Acetylated Histone H3K9 is associated with meiotic recombination hotspots, and plays a role in recombination redundantly with other factors including the H3K4 methylase Set1 in fission yeast

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ABSTRACT

Histone modifications are associated with meiotic recombination hotspots, discrete sites with augmented recombination frequency. For example, trimethylation of histone H3 lysine4 (H3K4me3) marks most hotspots in budding yeast and mouse. Modified histones are known to regulate meiotic recombination partly by promoting DNA double-strand break (DSB) formation at hotspots, but the role and precise landscape of involved modifications remain unclear. Here, we studied hotspot-associated modifications in fission yeast and found general features: acetylation of H3 lysine9 (H3K9ac) is elevated, and H3K4me3 is not significantly enriched. Mutating H3K9 to non-acetylatable alanine mildly reduced levels of the DSB-inducing protein Rec12 (the fission yeast homologue of Spo11) and DSB at hotspots, indicating that H3K9ac may be involved in DSB formation by enhancing the interaction between Rec12 and hotspots. In addition, we found that the lack of the H3K4 methyltransferase Set1 generally increased Rec12 binding to chromatin but partially reduced DSB formation at some loci, suggesting that Set1 is also involved in DSB formation. These results suggest that meiotic DSB formation is redundantly regulated by multiple chromatin-related factors including H3K9ac and Set1 in fission yeast.

INTRODUCTION

Histones package eukaryotic DNA into a highly condensed chromatin structure and impact all aspects of DNA-templated processes. Once incorporated into nucleosomes, histones sterically keep DNA-processing enzymes away from DNA and thereby inhibit DNA-related events. Consistently, cis-elements such as transcriptional promoters are often devoid of nucleosomes, and histones are transiently evicted from sites of transcription or repair (1). Histones also regulate the behaviour of chromatin-structured DNA through post-translational modifications by affecting histone–DNA interactions or by recruiting other proteins. Various modifications such as acetylation and methylation are known to date, and each modification is related to one or several DNA-templated reactions. For example, acetylation of histones and methylation of histone H3 lysine4 (H3K4), often observed in active chromatin regions, are involved in the activation of transcription (2).

Homologous recombination, a process in which genetic information is exchanged between homologous DNA duplexes, is markedly activated during meiotic prophase. Meiotic recombination contributes to ensuring proper chromosome segregation as well as conferring genetic diversity to gametes and is essential to most sexually reproducing organisms. Not surprisingly, its failure can lead to consequences like infertility or birth defects. Furthermore, as this type of recombination is triggered by programmed DNA double-strand breaks (DSBs) that can be detrimental to cells, it must be initiated in a strictly regulated manner. Therefore, it is important to fully understand how meiotic recombination occurs in vivo: how are meiotic DSBs formed in the chromatin structure? Notably, meiotic DSBs are introduced by the conserved type II topoisomerase-like protein Spo11 (3), whose access to DNA is influenced by chromatin structure (4), and hence, meiotic DSBs occur predominantly in discrete sites called recombination hotspots. Because of their importance in recombination regulation, hotspots have been intensively studied.

Several traits of histones are known to be associated with meiotic recombination hotspots. First, hotspots...
often reside in chromatin sites with less histone density such as nucleosome-depleted regions (4). Most hotspots in budding yeast coincide with transcriptional promoter regions (5) that contain fewer nucleosomes, and fission yeast hotspots are also devoid of histones (6). These observations would suggest that histones should be depleted around hotspots to allow the action of recombination factors including Spo11. This idea is supported by chromatin remodelling at the fission yeast hotspot ade6-M26. M26 is a G:T nonsense point mutation (7,8), which creates a cyclic AMP (cAMP) response element-related ‘M26-sequence’ (5′-ATGACGT-3′) that is bound by the activating transcription factor (ATF)/cAMP response element binding (CREB) family transcription factor dimer Atf1-Pcr1 (Figure 1A) (9,10). Recombination at this locus is dependent both on the M26 sequence as well as Atf1-Pcr1 binding and is also proposed to be facilitated by chromatin alteration, which exposes embedded DNA around ade6-M26 (11).

Another characteristic of histones near hotspots is an enrichment of post-translational modifications. For example, budding yeast hotspots are associated with histone H3 lysine 9 acetylation (H3K9ac) and H3K4 trimethylation (H3K4me3) (12,13). In particular, H3K4me3 has been more extensively analysed, and deleting the sole H3K4 methyltransferase gene SET1 results in a massive reduction in DSB formation (14). Similarly, a vast majority of hotspots carry H3K4me3 in mice (15,16) and, the meiotic H3K4 trimethylase PRDM9 is important for defining recombination hotspot distribution in mouse and human (17–19). Around the fission yeast ade6-M26 hotspot, histones are more acetylated than around its negative control locus ade6-M375, and such hyperacetylation promotes DSB formation (20).

Although studies on various model organisms report the involvement of histones and their modifications in meiotic recombination, several crucial points remain elusive. For instance, the exact functions of hotspot-associated histone modifications are not yet known. Although H3K4me3 is proposed to be a possible determinant of hotspots in budding yeast and mouse, it does not overlap with all hotspots and vice versa. In addition, at least some DSBs can still be detected in set1-deleted budding yeast and Prdm9−/− mice, where H3K4me3 associated with authentic hotspots is largely eliminated (12,21,22). Moreover, further scrutiny of H3K4me3 around budding yeast hotspots increased the possibility that the modification may be due to colocalization of promoters and hotspots in this organism (23). These observations lead to the assumption that additional factors such as other histone modifications may exist around hotspots (16). It is also unclear how conserved these hotspot-associated modifications are across species.

