited to hot spots by PRDM9 and is necessary for both histone modifications and DNA accessibility at hot spots. In male mice lacking HELLS, DSBs are retargeted to other sites of open chromatin, leading to germ cell death and sterility. Together, these data provide a model for hot spot activation in which HELLS and PRDM9 form a pioneer complex to create a unique epigenomic environment of open chromatin, permitting correct placement and repair of DSBs.

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In eukaryotic cells, most DNA is wrapped around an octamer of histone proteins to form nucleosomes, the basic unit of chromatin. Chromatin functions as a physical barrier to DNA, and regulating access to DNA is an essential aspect to normal cellular function. Numerous proteins have evolved to carry out this function; these include pioneer factors, chromatin remodelers, and epigenetic modifiers [Iwafuchi-Doi and Zaret 2014; Clapier et al. 2017]. Pioneer factors initiate formation of accessible chromatin by engaging partial recognition motifs present within nucleosomal DNA [Soufi et al. 2012; Zaret and Mango 2016]. Binding of the pioneer factor leads to increased accessibility accompanied by deposition of active histone modifications, ultimately enabling other DNA-binding proteins to gain access to regulatory elements [Iwafuchi-Doi and Zaret 2014]. Recruitment of chromatin remodeling enzymes by pioneer factors is a critical step in creating accessible DNA [King and Klose 2017]. The Snf2 family of chromatin remodelers use energy from ATP hydrolysis to exchange, evict, and reposition nucleosomes within chromatin [Clapier and Cairns 2009]. To date, most studies of pioneer factors have focused on their role as master regulators of cellular differentiation and lineage reprogramming [Soufi et al. 2012; Zaret and Mango 2016], leaving open their necessity in other cellular processes requiring access to DNA such as meiotic recombination.

Meiosis, which is essential for all sexual reproduction, involves major reorganization of chromatin, along with chromosome condensation, pairing, and, ultimately, genetic recombination. The outcome of recombination is the exchange of genetic material between parental chromosomes, resulting in the shuffling of alleles between generations. In mammals, fungi, and plants, recombination is concentrated at specialized sites termed hot spots [Baudat et al. 2013; Tock and Henderson 2018]. The physical exchange of DNA is initiated when the topoisomerase SPO11 is recruited to hot spots, where it induces the double-strand breaks (DSBs) required for DNA exchange between homologous chromosomes [Keeney 2001]. Significant chromatin remodeling is likely a prerequisite to overcome chromatin barriers at hot spots and allow the proper targeting of SPO11.

In most mammals, hot spot locations are determined by the DNA-binding protein PRDM9 [Baudat et al. 2010,
PRDM9 contains a SET domain, which catalyzes both histone H3 lysine 4 trimethylation (H3K4me3) and H3K36me3 (Buard et al. 2009; Eram et al. 2014), and a series of tandem C-terminal zinc finger domains that bind DNA. Prdm9 is highly polymorphic with the majority of differences in regions encoding the zinc finger domains (Berg et al. 2011; Kono et al. 2014). Allelic variants bind different DNA sequences, ultimately dictating the location of hot spots. PRDM9 binding occurs within nucleosome-depleted regions (NDRs) that are subsequently targeted for DSBs by SPO11 (Baker et al. 2014; Lange et al. 2016). Loss of Prdm9 results in the persistence of unrepaired DSBs, incomplete synopsis of homologous chromosomes, and pachytene arrest in meiotic prophase I (Hayashi et al. 2005; Sun et al. 2015). Interestingly, meiosis-specific loss of two Snf2 family chromatin remodeling factors, Hells (Lsh) and Ino80, each results in phenotypes similar to loss of Prdm9 (Zeng et al. 2011; Serber et al. 2016), indicating that these chromatin remodelers might play direct roles in nucleosome remodeling at recombination hot spots.

Given the broad similarity in requirements to create accessible DNA for both pioneer factor-mediated control of gene regulation and meiotic recombination, we hypothesized that PRDM9 functions as a pioneer factor to overcome chromatin barriers at hot spots by recruitment of chromatin remodeling enzymes.

**Results**

**Hot spots are marked by a unique epigenomic state**

To functionally annotate the chromatin landscape of male germ cells, we determined co-occupancy of histone modifications using ChromHMM (Ernst and Kellis 2012). ChromHMM uses a hidden Markov model to classify genomic regions into “states” by identifying combinatorial patterns of chromatin features, in this case histone modifications. Using ChromHMM, we solved an 11-state model using new and our previously published nucleosome-resolution ChIP-seq data of 10 histone modifications or histone variants (Fig. 1A, Supplemental Table 1). ChIP-seq was performed using enriched germ cells from 12- to 14-d postpartum (dpp) C57BL/6J (B6) mice. After birth, the first wave of meiosis occurs semisynchronously (Bellve et al. 1977), allowing enrichment of meiotic stages when PRDM9 is active (Ball et al. 2016). This germ cell model recapitulated many of the previously observed chromatin states found in other cell types, including promoters and insulators (states 1), enhancers (states 2–5), transcriptionally active regions (state 6), Polycomb repressive complex-repressed (state 8), and heterochromatin (states 9 and 10) (Fig. 1A, B).

Of these 11 chromatin states, only state 7 was enriched for PRDM9-binding sites; it is distinguished by a unique combination of histone modifications. In addition to H3K4me3 and H3K36me3, state 7 is characterized by H3K4me1 and H3K9ac (Fig. 1A, B). In contrast to other acetylation marks often found at enhancers (i.e., H3K27ac and H2BK120ac), only H3K9ac is enriched at hot spots. The majority (78%, 14,773/18,900) of all state 7 locations overlap with previously reported locations of B6 meiosis-specific DSBs (Smagulova et al. 2016) and PRDM9-dependent H3K4me3 modification (Fig. 1A, B), highlighting that state 7 represents recombination hot spots. Interestingly, compared with other phyla (Choi et al. 2013; Yamada et al. 2018), we did not detect H2A.Z at hot spots in mice. Together, these data identify a unique combination of histone features that biochemically distinguish recombination hot spots from
other regulatory elements and may be involved in creating chromatin accessibility for DSB formation.

**Hot spots are sites of accessible chromatin**

Given that recombination hot spots are marked by histone modifications associated with active chromatin, we measured DNA accessibility in germ cells using the assay for transposase-accessible chromatin [ATAC-seq] (Buenrostro et al. 2013). Biological replicates proved highly reproducible ($r = 0.96$), showed enrichment of open chromatin at promoters, and revealed typical nucleosome binding patterns (Supplemental Fig. 1A–D), all indicators of high-quality libraries. At PRDM9-dependent H3K4me3 sites, we detected increased DNA accessibility overlapping with PRDM9 motif locations (Fig. 1D–F). Overall, ATAC-seq identified fewer hot spots compared with H3K4me3 ChIP-seq (2902 ATAC peaks overlapping 13,498 PRDM9-dependent H3K4me3). Hot spots with greater PRDM9 binding and histone modification generally have more open chromatin (Fig. 1D). On average, the location of accessible DNA showed an inverse relationship to nucleosome positions at hot spots (Fig. 1E,F). Filtering ATAC-seq reads for those that fall within NDRs (i.e., <120 bp) showed that open chromatin was highest where PRDM9 binds. Together, these data build on an earlier observation that detected increased DNase hypersensitivity at a single hot spot (Shenkar et al. 1991), and show that generally hot spots are sites of open chromatin.

**PRDM9 acts as a meiosis-specific pioneer factor to create open chromatin**

To determine whether the unique epigenomic state at hot spots is PRDM9-dependent, we used a strategy to maintain the same genetic background while changing Prdm9 alleles. To do so, we used a “knock-in” (KI) mouse strain that exchanges the zinc finger domain of the endogenous B6 Prdm9Dom2 allele with the zinc finger domain from CAST/EiJ mice [Prdm9Cst] (Supplemental Fig. 1A; Baker et al. 2014). We measured chromatin accessibility and H3K9ac in enriched KI germ cells (Supplemental Fig. 1E,F), combined with H3K4me3 (Baker et al. 2014), and compared these with the B6 results (Fig. 2A,B). Quantifying differences between B6 and KI identified 5775 ATAC peaks and 14,249 H3K9ac peaks increased in KI (FDR < 0.01). Of these, 92% of the ATAC peaks ($n = 5305$) and 94% of the H3K9ac peaks ($n = 13,408$) overlap with reported PRDM9-dependent H3K4me3 peaks (Baker et al. 2014). Reciprocally, 1756 ATAC peaks and 7049 H3K9ac peaks were increased in B6 compared with KI, with 94% of the ATAC peaks ($n = 1643$) and 91% of the H3K9ac peaks ($n = 6431$) overlapping known PRDM9Dom2-dependent H3K4me3 peaks. Similar to the case with B6, open chromatin at hot spots in the KI strain is highest at PRDM9-binding sites (Supplemental Fig. 1H). These data show that hot spot chromatin that is accessible in B6 is not accessible in the KI. Instead, in the KI there are unique sites of accessible chromatin that overlap with known Prdm9Cst hot spots, sites that are not open in B6.

Next, if PRDM9 binding facilitated open chromatin, we reasoned that variants that disrupt PRDM9 binding should change DNA accessibility. B6 and DBA/2J (D2) strains share the Prdm9Dom2 allele, thus providing a natural experiment to determine the effect of genetic variation on chromatin accessibility. Recently we mapped quantitative trait loci (QTL) that regulate H3K4me3 levels in a genetic reference population whose genomes are homozygous mosaics of B6 and D2 (Baker et al. 2019). That study identified 1331 hot spots where the H3K4me3 level is regulated by local variation (locational QTL). We performed ATAC-seq on D2 mice and compared H3K4me3 levels and open chromatin at local-QTL to that from B6 (Fig. 2C). Hot spots with biased H3K4me3 levels also had biased open chromatin corresponding to increased frequency of single-nucleotide variants near PRDM9-binding sites. At each hot spot, we separately calculated scores for the PRDM9 motif for both B6 and D2 sequences. In general, the strain with the highest motif scores had the highest chromatin accessibility (Fig. 2C, right).

The programmed DSBs in meiosis are repaired primarily using the homologous chromatid as a template. This enabled us to determine whether the unique epigenomic landscape at hot spots occurs on both chromatids or only on the initiating chromatid. We performed ATAC-seq and H3K9ac ChIP-seq on spermatocytes from (B6xCAST)F1 hybrids and compared them with our published PRDM9 and H3K4me3 ChIP-seq data (Baker et al. 2015a). At asymmetric hot spots—i.e., those sites where PRDM9 preferentially binds one parental chromatid (Baker et al. 2015a; Smagulova et al. 2016)—both acetylation and chromatin accessibility are largely restricted to the parental chromatid to which PRDM9 binds (Fig. 2D,E). These data show that open chromatin is concordant with PRDM9 binding. Interestingly, however, they also suggest that hot spot chromatin is not open on the homologous chromosome, implying another mechanism is required to facilitate DNA accessibility for repair.