Seeking clues to understand the relation between hotspots and modified histones, we used fission yeast to examine hotspot-associated histone modifications and their possible roles. From analyses focused on several M26-sequence-dependent hotspots as well as the whole genome, we found that hotspots are generally enriched with H3K9ac. Eliminating H3K9ac partially decreases Rec12 levels and DSB formation at most hotspots. We also found that hotspots in fission yeast are not associated with H3K4me3, but that deletion of the H3K4 methyltransferase gene set1 causes increased Rec12 binding to chromatin and reduced DSB formation at some loci. These results point to a possibility that H3K9ac and Set1 are both, but differently, involved in meiotic DSB formation. They also suggest that other factors are likely to play a part in meiotic recombination, given the partial defects in the mutants of H3K9ac and Set1. We speculate that multiple factors including H3K9ac and Set1 play various roles in meiotic DSB formation in fission yeast. Our present results will provide novel insights into the mechanism of meiotic recombination.

**MATERIALS AND METHODS**

**Yeast strains and general methods**

Strains used in this study are listed in Supplementary Table S1. Procedures for strain constructions are described in Supplementary Materials and Methods. Spore viability was measured by micromanipulating spontaneously released spores with the SINGER MSM system (Singer), and meiotic recombination frequency was measured by random-spore analyses. The binomial test was used for Figure 4D, and the t-test was used for all other statistical analyses. Throughout the article, statistical significance is indicated as **P < 0.05, ***P < 0.001.**

**Detection of meiotic DSBs and Rec12-oligonucleotides**

Meiotic DSBs except for those at hsp10 were detected as previously described (24,25). DSBs at hsp10 were detected by digesting genomic DNA with Nru I followed by southern hybridization. The hybridization probe was polymerase chain reaction (PCR) amplified from Schizosaccharomyces pombe genomic DNA using the primers 5′-GTCAGCATGCTCTAGTAGGGTTATTGG-3′ and 5′-TCGTTTAAGAAGCTGTGATTGTTCACG-3′. Rec12-oligonucleotides complexes were detected as in (26).

**Chromatin immunoprecipitation quantitative real-time PCR**

Chromatin immunoprecipitation (ChIPs) were performed as previously described (20). Antibodies used are described in Supplementary Materials and Methods, and the specificities of anti-H3K9ac and -H3K4me3 antibodies were confirmed by western blotting (data not shown). To calculate histone modification levels, DNA immunoprecipitated with anti-modified histones and histone H3 was analysed by quantitative real-time PCR (qPCR) using the Applied Biosystems 7500 Real-Time PCR system. The results were analysed as follows. First, signal intensities obtained with modified histones were divided by those with histone H3. For example, H3K9ac at ade6-M26 was calculated according to the formula below.

\[ K9ac@M26/H3cter@M26 \]

Also calculated were relative histone modification levels at the analysed loci normalized to those at the internal...
control locus, prp3⁺ promoter region. For example, fold enrichment of H3K9ac at ade6-M26 was calculated according to the formula below.

\[
\text{K9ac}_\text{ade6-M26} = \frac{\text{H3cter}_\text{ade6-M26}}{\text{H3cter}_\text{prp3}}
\]

To calculate Rec12 levels, the formula below was used.

\[
\text{Rec12}_\text{ade6-M26} = \frac{\text{Rec12}_\text{ade6-M26}}{\text{Rec12}_\text{prp3}}
\]

Used primers are listed in Supplementary Table S2. All experiments were performed three times from independent cell cultures.

**ChIP-chip**

ChIPs were performed as previously described (20). DNA from whole-cell extracts, and ChIP fractions were amplified and end-labelled with the in vitro transcription (IVT) Amplification Method described elsewhere (27). Each sample was analysed using a GeneChip® S. pombe Tilling 1.0 FR Array (P/N 900647, Affymetrix) as described in the Affymetrix® Chromatin Immunoprecipitation Assay Protocol (P/N 702238 Rev. 3). Signals were quantile normalized and smoothed with a half window size of 250 bp (histones) and 375 bp (Rec12) using Two Sample
Comparison Analysis of an Affymetrix set1 and the MA CisGenome (28) [One Sample Comparison, (W) = 0, (Region Boundary Cutoff, MA >) = 0.3, (Maximum Gap within a Region) = 250, (Maximum Run of Insignificant Probes within a Region) = 5, (Minimum Region Length) = 250, (Minimum Number of Significant Probes within a Region) = 15].

(Histones) ChIP signals obtained with the anti-histone H3ctet antibody were normalized to those with DNA in whole-cell extract. These scores were then used to normalize ChIP signals obtained with the anti-histone H3K9ac, H3K14ac and H3K4me3 antibodies. Sites with enriched modified histones were determined by using MA of CisGenome (28) [One Sample Comparison, (W) = 0, (Window Boundary) = 250, (Region Boundary Cut-off, MA >) = 0.3, (Maximum Gap within a Region) = 250, (Maximum Run of Insignificant Probes within a Region) = 5, (Minimum Region Length) = 250, (Minimum Number of Significant Probes within a Region) = 22.5].

The region around the ade6 gene (chr3: 1312712-1325203) and the set1 gene (chr3: 412000-417000), 100 kb regions from each end of every chromosome, centromeres (chr1: 3752000-3792000; chr2: 1595000-1650000; chr3: 1060000-1150000) and mating-type loci (chr2: 2110000-2140000) and SPBPJ4664.02 gene (chr2: 688616-700531), which were excluded from analyses in a previous study (29) owing to the difference in genotype and the low density of probes used in ChIP-chip data, were not analysed in this study either. Two closely positioned binding sites were merged, provided that the corresponding region in another strain contains a single binding site.