**Formation of open chromatin at hot spots precedes meiotic DSBs**

In somatic cells, DSBs lead to nucleosome remodeling and increased chromatin accessibility (Price and D’Andrea 2013). To determine whether this is also true of meiotic DSBs or whether chromatin remodeling precedes DSB formation, we tested whether the epigenomic state at hot spots is dependent on DSB formation. We performed ATAC-seq and ChIP-seq for H3K9ac and H3K4me3 on spermatocytes collected from 12- to 14-dpp mice lacking SPO11 (Baudat et al. 2000). In the absence of SPO11, we found wild-type levels of open chromatin and H3K9ac that show no quantitative differences compared with heterozygous littermate controls (Fig. 2F–H). These data agree with previous observations showing that H3K4me3 and H3K36me3 modifications at hot spots are independent of and therefore precede SPO11 binding and subsequent DSB formation (Grey et al. 2017).
PRDM9 overcomes chromatin barriers to transiently bind DNA

Pioneer factors recognize partial DNA-binding motifs within nucleosomes (Soufi et al. 2012). Based on DNA sequence, PRDM9Dom2-binding sites (Smagulova et al. 2011) and PRDM9Cst-binding sites (Supplemental Fig. S2A) are predicted to be occupied by nucleosomes. To measure nucleosome occupancy in the absence of PRDM9 binding in vivo, we combined data from a total of eight MNase-seq libraries from four Mus musculus domesticus mouse strains (B6, D2, [B6xD2]F1, and WSB/EiJ) that do not carry the Prdm9Cst allele, resulting in >328 million reads. Hot spots were sorted based on MNase signal strength across the central 100-bp overlapping PRDM9Cst-binding sites (Supplemental Fig. S2B). We found that ∼50% of

**Figure 2.** Open chromatin at hot spots is dependent on PRDM9 but not on DSBs. (A, top) Graphic comparing B6 versus KI strains sharing the same genetic background and differing only in Prdm9 allele. Paired horizontal bars indicate homologous chromosomes, and height of the peaks suggests the level of open chromatin at the hot spot. (Bottom) Normalized [cpm] profiles of histone modifications and chromatin accessibility at two hot spots activated by different Prdm9 alleles. (B) MA plots comparing ATAC-seq (top) and H3K9ac (bottom) between B6 and KI. Prdm9Dom2 (blue) and Prdm9Cst (red) hot spot annotations are from Baker et al. (2014). (C, top) B6 and D2 strains represent different genetic backgrounds sharing the same Prdm9Dom2 allele. (Bottom) Heat maps showing H3K4me3, ATAC, single-nucleotide variant locations, and PRDM9 motif scores at 1331 local-QTL. (D, top) Graphic outlining genetic makeup of heterozygous (B6xCAST)F1 hybrids with two Prdm9 alleles. (Bottom) Allele-specific profiles of H3K4me3, H3K9ac, ATAC, and PRDM9 [reads uniquely mapped to either the B6 or CAST haplotype]. (E) Scatter plots of haplotype-specific PRDM9 binding (n = 3786) versus H3K9ac (left) or ATAC (right). (F, top) Open chromatin at hot spots occurs before programmed DSBs. (Bottom) H3K4me3, H3K9ac, and ATAC profiles in Spo11−/− mutant and H3K4me3 and ATAC profiles in heterozygous control at a representative hot spot (Pbx1). (G) Metaprofile of H3K4me3 and ATAC (top) and H3K4me3 and H3K9ac (bottom) at recombination hot spots from Figure 1D from Spo11−/−. (H) MA plot of ATAC-seq from Spo11−/− [mut; n = 2] and Spo11+/- [het; n = 1].
PRDM9\textsuperscript{Cst}-binding sites have nucleosome signals at the PRDM9-binding site and that these nucleosomes are remodeled in spermatocytes that express PRDM9\textsuperscript{Cst} to become nucleosome-depleted regions. These data are in agreement with a recent study [Hinch et al. 2019] that found higher levels of nucleosome occupancy at PRDM9-binding sites in the absence of PRDM9. In addition to exploring PRDM9 in vivo, expression of PRDM9 ex vivo recapitulates aspects of hot spot activation, including allele-specific H3K4me3 deposition [Baker et al. 2015b]. To further investigate whether PRDM9 binds nucleosomal DNA in an orthologous system, we aggregated MNase-seq signal from HEK293 cells expressing different PRDM9 alleles [Supplemental Fig. 2C; Baker et al. 2015b]. HEK293 cells that either do not express PRDM9 or are transfected with the PRDM9\textsuperscript{A} allele have no obvious nucleosome pattern at PRDM9\textsuperscript{Cst} hot spots. In contrast, these same genomic regions have depleted nucleosomes at PRDM9\textsuperscript{Cst}-binding sites upon expression of PRDM9\textsuperscript{Cst} [Supplemental Fig. 2C]. These observations support the germ cell data and suggest that nearly half of PRDM9-binding sites must be buried within nucleosomes.

Residence time of DNA-binding proteins can be estimated in vivo using nuclease protection assays such as ATAC-seq and DNAse hypersensitivity [Sung et al. 2014]. Proteins with long residence times will protect DNA from nucleases, resulting in a characteristic footprint observed by decreased cutting frequency at binding sites. In vitro, PRDM9 has a long residency time when bound to synthetic DNA oligonucleotides [Striedner et al. 2017]. Footprint analysis of ATAC-seq data from germ cells found that PRDM9\textsuperscript{Cst} motif locations showed little evidence for nuclease protection [Supplemental Fig. 2D,E], relative to long-resident proteins like CTCF [Supplemental Fig. 2F,G], similar to other transiently binding pioneer factors [Swinstead et al. 2016]. Together, these data suggest that PRDM9\textsuperscript{Cst} can access target motifs even within nucleosomal DNA, and the lack of a clear footprint is consistent with PRDM9 having a short residency time in vivo.

The chromatin remodeling factor HELLS is required for proper synapsis

Our observations that hot spots are marked by histone modifications associated with active chromatin and show PRDM9-dependent formation of accessible chromatin all point to an unknown mechanism controlling chromatin remodeling. To identify candidate remodeling complexes, we reasoned that if chromatin remodeling is required for hot spot activation, loss of the remodeling enzyme would result in a phenotype similar to loss of Prdm9. Loss of either Ino80 [Serber et al. 2016] or Hells [Zeng et al. 2011] fulfills these criteria. Given Ino80 plays a major role in histone variant exchange, specifically H2A.Z and H2A [Papamichos-Chronakis et al. 2011], and we found that recombination hot spots in mice lack H2A.Z [Fig. 1A], we focused our attention on Hells.

To examine HELLS localization during spermatogenesis, we used immunohistochemistry to detect protein abundance in seminiferous tubules [Fig. 3A]. HELLS showed high expression in both spermatogonia and meiotic cells, similar to a previous result [Zeng et al. 2011]. HELLS expression overlaps with phosphorylated H2AX (\gamma\text{H2AX}), a marker of DSBs, with broad temporal colocalization in leptotene and zygotene stages. During pachytene, both HELLS and \gamma\text{H2AX} are sequestered to the sex body [Fig. 3A, arrow]. In addition, HELLS is coexpressed with PRDM9 in spermatocytes [Fig. 3A]. The timing and colocalization of PRDM9 and HELLS are supported by recent single-cell transcript analysis [Jung et al. 2019] showing individual leptotene cells coexpress Hells and Prdm9 [Supplemental Fig. 3]. Together, these data show that HELLS and PRDM9 are both present when recombination hot spots are activated.

Next, we used a conditional strategy to study the meiosis-specific function of HELLS. We used Stra8\textsuperscript{-}\text{icre} [Sadate-Ngatchou et al. 2008] to create male mice with a homozygous loss of Hells at the onset of meiosis [Hells CKO]. Adult CKO mice had reduced testis weight [Fig. 3B] with extensive loss of postmeiotic germ cells [Fig. 3C], while testes from Hells heterozygous littermates [control] appear normal. This block in germ cell development recapitulates an earlier observation using allografts of testis tissue between Hells\textsuperscript{-/-} mice and wild-type donors [Zeng et al. 2011]. Cross sections of adult Hells CKO seminiferous tubules show loss of HELLS staining [Supplemental Fig. 4A]. The loss of later stage germ cells correlates with a large increase in germ cell apoptosis in Hells CKO compared with heterozygous littermate controls [Fig. 3C,D]. Finally, Hells CKO male mice did not sire any offspring when mated to normal B6 females. In summary, we confirm that HELLS is required for sperm production and fertility of male mice.

Using surface-spread meiotic chromatin in combination with immunohistochemistry, we found that HELLS is diffusely localized in leptotene and zygote spermatocytes [Fig. 3F], similar to localization of PRDM9 in the same stages [Parvanov et al. 2017]. Meiotic spreads showed that later in prophase I, after repair of the majority of autosomal DSBs, HELLS is restricted to the sex body. Staining for HELLS is absent from Hells CKO mice [Fig. 3F]. To determine at which stage meiotic progression is blocked in Hells CKO mice, we staged spermatocytes using spreads stained for both \gamma\text{H2AX} as a marker of DSBs and SYCP3 to characterize chromosome synapsis in adult male mice [Supplemental Fig. 4B]. We found that Hells CKO mice have an increased proportion of spermatocytes in leptotene and zygote stages and a nearly complete loss of normal pachytene and diplotene stages [Fig. 3F]. Hells CKO cells scored as pachytene consistently show persistent \gamma\text{H2AX} phosphorylation and incomplete synapsis absent from littermate controls [Supplemental Fig. 4B]. These pachytene-like cells are negative for the testis-specific histone variant H1t [Cobb et al. 1999], a marker for late pachytene [Supplemental Fig. 4C]. Overall, our data confirm that loss of Hells leads to meiotic arrest at the late zygote to early pachytene stage [Zeng et al. 2011], with incomplete synapsis and persistent DSBs leading to spermatocyte apoptosis.
In Hells-null mice, meiotic DSBs are targeted to other functional elements

Because loss of Hells leads to incomplete synopsis and persistent γH2AX, we next determined the levels and locations of meiotic DSB in mutant spermatocytes. To characterize DSBs cytologically, we used spread meiotic chromatin labeled with SYCP3 and DMC1. DMC1 is a meiosis-specific recombinase that binds to single-stranded DNA at the sites of DSBs (Neale and Keeney 2006). While zygotene spermatocytes from Hells CKO overall had similar levels of DMC1 foci as littermate controls (Fig. 4A), these foci persist in Hells CKO pachytene-like cells on synapsed chromosomes (Fig. 4B), indicating incomplete DSB repair.

While the number of DSBs were similar between Hells CKO and control, their genomic locations were dramatically different. To determine locations of meiotic DSBs, we identified genomic regions bound by DMC1 (Khil et al. 2012) in testes from adult CKO and littermate controls. In total, we identified 13,643 DMC1 peaks in Hells CKO and 15,718 in heterozygous littermates. Overall, Hells CKO germ cells lost DSBs at canonical hot spots and gained DSBs at other sites (Fig. 4C).

For example, loss of Hells resulted in a 12-fold increase in DSBs at promoters and a concomitant decrease in DSBs at distal sites (Fig. 4D). Nearly all DMC1 peaks identified in Hells CKO germ cells overlap with the location of DSBs in Prdm9−/− (93.7%, 13,206/13,643), while only 15.7% (2475/15,718) of DMC1 sites identified in control germ cells overlap Prdm9−/− DMC1 peaks (Fig. 4E).