*S. pombe* sequence information was according to the Wellcome Trust Sanger Institute (September 2004). The raw and processed data for the ChIP-chip analyses are deposited at the Gene Expression Omnibus (GEO accession number GSE31648).

**RESULTS**

**Detailed histone modifications around the ade6-M26 recombination hotspot**

To gain clues for globally analysing hotspot-associated modifications, we first focused on several M26-sequence-dependent hotspots to test which lysine residues are specifically acetylated and whether H3K4 is methylated. Modification levels at hotspots and their respective non-hotspot control loci were compared by ChIP. For accurate detection of subtle difference associated with meiotic recombination, we designed the experimental system as follows. (i) The highly synchronous pat1-114 haploid meiosis system was adopted (For details, see Supplementary Materials and Methods). As we previously observed that the difference in acetylation levels between M26 and M375 is more prominent in early meiosis (20), cells harvested 1 h after meiosis induction were analysed. (ii) A limited region (~100 bp) encompassing M26 or M375 was analysed by qPCR. (iii) To correct for differences in nucleosome occupancy among analysed loci, ChIP experiments were also performed using an antibody against the C-terminal domain of histone H3 (H3ctet). The modified histone signals were normalized to H3ctet signals.

We observed that both ade6-M26 and ade6-M375 are located in a region with less nucleosomes (Supplementary Figure S1; see later in the text and Supplementary Figure S12 for details) and did not show a significant difference in histone H3 levels (Figure 1B). Remarkably, H3K9 was more acetylated around M26 than around M375 (Figure 1C, t-test, *P* = 0.048), whereas H3K14 was acetylated at comparable levels between the two loci (Figure 1D). Four lysines of histone H4 were also tested for acetylation: lysine5 (H4K5), 8 (H4K8), 12 (H4K12) and 16 (H4K16). Although the lysines other than H4K5 seemed to be more acetylated at M26 than at M375, the differences were not significant (Supplementary Figure S2A–D). Extending our analysis to other modifications, we examined the distinct H3K4 methylation states; mono (H3K4me1), di (H3K4me2) and H3K4me3 levels were similar between ade6 and prp3, mono (H3K4me1), di (H3K4me2) and H3K4me3 levels were similar between M26 and M375. However, H3K4me3 was less enriched around M26, the hotspot locus, than around M375, the control locus (*P* = 0.034).

ChIP consists of many steps that can intrinsically cause variability between samples. Such sample-to-sample difference can be considered by simultaneous analyses of an internal control locus to discuss histone modification patterns between hotspots and their control sites. For this purpose, a fragment of the promoter region of prp3, which is on a different chromosome from the ade6 gene (prp3 is on the Chromosome I and ade6 is on the Chromosome III), was mainly used. This region was in a similar chromatin status between hotspot-containing strains and their control strains (Supplementary Figure S3). Relative modification levels at the hotspot (or control) loci were calculated for individual experiments by normalizing the modified histone/H3ctet ratio of ade6 fragments to that of prp3. Then, the averages and standard deviations were calculated (Figure 1H–J). By this analysis, we confirmed that histone H3K9ac is more enriched at M26 than at M375 (*P* = 0.0046) and that H3K4me3 is lower at M26 than at M375 (*P* = 0.00016). Such a tendency of H3K4me3 was constantly observed throughout early meiosis, as the modification levels remained constant both at M26 and M375 during the time course (Supplementary Figure S4). We also tested, aside from prp3 aforementioned, the ORFs of act1+ and lys1+ as an internal control, and these two loci gave indistinguishable results from Figure 1H–J (Supplementary Figure S5). Taken together, we
hypothesized that a high level of H3K9ac and a low level of H3K4me3 might define chromatin around the ade6-M26 hotspot.

Histone modifications around an M26-sequence-dependent hotspot close to the 3'-end of the ade6 gene

Because M26 is close to the promoter of the ade6 gene (Figure 1A), and histone modification patterns are influenced by their relative locations along ORFs (2), we analysed another M26-sequence-dependent hotspot ade6-3049, which is located near the 3'-end of the ade6 gene (Figure 1K). This time, ade6-3057 was used as a non-hotspot control. Histone H3 was less associated with the 3049 hotspot than the 3057 control locus (Figure 1L and R). The modification levels normalized to histone occupancy demonstrated that acetylation patterns at ade6-3049 are similar to those at ade6-M26: among the six acetylated lysines tested, H3K9ac was the most enriched at the hotspot (Figure 1M, N and S and Supplementary Figure S2E–H). We also checked H3K4 methylation and found that H3K4me1 and H3K4me3 were higher at ade6-3057 than at ade6-3049, and that the H3K4me2 level between both loci was similar (Figure 1O, P, Q and T). This observation indicates that the 3049 hotspot did not show any degree of higher H3K4me compared with the negative control locus. The results on M26 and 3049 highlight two findings about histone modifications that could characterize M26-sequence-dependent hotspots. First, H3K9ac is more tightly associated with these two hotspots than all other lysine acetylations tested. Second, in contrast to observations in budding yeast and mammals, H3K4me3 levels are not elevated around these recombination hotspots.