To determine the epigenomic landscape targeted for DSBs in Hells CKO, we calculated enrichment of DMC1 sites and open chromatin within the ChromHMM states (Fig. 4F). While hot spots (state 7) were enriched for DSBs in the Hells control, in both Hells and Prdm9 mutants, DSBs were enriched in states 1 and 2, which are annotated as promoters, insulators, and enhancers. Interestingly, although H3K4me1, H3K4me3, and H3K9ac are present in states 1 and 2 (Fig. 1A), state 3 had a combination of histone modifications similar to classically defined enhancers that lack H3K4me3, and was less enriched for DSBs. States 1 and 2 were enriched for PRDM9-independent accessible chromatin; whereas state 7 only had PRDM9-dependent accessible chromatin in B6 compared with KI (Fig. 4F). Notably, unlike hot spots, states 1 and 2 were not enriched for H3K36me3. Furthermore, in Hells CKO, in which DSBs largely occur at state 1 and 2 locations, spermatocytes undergo apoptosis similar to loss of Prdm9. Together, these data suggest that in the absence of Hells, DSBs are retargeted to regions of open chromatin that lack the proper epigenome to become competent hot spots.
HELLS is required for epigenomic activation of recombination hot spots

Because DSBs were retargeted in Hells CKO germ cells, we next examined the epigenome of the mutant spermatocytes. We first confirmed loss of HELLS in 12-dpp whole testes [Fig. 5A], when PRDM9 is active but prior to the Hells CKO meiotic arrest. ChIP-seq for H3K4me3 and ATAC-seq on enriched germ cell populations from 12-dpp male mice identified a significant reduction of epigenomic modification in Hells CKO mice at recombination hot spots (Fig. 5B,C; Supplemental Fig. 5). While 11,812 H3K4me3-modified sites are significantly reduced in Hells CKO compared with control (log2 fold change < -1, FDR < 0.01), only 73 sites showed higher modification in Hells CKO. Annotating peaks using state 7, we found that both H3K4me3 (n = 11,125) (Fig. 5D) and chromatin accessibility (n = 1851) (Fig. 5E) sites at hot spots were reduced in Hells CKO. In contrast, among peaks overlapping HMM state 1 (n = 31,161 H3K4me3 peaks), there were virtually no sites that changed. Histological sections of 12-dpp seminiferous tubules from both Hells CKO and heterozygous control littermates showed similar stages in development (Supplemental Fig. S6A). Although measuring PRDM9 in whole testes from CKO mice by Western blot detected a modest reduction in protein abundance (Supplemental Fig. 6B,C), PRDM9 was readily detected in individual leptotene and zygotene spermatocytes (Supplemental Fig. 6D). These data show that HELLS is required for establishment of the epigenomic state and chromatin accessibility at hot spots.

HELLS forms a complex with PRDM9

To develop a model for temporal molecular characterization of hot spot activation, we chose a human cell line

Figure 4. Loss of HELLS leads to redirection of programmed DSBs to other functional elements. [A] Quantification of DMC1 foci from chromatin spreads at zygotene/early pachytene stage spermatocytes in Hells CKO and control [Ctrl] animals [mean ± SEM]. [B] Representative chromatin spreads of a pachytene-like spermatocyte from Hells CKO and pachytenone spermatocyte from Hells control. Spreads were immunolabeled with anti-SYCP3 [magenta] and anti-DMC1 [green]. Scale bar, 10 µm. [C] Profiles of DMC1 signal and H3K4me3 level for representative locus in Hells CKO and control spermatocytes. ChromHMM state annotations are shown below profile tracks. [D] Distribution of DMC1 ssDNA regions relative to transcription start sites [TSs]. (E) Intersection of DMC1 locations identified in spermatocytes from Prdm9−/− and Hells control and CKO mice. (F) Heat map showing fold enrichment of DMC1 locations and ATAC in ChromHMM states from Figure 1A.

Figure 5. HELLS is required for histone modification and chromatin accessibility specifically at hot spots. [A] Western blot of whole-testis protein extract from B6 [WT], Prdm9−/− [KO], and Hells heterozygous [Het] and CKO [β-Tubulin was used as a loading control]. [B] Profile of ATAC and H3K4me3 levels from 12-dpp Hells CKO and control spermatocytes. HMM annotations are below. (C) Heat map of H3K4me3 and ATAC signal at all hot spots from Figure 1D (cpm in 10-bp bins). [D] MA plots of H3K4me3 levels comparing Hells CKO (n = 3) and homozygous controls (n = 3) for all H3K4me3 sites [left]. HMM state 7 [middle, filtered for overlap with DSBs and PRDM9-dependent H3K4me3], and HMM state 1 [right]. [E] MA plot of ATAC signal [blue indicates HMM state 7; n = 2 control; n = 3 CKO].
that expresses HELLS and created a stably integrated Flag-PRDM9C allele under inducible control of the tetracycline promoter [HEK293-P9C] (Fig. 6A). Addition of doxycycline led to robust expression of Flag-PRDM9C and increased H3K4me3 at PRDM9C hot spots (Fig. 6A,B). PRDM9C expression also increased MNase sensitivity at PRDM9C binding sites compared with adjacent regions (Fig. 6C), indicating nucleosome remodeling. Reciprocal coimmunoprecipitation (co-IP) using antibodies directed to either HELLS or PRDM9 [Flag] identified a doxycycline-dependent interaction between the two proteins (Fig. 6A right). We confirmed PRDM9–HELLS interaction in vivo by performing co-IP on protein lysates prepared from 12-dpp mouse testes. We detected a HELLS–PRDM9 interaction in testes from B6 mice and littermate controls but not in Hells CKO [Fig. 6D] or Prdm9+/− [Fig. 6E]. To test whether PRDM9–HELLS interaction requires assembly on chromatin, co-IP was repeated using a mouse strain expressing a PRDM9 variant that lacks the DNA-binding domain [PRDM9ΔZF] (Parvanov et al. 2017). We found that HELLS did interact with PRDM9ΔZF [Fig. 6E], suggesting that this interaction is independent of PRDM9-directed DNA binding.