Histone modifications around a natural M26-sequence-dependent hotspot and in meiotic diploid cells

We wanted to know whether the aforementioned findings apply to more physiological conditions. First, we focused on the natural M26-sequence-dependent hotspot cds1-M26hg-I3, which is located in an intron of the cds1+ gene and is activated by Atf1-Per1 (25). A point mutation (cds1-2) within the M26 sequence abolishes recombination activity (Figure 2A) (25). Using the pat1-114 meiosis system, we compared the levels of histone H3 (Figure 2B), H3K9ac, H3K14ac and three degrees of histone H3 modification patterns at the cds1-M26hg-I3 hotspot and cds1-2. As shown in Figure 2C, H3K9 was more acetylated at the hotspot than at the control locus (P = 0.017), whereas the levels of H3K14ac were similar. Moreover, H3K4me3 levels at all degrees were lower around the hotspot than around the cds1-2 control locus (Figure 2D) (P = 0.0051 for me1; P = 0.017 for me2; P = 0.0060 for me3). Therefore, a natural M26-sequence-dependent hotspot showed similar H3K9ac and H3K4me patterns to those around ade6 hotspots.

In pat1-114 cells, meiosis is synchronously induced by activation of Mei2, a master regulator of meiosis entry, through heat inactivation of the meiosis-inhibiting Pat1 kinase (30). Although this system is widely used to analyse meiotic recombination in S. pombe, it might be possible that histone modification patterns are affected by the artificial meiosis. To exclude this possibility, we performed similar experiments in h+/h− diploid cells induced into meiosis by nitrogen withdrawal and harvested 3 h after the induction. Histone H3 and its modification levels were assessed by ChIP as in Figure 1H–J. (A) Positions of cds1-M26hg-I3 and cds1-2 within the cds1 gene and sequences around the two loci. The rectangle and white lettering indicate the M26-sequence at I3. (B) Histograms of histone occupancy demonstrated that acetylation patterns at ade6-3049 are similar to those at ade6-M26: among the six acetylated lysines tested, H3K9ac was the most enriched at the hotspot (Figure 1M, N and S and Supplementary Figure S2E–H). We also checked H3K4 methylation and found that H3K4me1 and H3K4me3 were higher at ade6-3057 than at ade6-3049, and that the H3K4me2 level between both loci was similar (Figure 1O, P, Q and T). This observation indicates that the 3049 hotspot did not show any degree of higher H3K4me compared with the negative control locus. The results on M26 and 3049 highlight two findings about histone modifications that could characterize M26-sequence-dependent hotspots. First, H3K9ac is more tightly associated with these two hotspots than all other lysine acetylations tested. Second, in contrast to observations in budding yeast and mammals, H3K4me3 levels are not elevated around these recombination hotspots.
lower H3K4me3 were associated with M26 rather than M375 (Figure 2E–G) \((P = 0.0051\) for K9ac; \(P = 0.0088\) for K4me3). Therefore, pat1-114 haploid meiosis and diploid meiosis gave similar results, supporting the relevance of the histone modification patterns obtained with the pat1-114 system. Collectively, we conclude that high H3K9 acetylation, but not H3K4 trimethylation, is a characteristic of M26-sequence-dependent hotspots.

Interestingly, these patterns were already established at ade6-M26 before entering meiosis (Supplementary Figure S6), similarly to what was previously observed at hotspot-associated H3K4me3 in budding yeast (12). Along with recent reports that nucleosome-free regions around hotspots are already seen in growing budding and fission yeast cells (5,6,13), hotspot-associated chromatin traits in these yeasts are not consequences caused by meiotic recombination, but rather, may be determined by factors present under pre-meiotic growth conditions.

**Histone modification patterns observed around M26-sequence-dependent hotspots are distinct from those at an M26-sequence-dependent transcriptional promoter of ctt1**

The M26-sequence and Atf1-Per1 also regulate the transcription of various genes, such as those involved in stress responses (31). Transcription is initiated also from ade6-M26 and ade6-3049, but not from their corresponding sites in wild-type and negative control cells (32) (S. Y. K. O. and T. Y. unpublished observation), although its biological significance is unknown. These facts raise a possibility that the ade6-M26 and ade6-3049 hotspots can function as transcriptional promoters, and the histone modifications observed at M26-sequence-dependent hotspots may be shared with transcription, and not specific to recombination. To test this postulation, we focused on M26-dependent transcription that is not accompanied by recombination. The ctt1 promoter is a good model for the following reasons. (i) It is induced by a high concentration of \(\text{H}_2\text{O}_2\) in an Atf1-dependent manner (33). (ii) We found that mutations in the M26-sequence of its promoter (hereafter referred to as ctt1-mut) severely impaired Atf1 binding to the sequence and \(\text{H}_2\text{O}_2\)-induced transcription of ctt1 (Figure 3A and Supplementary Figure S7A and B). (iii) The nearest Rec12 (the fission yeast homologue of Spol1)-DNA linkage site (29) and Rec12 binding site (Supplementary Figure S7C, see later in the text for the difference of Rec12-linkage sites and Rec12 binding sites) are both \(~4\) kb away from the ctt1 M26-sequence, indicating that this site is not close to meiotic recombination hotspots. We therefore activated transcription of ctt1 by treating vegetatively growing cells, in which meiotic recombination proteins such as Rec12 are not expressed, with \(1\) mM \(\text{H}_2\text{O}_2\) for an hour. Histone modification levels were measured at its promoter (Pctt1) and at the mutated promoter (Pctt1-mut).

Under non-stressed conditions, we did not observe a substantial difference in histone H3 and its modification levels between Pctt1 and Pctt1-mut, although acetylation levels at K9 and K14 were slightly higher at Pctt1-mut (Supplementary Figure S7D–F). After \(\text{H}_2\text{O}_2\) treatment, however, histone levels plummeted at Pctt1 but stayed constant at Pctt1-mut (Figure 3B and Supplementary Figure S7D). Remarkably, in \(\text{H}_2\text{O}_2\)-treated cells, H3K9ac \((P = 0.00031)\), H3K14ac \((P = 0.0028)\) and H3K4me3 \((P = 0.028)\) were all higher at Pctt1 than at Pctt1-mut (Figure 3C and D). These patterns are different from those observed at M26-sequence-dependent hotspots, as hotspots are not enriched with H3K4me3 compared with their respective controls. In summary, we infer that the modifications we observed at M26-sequence-dependent hotspots may not be caused solely by transcription from M26-sites but may be related to recombination.

**Genome-wide analyses of histone modifications around meiotic recombination hotspots**

Although the results described so far demonstrate a good correlation between histone modifications and recombination at M26-sequence-dependent hotspots, nearly 90% of fission yeast hotspots do not carry M26-sequence(s) around them (29). We therefore tested whether the modification patterns observed at M26-sequence-dependent hotspots are the general case by mapping histone H3, H3K9ac, H3K14ac, H3K4me3 and Rec12 across all three chromosomes of *S. pombe*.

To analyse the distribution of histone H3 and modified histones, *pat1*-114 cells were harvested 1h after meiosis...
induction and were subjected to ChIP-chip experiments. In general, H3K9ac, H3K14ac and H3K4me3 localized at many sites where histone H3 level was reduced (Figure 4A–C). This finding is consistent with various prior observations that all these modifications are often found in open chromatin domains (2). In these domains, histone H3, present at lower level than the genome average and/or surrounding nucleosome-depleted regions, would be marked with the three modifications.

To map meiotic recombination hotspots, pat1-114 haploid cells expressing Rec12 whose C-terminus is tagged with FLAG (Rec12-FLAG) were harvested 5 h after meiosis induction and processed for ChIP-chip analyses using anti-FLAG antibodies. This experiment was performed in the rad50S background, in which Rec12-induced DSBs accumulate so that we could avoid losing chromosome-bound Rec12 signals owing to prompt repair. We note that Rec12-FLAG is fully functional, as losing chromosome-bound Rec12 signals owing to prompt Rec12-induced DSBs accumulate so that we could avoid losing chromosome-bound Rec12 signals owing to prompt repair. We note that Rec12-FLAG is fully functional, as

The immunoprecipitation ratio between modified histones and H3cter, usually a good indicator for histone modification levels, might not be relevant to evaluate modifications at hotspots, as hotspots are

Figure 4. Genome-wide analysis of histone modifications around meiotic recombination hotspots. The pat1-114 cells were induced into meiosis and harvested as in Figure 1. ChIP was performed using anti-histone H3, H3K9ac, H3K14ac and H3K4me3 antibodies, and the resultant DNA was analysed by GeneChip S. pombe Genome-wide analysis of histone modifications around meiotic recombination hotspots. The pat1-114 cells were induced into meiosis and harvested 5 h after the induction. ChIP was performed using anti-FLAG antibody, and the resultant DNA was subsequently used for ChIP-chip analysis. The pat1-114 rad50S rec12+-FLAG cells were induced into meiosis and harvested 5 h after the induction. ChIP was performed using anti-FLAG antibody, and the resultant DNA was similarly analysed. (A–C) Examples of ChIP-chip data. The x-axis shows the chromosomal coordinates in bp, and the y-axis shows the log2 of signal strength. The vertical dotted lines indicate Rec12 binding (i.e. DSB) sites. Genes are shown as filled boxes at the bottom of the figure. Comparison between Rec12 binding sites (Rec12) and histone H3 (H3cter; A), acetylated histone H3 (H3K9ac and H3K14ac; B) or trimethylated histone H3K4 (H3K4me3; C). Representative results are shown. Note that the levels of modified histones (B and C) are normalized to those of histone H3. (D) Venn diagrams showing the overlap between Rec12 binding (DSB) sites and modified histones calculated as described in ‘Materials and Methods’ section. (E–K) Distribution of histone H3 (E), H3K9ac (F and I), H3K14ac (G and J) and H3K4me3 (H and K) around meiotic recombination hotspots. The charts were created by a moving average method with a window size of 1 kb and a step size of 0.1 kb. The y axis shows the log2 of signal strength. The lines indicate the average of all hotspots. (F–H) Histone modification levels normalized to whole-cell extract (WCE) and (I–K) Histone modification levels normalized to whole-cell extract were presented. (I–K) Histone modification levels normalized to whole-cell extract were presented.
generally associated with less nucleosomes. To consider this point, we normalized signals of modified histone immunoprecipitates to those of whole-cell extracts. These analyses revealed that H3K9ac (Figure 4I), but not H3K14ac (Figure 4J), is enriched in the centre of hotspots, and that the amount of H3K4me3 (Figure 4K) is reduced around hotspots.

These results provide important insights into fission yeast hotspot-associated modifications in general. (i) H3K9ac is associated with hotspots. (ii) H3K4me3 does not necessarily mark meiotic recombination hotspots. Therefore, histone modification patterns found in M26-sequence-dependent hotspots are not limited to M26-sequences; rather, they are generally associated with fission yeast hotspots.