We next showed that HELLS is required for robust PRDM9 binding at hot spots [Fig. 6F–H]. HELLS expression in HEK293-P9C cells was reduced using small interfering RNA [siRNA], followed by induced expression of PRDM9C (Fig. 6G). ChIP for Flag-PRDM9C in HEK293-P9C cells successfully detected robust binding at C-dependent hot spots and no binding at a PRDM9A hot spot or at the GAPDH promoter [Fig. 6H]. Critically, upon loss of HELLS, PRDM9C binding at hot spots was reduced to background levels [Fig. 6H]. Together, these data show that PRDM9 and HELLS form a complex in vivo and, by extension, suggest that active chromatin remodeling is required for robust PRDM9 binding at hot spots.

**HELLS is recruited to recombination hot spots through PRDM9**

Our experiments indicate that HELLS and PRDM9 interact and open chromatin at hot spots. This suggests a model in which PRDM9 recruits HELLS to hot spots to facilitate nucleosome remodeling for DSBs. To confirm this, we first tested whether HELLS binding at hot spots requires the presence of PRDM9 using our HEK293-P9C cells. In the absence of PRDM9C, ChIP for HELLS did not identify binding at any of the five genomic locations tested by qPCR. In contrast, upon expression of PRDM9C, we observed significantly increased binding of HELLS only at C-hot spots and no change at negative controls [Fig. 7A], suggesting HELLS binding at hot spots is PRDM9 dependent.

To test for HELLS binding of hot spots in vivo, we performed ChIP using enriched germ cells from 12-dpp KI mice. Biological replicates were highly correlated [Pearson’s r = 0.91] [Fig. 7B] and identified 2675 HELLS-binding sites. Visually, we detected clear HELLS ChIP signal at recombination hot spots [Fig. 7C] and promoters [Supplemental Fig. 7A,B]. HELLS peaks were annotated based on overlap with PRDM9-dependent H3K4me3 peaks from KI mice (Baker et al. 2014) and TSSs representing promoters [Fig. 7D]. The largest class of HELLS-binding sites...
act with nucleosomal DNA, and (2) the ability to catalyze chromatin remodeling. Here we show that to-gether HELLS and PRDM9 fulfill these criteria at hot spots. PRDM9 brings two of the salient features of canon-ical pioneer factor-mediated chromatin reorganization into one molecule: (1) a sequence-specific targeting domain that provides locational specificity and can inter-act with nucleosomal DNA, and (2) the ability to catalyze increased chromatin accessibility. This suggests that the ability for PRDM9 to function as a pioneer factor is independent of chromatin structure—much like HELLS. Interestingly, the ability to target DSBs to open chromatin is critical for meiotic recombination. In mice, meiotic DSBs are concentrated within hot spot NDRs. In the absence of either HELLS or PRDM9, crossovers identified in KI germ cells at stages earlier or later than leptotene/zygote did not detect open chromatin at hot spots (Maezawa et al. 2018). This observation supports the role of a chromatin remodeler (PRDM9 or HELLS, and IgG signals ±1 kb from PRDM9Cst motif. (H) Metaprofile of H3K4me3 [red] and HELLS [gray] signals at hot spots from G (all HELLS signals are from merged replicates). (F) Box plot comparing distribution of open chro-matin at hot spots with (gray; n = 1230) or without (black; n = 4529) a HELLS ChIP-seq peak. [P-values were from Welch’s two-sided test]. (E) Scatter plot of replicate HELLS ChIP-seq from mouse germ cell (n = 2675 peaks). [C] Profile of H3K4me3, ATAC, HELLS, and IgG control from germ cells of KI mice at the Hlx1 PRDM9Cst hot spot. (D) Pie chart annotating HELLS ChIP-seq peaks. [E] Box plot comparing distribution of HELLS binding (n = same as in D, P-values from Welch’s two-sided test). HS Hot spots (red); TSS promoters (blue); [other] unknown elements (black). (F) Box plot comparing distribution of open chromatin at hot spots with [gray; n = 1230] or without [black; n = 4529] a HELLS ChIP-seq peak [P-values were from Welch’s two-sided test]. (G) Heat maps of H3K4me3, ATAC, HELLS, and IgG signals ±1 kb from PRDM9Cst motif. (H) Metaprofile of H3K4me3 [red] and HELLS [gray] signals at hot spots from G (all HELLS signals are from merged replicates). (I) Model for PRDM9-dependent recruitment to recombination hot spots. PRDM9 and HELLS bind before interacting with chromatin. Nucleosome remodeling creates open chromatin surrounding PRDM9-binding sites.

Discussion

Pioneer function is defined by three features: (1) the ability to recognize partial motifs within nucleosomal DNA, (2) acquisition of active histone modifications, and (3) increased chromatin accessibility. Here we show that together HELLS and PRDM9 fulfill these criteria at hot spots. PRDM9 brings two of the salient features of canonical pioneer factor-mediated chromatin reorganization into one molecule: (1) a sequence-specific targeting domain that provides locational specificity and can interact with nucleosomal DNA, and (2) the ability to catalyze epigenetic marks associated with active chromatin. Both of these features are required for meiotic recombination [Parvanov et al. 2017; Diagouraga et al. 2018]. While the number of recombination hot spots are generally depleted from repressive regions of chromatin marked by H3K9me2 (Walker et al. 2015; Patel et al. 2019), hot spots that are found in these domains show nearly similar levels of PRDM9-dependent histone modification as those found outside of H3K9me2/3 domains, supporting the idea that even within heterochromatin, PRDM9 and HELLS have the capacity to create open chromatin. Additionally, a previous experiment characterizing the chromatin landscape using purified populations of male germ cells at stages earlier or later than leptotene/zygotene did not detect open chromatin at hot spots [Maezawa et al. 2018]. This observation supports the role of a PRDM9–HELLS complex in creating de novo open chromatin at hot spots during a developmentally narrow window. Further evidence for a pioneer factor role for PRDM9 comes from our ex vivo analyses. Ectopic expression of PRDM9 in cells expressing HELLS recapitulates allele-specific histone modification and nucleosome remodeling. This suggests that the ability for PRDM9 to function as a pioneer factor is independent of chromatin structure that might be unique to meiosis.