Effects of mutation in H3K9 and deletion of set1 on meiosis

Genome-wide histone modification analyses led us to assess the contribution of H3K9ac to meiotic recombination. We also analysed possible roles of H3K4me3, despite its absence at fission yeast hotspots, given its high occurrence at hotspots in mice and budding yeast. To this end, cells lacking either one of these modifications were created. A strain without H3K9ac was constructed by mutating all three histone H3 genes to change H3K9 to alanine (H3K9A) because multiple enzymes can acetylate H3K9. An H3K4me3-deficient strain was obtained by deleting the set1+ gene, which codes for the sole H3K4 methyltransferase (35). These strains were first tested for meiotic phenotypes.

Both H3K9A and set1Δ haploid cells in pat1-114 h+ background arrested at G1 in response to nitrogen starvation, entered meiosis, and completed ensuing DNA replication without any abnormality (Supplementary Figure S9A), suggesting that neither H3K9ac nor H3K4me3 is required for G1 arrest followed by meiosis entry and pre-meiotic DNA synthesis. This result indicates that, in contrast to budding yeast, Set1 is dispensable for normal pre-meiotic DNA replication in fission yeast (14). Spore viability was also tested, and we found that it was reduced to about two-thirds of the wild-type level by H3K9A mutation (P = 0.000012), while it was largely unaffected by the set1 deletion (Supplementary Figure S9B).

Meiotic recombination frequency was next analysed in the H3K9A and set1Δ mutants. We found that the H3K9A mutation, but not set1 deletion, increases the frequency of conversion from h+ cells to M cells (data not shown). This phenotype might be because the mutation disrupted heterochromatin structure at the mating-type locus. As such a defect hampers the analysis of recombination frequency, we crossed, instead of h+ cells, P cells whose entire mat2/3 interval is removed (mat1-P mat2.3::LEU2) (36) to h− cells. Gene conversion frequencies at ade6-M26 were first tested by checking adenine prototrophy of spores derived from ade6-M26 and ade6-M210 parental strains. The analyses revealed that the H3K9A mutation reduced recombination frequency to less than half of wild-type cells (Supplementary Figure S9C). This result, along with the finding that H3K9ac is enriched at ade6-M26 (Figure 1C and J), suggests that this modification would positively regulate recombination. Intriguingly, deletion of set1 gene also decreased gene conversion at M26 to a similar extent with H3K9A. As the H3K4me3 level is not elevated at M26 than its control locus (Figure 1G and J), Set1 would facilitate recombination independently of H3K4me3. Next, the genetic distance between ade6-arg1 (~299 kb region containing seven Rec12 binding sites, Supplementary Figure S12) was measured by crossing ade6-M210 and arg1-230 in wild-type, H3K9A and set1A backgrounds. We observed a partial, but significant, reduction in recombination activity in the H3K9A mutants (P = 0.0018) and the set1Δ mutants (P = 0.020) (Supplementary Figure S9D). The effects of H3K9A were stronger than those of set1Δ. These results show that both H3K9ac and Set1 are involved in meiotic recombination.

Effects of mutation in H3K9 and deletion of set1 on Rec12 binding to chromatin

The aforementioned results, showing that H3K9A mutation and set1Δ deletion reduced recombination at several sites, prompted us to analyse the effects of the mutations more thoroughly. To this end, we focused on early recombination events such as the loading of Rec12 to hotspots and meiotic DSB formation.

Distribution of Rec12 binding sites was tested by ChIP-chip analyses, as in Figure 4. Figure 5A compares the Rec12 binding maps of wild-type, H3K9A and set1Δ cells. Remarkably, the H3K9A mutation mildly reduced Rec12 signals at most of the hotspots without changing overall hotspot distribution patterns (Figure 5B). The average magnitude of this reduction was ~20% (Figure 5D). Although the impact of the H3K9A mutation was relatively modest, they were significant because two independent cultures of wild-type cells showed only a slight difference in hotspot-associated Rec12 levels (Supplementary Figure S10A–C). To our surprise, set1Δ deletion generally increased Rec12 binding to hotspots (Figure 5C), and analyses of total hotspots revealed that Rec12 ChIP signals were 20% higher in the set1Δ mutant than in wild-type cells (Figure 5D).

To complement the genome-wide analyses, ChIP-qPCR at several loci (Figure 5E–I) were performed. At hsp10 and moc3 hotspots (Figure 5E and F), Rec12 levels were significantly reduced in the H3K9A mutant (P = 0.0098 at hsp10; P = 0.027 at moc3), confirming that H3K9ac positively regulate Rec12 localization at these hotspots. At mbs1 locus (Figure 5G), we did not see an apparent change in Rec12 levels in the H3K9A mutants. However, we speculate that several acetylated-lysines including H3K9ac might redundantly function at this site, as lack of Gcn5, which acetylates multiple lysines on histones, decreased Rec12 levels (Supplementary Figure S10D). We also ascertained that set1Δ deletion increases Rec12 binding at some sites, which are not categorized as hotspots in wild-type cells such as vht1 (Figure 5H) and mug160 (Figure 5I). It could be possible that Rec12 protein levels differ between wild-type and the mutant cells and affect interpretation of ChIP-chip results. However, we observed that comparable
amounts of Rec12 were expressed and immunoprecipitated from wild-type, H3K9A and set1Δ cells (Supplementary Figure S10F). Collectively, our analyses suggest that H3K9ac play some roles to facilitate Rec12 binding to hotspots and could have the potential to directly activate recombination. Set1 seems to restrict the access of Rec12 to a majority of hotspots, despite the fact that H3K4me3 is not enriched at hotspots.

**Effects of mutation in H3K9 and deletion of set1 on DSB formation**

Finally, we set out to test the roles of H3K9ac and Set1 in meiotic DSB formation. Genomic DNA was extracted during meiotic progression and analysed by pulsed-field gel electrophoresis. For a rough estimation, DSBs formed on chromosomes were visualized by ethidium bromide staining (Figure 6A). In wild-type cells, smears reflecting Rec12-induced DSBs were observed ~3.5 h after meiosis induction. They intensified as meiosis proceeded, whereas signals of unbroken three chromosomes concomitantly disappeared. Intriguingly, the H3K9A mutation and set1Δ deletion partially reduced DSB formation, as evidenced by the presence of intact chromosomes at 4.5 and 5 h (Figure 6A, 4.5 and 5 h in H3K9A and set1Δ). We also noticed that DSB signals at 3.5 h in the H3K9A mutant is reproducibly weaker than in wild-type cells (Figure 6A, 3.5 h; arrowheads), implying that DSB formation may be delayed by the H3K9A mutation. Consistent with the result in Figure 6A, Rec12-oligonucleotide complexes, another measure of the relative frequency of meiotic DSB (37), were less abundant in the H3K9A and the set1Δ mutants than in wild-type cells (Figure 6B, Supplementary Figure S11A and B), whereas Rec12 proteins were equally expressed and immunoprecipitated in wild-type and mutant cells. These results further indicate that H3K9ac and Set1 can facilitate DSB formation. It is also noteworthy that the complexes were consistently less detected in the set1Δ cells than the H3K9A cells at later stages, suggesting that Set1 might also function in other processes than DSB formation, such as releasing Rec12-oligonucleotides.

To quantitatively evaluate the DSB levels in the mutants, DSBs formed around several hotspots were analysed at a higher resolution using genomic DNA purified as in Figure 6A. The DSB signals at mbs1 and mbs2 were weaker in H3K9A mutants than in wild-type cells, and the defect was more obvious at the onset of DSB formation (Figure 6C and D). This result, together with our ChIP-chip data showing enrichment of H3K9ac at mbs1 and mbs2 (Supplementary Figure S8C), suggests that H3K9ac may be directly involved in DSB formation at hotspots. At the cds1Δ hotspot, DSB levels were reduced in the H3K9A mutant to <50% of wild-type cells (Figure 6E and F). Similarly, DSB formation was substantially impaired at the hsp10 (Supplementary Figure S11C and D) and the ade6-M26 (data not shown) hotspots. Like mbs1 and mbs2, H3K9ac was associated with these hotspots (Figures 1C and I, 2C and Supplementary Figure S8C) and may at least partly facilitate DSB formation. In addition, these experiments also suggest that factors other than hotspot-associated H3K9ac are involved in DSB formation, as the effects of the H3K9A mutation were partial in many cases.

The set1Δ cells showed more intricate phenotypes. DSB levels in this mutant were comparable with wild-type cells at mbs2 and at one site of cds1, but lower at mbs1, the other site of cds1, and hsp10 (Figure 6C–F and Supplementary Figure S11C and D), indicating that the effects of set1Δ deletion may vary among hotspot loci. Remarkably, as H3K4me3 was not elevated around mbs1, hsp10 (Supplementary Figure S8C) and cds1 (Figure 2D), Set1 would contribute to DSB formation at these loci independently of H3K4me3. Taken together, we infer
that H3K9ac at hotspots may play some roles in DSB formation, and that Set1 may also enhance the process in some loci by a mechanism distinct from H3K9ac.

**Discussion**

Recent studies showed that meiotic recombination hotspots are enriched with various histone modifications (12,16,20). However, hotspot-associated modifications are not fully identified yet, and their roles in recombination activation remain obscure. Here, using fission yeast, we studied hotspot-associated modifications and examined their possible roles in meiotic recombination.

**Hotspot-associated histone modifications in fission yeast and other species**

One of the most important findings in this study is that H3K9ac is a major hotspot-associated modification in fission yeast. Although this mark has not been extensively studied in other organisms, it is conceivable that H3K9ac may be universally associated with hotspots in a broad range of species. In this regard, hotspots in budding yeast are generally enriched with H3K9ac (13), and H3K9 is also highly acetylated at the mouse hotspot Psmb9 (15).

In contrast, H3K4me3 was not elevated around fission yeast hotspots. This differs from budding yeast and mice, in which a vast majority of hotspots has H3K4me3. Although the reason for this difference is currently unknown, the relation between hotspots and H3K4me3 may vary among species. For instance, between budding yeast and mouse, there is a striking difference in the distribution of hotspot-associated H3K4me3: very often found around transcription start sites in the former, but virtually not in the latter (16). Moreover, several higher eukaryotes such as plants and dogs do not contain a...
functional Prdm9 gene in their genomes (38). These observations point to the importance of testing the presence of H3K4me at hotspots in various organisms.

Other modifications are also likely to be involved in meiotic recombination. Indeed, H3K9 methylation and histone H2A lysine5 acetylation levels are related to meiotic DSB formation in Caenorhabditis elegans (39,40). Comprehensive investigation is important to thoroughly understand the association of modified histones with meiotic recombination hotspots.