The ability to target DSBs to open chromatin is critical for meiotic recombination. In mice, meiotic DSBs [Baker et al. 2014; Lange et al. 2016] and crossovers identified through single-sperm sequencing [Hinch et al. 2019] are concentrated within hot spot NDRs. In the absence of open chromatin at hot spots, loss of either HELLS or
PRDM9 (Brick et al. 2012), SPO11 preferentially targets other regions of accessible chromatin to create DSBs. Hot spots are marked by a unique combination of histone modifications not found at other regulatory elements, specifically H3K4me1, H3K4me3, H3K36me3, and H3K9ac. These observations support a precedent set by an early study that found enrichment of H3K9ac at an individual hot spot (Buard et al. 2009), and a recent survey of histone modifications in purified populations of male meiotic cells found that out of six histone acetylation antibodies tested, only H3K9ac showed high enrichment at hot spots (Lam et al. 2019). It is possible that this unique combination of histone modifications provides an epigenomic addressing system for directing placement or repair of DSBs through recruitment of an epigenetic reader. A plausible candidate for such a reader is ZCWPW1 (Jung et al. 2019; Li et al. 2019), which is coexpressed with PRDM9 during the leptotene/zygotene phase and carries two protein domains that recognize H3K4me3 and H3K36me3. While the redirection of DSBs to other regulatory elements usually results in a meiotic block and infertility, notably, the severity of the infertility phenotype caused by loss of PRDM9 depends on the genetic background (Mihola et al. 2019), suggesting other components in action. Finally, through creation of aberrant sites of open chromatin, the pioneer function of the HELLS–PRDM9 complex could provide a molecular mechanism by which particular alleles are associated with genomic instability in certain cancers (Houle et al. 2018).

Here we identified that the chromatin remodeling enzyme HELLS is a second critical component of recombination initiation. Chromatin accessibility and histone modification at hot spots require HELLS, and furthermore, HELLS localizes at hot spots through interaction with PRDM9. HELLS is a member of the SNF2 family of ATP-dependent chromatin remodeling enzymes that use the energy of ATP to reposition, remodel, and remove histones from DNA substrate (Clapier and Cairns 2009). HELLS is misregulated in human tumors (von Eyss et al. 2012) and mutated in patients with immunodeficiency–centromeric instability–facial anomalies syndrome (Thijssen et al. 2015). Mice with systemic loss of HELLS exhibit prenatal lethality (Geiman et al. 2001); therefore, most functional studies of HELLS have required ex vivo cell culture. Notable exceptions are two studies showing that loss of HELLS in both male and female germ cells results in errors in synopsis and DSB repair during meiosis (De La Fuente et al. 2006; Zeng et al. 2011). Here, using conditional loss of function, we confirmed the requirement of HELLS in meiotic progression and further defined the mechanistic role of HELLS in chromatin remodeling at hot spots.

HELLS has been implicated in the establishment of genome-wide methylation patterns and chromatin repression (Yu et al. 2014; Termanis et al. 2016). HELLS ATPase domain is required for these functions (Ren et al. 2015; Termanis et al. 2016), and ATP is necessary for nucleosome remodeling in vitro (Jenness et al. 2018). If HELLS functioned to close chromatin in meiotic cells, we would have expected to see an increase in open chromatin in CKO mice; instead, we detected a near universal closing of chromatin at hot spots upon loss of HELLS. Given that our CKO strategy ablated HELLS primarily in meiotic cells, these epigenomic experiments are not optimal for determining the consequences of loss of HELLS in other cell types but suggest the idea that the primary role of HELLS in meiosis is creating open chromatin at hot spots. In contrast, ChIP for HELLS was performed on a population of male germ cells, including spermatogonia. In addition to hot spots, we found that HELLS is enriched at promoters associated with higher gene expression, in agreement with previous results in fibroblasts (von Eyss et al. 2012). However, like many cell types, promoters are constitutively open during spermatogenesis (Maezawa et al. 2018), suggesting the idea that HELLS role in opening chromatin in prophase I is specific to hot spots. Together these data show that, in addition to its well-characterized role in generating repressive chromatin, HELLS is associated with increased chromatin accessibility. These disparate functions likely depend on HELLS protein binding partners.

Our evidence suggest that HELLS is recruited to hot spots through PRDM9, rather than being recruited as a consequence of PRDM9-dependent epigenomic modification. The HELLS–PRDM9 interaction persists in cells with a mutated PRDM9 lacking zinc fingers, supporting the idea that their interaction is independent of DNA binding by PRDM9. In addition, on average the maximum HELLS enrichment is found in the center of NDR at the PRDM9-binding site, rather than at the flanking nucleosomes. These observations support a model in which HELLS is recruited by direct interaction with PRDM9, and agree with the previous observation that HELLS-mediated chromatin remodeling in vitro requires interaction with the zinc finger protein CDC47 (Jenness et al. 2018).

Overall, data reported here give rise to a model (Fig. 7I) in which HELLS and PRDM9 form a meiosis-specific pioneer complex to create open chromatin at recombination hot spots. This model not only expands our knowledge about diversity in function of pioneer complexes in general but, more specifically, also poses interesting challenges with respect to meiotic mechanisms. Clearly further work is required to determine how the HELLS–PRDM9 complex is temporally regulated, how the epigenomic environment and open chromatin at hot spots recruits programmed DSBs, and how chromatin barriers are overcome on the template chromatid used to repair these breaks.

Materials and methods

Mice

C57BL/6J [B6, stock no. 000664], DBA/2J [D2, 000671], CAST/Eij [CAST, 000928], B6.129X1-Spo11tm12Hou/J [Spo11–/–, 019117], Tg [Str8-iCre]Reb/J [Str8-iCre, 008208], B6.Cg-TgACTFLPe 9205Dym/J [ACTFLPe, 005703], and B6.129P2-Prdm9tm1.1Kpgn/J [Prdm9–/–, 010719] are all available through The Jackson Laboratory. Mice carrying various Prdm9 alleles B6.C57Bl/6J-Kpgn [Kpgn] / Kpgn (Baker et al. 2014) and B6.Cg-Prdm9tm3.1Kpgn / Kpgn
Frozen embryos of the EUCOMM allele C57BL/6NTac-Flag-PRDM9C expression was induced by replacing culture media with buffer (50 mM Hepes-KOH at pH 7.4, 150 mM NaCl, 0.4% NP-40), and eluted with 20 µL of 2× sample loading buffer [50 mM Tris-HCL at pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.05% bromophenol blue] heated for 10 min at 95°C. Primary antibodies for Western blots and immunoprecipitation included anti-Flag M2 (Millipore/Sigma F3165), anti-PRDM9 [custom] (Parvanov et al. 2017), anti-HELLS (Millipore/Sigma 07-436, lot 2289129), anti-H3K4me3 (Millipore/Sigma 07-473, lot 3018770), anti-H3K9ac (Active Motif 39137, lot 09811002), anti-H3K27ac (Abcam ab4729, lot GR211893-1), anti-H3K27me3 (Millipore/Sigma 07-449, lot 2475696), anti-H2BK120ac (Active Motif 39120, lot 01008001), and anti-H2AZ (Millipore/Sigma 07-594, lot 2455725). For ChIP of H3K9ac, H3K27ac, and H2BK120ac used in the ChromHMM analysis, germ cell isolation and ChIP buffers were supplemented with 20 mM sodium butyrate.

Protein extracts for Western blots from cultured HEK293-P9C cells were prepared as described previously (Baker et al. 2015b). For co-IP, protein lysates were diluted to 500 µL using HEPES buffer and treated with 4 µL antibody and 20 µL of Protein-A/G reagent with DAPI (Invitrogen P36935).
genes were annotated as described (Berger et al. 2014) using TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 and removing Nextera adapters. For paired-end ATAC-seq samples, trimmed sequences were aligned using bowtie (version 0.12.9) [Langmead et al. 2009] with the following settings:--chunkmbs 2000 -S X2000 -m1. Mapped reads were adjusted for Tn5 insertion site as described [Buenrostro et al. 2013]. For single-end ATAC-seq, trimmed sequences were aligned using bwa mem (version 0.7.15) [Li and Durbin 2009] with default settings. Picard Tools was used to remove duplicates and calculate insert size metrics. ATAC-seq alignments were filtered to remove reads with MAPQ < 10 and mitochondrial reads.

ChIP libraries were aligned using bwa mem with default settings [version 0.7.15] [Li and Durbin 2009]. D2 ChIP-seq and ATAC-seq data were aligned to an in silico pseudogenome incorporating known variants [R78-REL1505] [Wu et al. 2010] and converted to mm10 reference coordinates using G2Gtools (accessible online at https://github.com/churchill-lab/g2gtools). Allele-specific ChIP analysis was performed using variant-aware alignment strategy EMASE [Raghupathy et al. 2018]. To identify hot spots and ensure robust haplotype calls, peaks were selected that overlap with [BxCI]F1 PRDM9 ChIP-seq summits and filtered for > 30 reads that map uniquely to either B6 or CAST.

Both ChIP-seq and ATAC-seq peaks were called using MACS version 1.4.2 [Zhang et al. 2008] using a cutoff of 10^{-5}. Duplicate reads were retained for ChIP against histone modifications and removing for HILLS ChIP. To build reference peakomes for each experiment, peaks were called on merged bam files from replicate experiments. Peak files across different strains or Prdm9 alleles were concatenated, sorted, and merged using bedtools [Quinlan and Hall 2010]. Peaks overlapping ENCODE blacklist regions were removed for subsequent analysis. Read counts for each reference peakome were extracted from sample bam files using BedTools multicoverage and are available as processed data at NCBI GEO (GSE135896). Genomic profiles were visualized using the UCSC browser.

Statistical analysis was performed using R [release 3.4.1]. For differential analysis, read counts were normalized using the trimmed mean of M-values method and differential analysis performed using edgeR [Robinson et al. 2010]. Genome-wide false discovery rates (FDRs) were calculated by adjusting P-values following the Benjamini-Hochberg method. Annotation of peaks using genomic locus overlap was performed with bedtools intersect or HOMER mergePeaks functions [Heinz et al. 2013] and visualized using the UpSet package [Lex et al. 2014]. Read matrices of heat maps were generated using the CoverageView R package after RPM normalization with 10-hp bins and visualized using Java TreeView [Saldanha 2004]. ATAC-seq footprints were aggregated and normalized using ATACseqQC [Ou et al. 2018]. Calculation of motif scores for predicted PRDM9-binding sites was performed by generating a position weighted matrix as outlined [Wasserman and Sandelin 2004].

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