Mechanism of histone modifications around hotspots in fission yeast and other species

M26-sequence-dependent hotspots were more tightly associated with H3K9ac than with all other acetylated lysines tested and were not enriched with any degrees of H3K4me compared with their control sites. Such two characteristics may be established by a mechanism involving M26-sequence and Atf1-Pcr1, as modification levels were largely not affected by their relative location within the ORF (Figure 1I, J, S and T compare M26 and 3049). Most likely, as we proposed previously, Atf1-Pcr1 binding to the hotspot would recruit histone acetyltransferases to acetylate H3K9 (20). There may be other proteins that contribute to histone modifications at recombination hotspots, similarly to Atf1-Pcr1. For instance, two sequence-specific DNA-binding factors, CCAT-binding factor and Rst2, were recently found to regulate the activity of so-called ‘CCAAT’ hotspots and ‘oligo-C’ hotspots, respectively (41).

Other organisms may also exploit sequence-specific DNA-binding factors to modify histones around hotspots. Seventy-three percent of mice hotspots (16) and ~40% of human hotspots (17) possess a binding sequence for the zinc finger domain of the histone methylase Prdm9, a strong candidate for introducing sequence for the zinc finger domain of the histone H3K4me3 (42). In Saccharomyces cerevisiae, earlier works pointed out that sequence-specific DNA-binding transcription factors are involved in meiotic recombination (43). Therefore, as discussed by Wahls’ laboratory, it may be a general feature among species that specific sequences and their cognate-binding factors regulate recombination through chromatin structure (44,45).

In fission yeast, hotspots associated with protein binding are thought to constitute only a fraction of the total hotspots (25,41), and, for most of the remaining hotspots, no motif sequences have been found so far. Nevertheless, fission yeast hotspots are generally enriched with H3K9ac rather than with H3K14ac and H3K4me3 (Figure 4). These observations may imply a possibility that modification patterns can be established in a sequence-independent manner. In this case, as histone acetylases and deacetylases control not only targeted acetylation at specific sites but also global acetylation of the whole genome (46), this kind of ‘global’ system may contribute to modifications observed at recombination hotspots. Alternatively, as Borde et al. (12) proposed for H3K4me3 at budding yeast hotspots, the low-level transcription around hotspots may facilitate acetylation of H3K9. Such notion is consistent with the presence of non-coding RNA emanating from significant populations of meiotic recombination hotspots (47). This ‘sequence-independent’ mechanism may be important for other organisms as well, as the binding of transcription factors, in S. cerevisiae, per se is not sufficient to predict DSB hotspots and other factor(s) are in all likelihood involved (5).

Roles of histones and their modifications in meiotic recombination

This study revealed several chromatin-related factors that are associated with hotspots and/or facilitate meiotic recombination. In this section, three of them are discussed in terms of their possible roles in recombination.

Histone H3 levels were generally low around hotspots. This finding is consistent with previous studies (6,11,48) and supports a notion that chromatin structure is less condensed at hotspots to allow the access of recombination factors. However, our present study provides additional insights into this concept. For example, the hotspot-surrounding nucleosome-less region spreads over a ~3-kb region (Figure 4E). This may reflect the fact that meiotic DSBs are clustered in a wide region often spanning 1–2 kb (6,49). Another finding is that the reduction of nucleosome occupancy is relatively modest (~25% lower than the genome average) (Figure 4E), which is at a similar level to budding yeast hotspots (5,13). This observation may imply that recombination activation requires only mild decrease of nucleosome levels. However, because DSB is formed only in a small fraction of cells in a population, we must also consider the possibility that reduction of hotspot-surrounding nucleosomes might be underestimated owing to the majority of cells, which do not undergo DSB formation. Moreover, previous studies demonstrated that MNase sensitivities around hotspots increase as meiosis proceeds. Therefore, at hotspots where DNA is actually cut, nucleosome depletion might be more prominent than we observed in this study. Further exploration is necessary to understand nucleosome positioning at hotspots.

Mutating H3K9 to alanine reduced Rec12 ChIP signals at most hotspots and impaired DSB formation, albeit its effects were not very strong. We infer that H3K9ac may at least partly stabilize and/or facilitate the association of Rec12 with hotspots to induce DSBs. In addition, considering that the H3K9A mutation reduced DSB at mbs1 in which it did not affect Rec12 levels (Figures 5G and 6C and D), H3K9ac may also target DSB-inducing proteins other than Rec12. As H3K9ac binds the bromodomain (50), there are several possible mechanisms for how this modification exerts such effects. For example, chromatin-modifying proteins, many of which possess the domain, may interact with H3K9ac and create open chromatin regions so that Rec12 can stably interact with hotspots. In this scenario, low level of H3K4me3 at hotspots (Figures 1G, J, Q, T, 2D, G and 4K) may be important for chromatin modifiers to discriminate between hotspots and transcriptional promoters, as the latter is usually associated with both acetylated histones.
and H3K4me3. Revealing the H3K9ac interacting factors would be a key step to understand the roles of the modification in meiotic recombination.

Given the high association of H3K9ac with hotspots, the modest phenotype of the H3K9A mutant is puzzling. These observations, however, may indicate that several redundant factors and/or back-up pathways are involved in meiotic recombination. In this respect, nucleosome level is decreased around hotspots (Figure 4E) (6). Moreover, lack of Gcn5, an acetyltransferase targeting multiple lysines on histones, causes apparent reduction of Rec12 levels at mbs1 (Supplementary Figure S10D). Therefore, additional factors including reduced nucleosome levels or other modifications might compensate for the loss of H3K9ac in fisson yeast. Identifying factors that function in parallel with H3K9ac will be crucial to understand fission yeast meiotic recombination. Such situation may be similar to the cases in Prdm9/C0 fission yeast meiotic recombination. Such situation may be crucial to understand H3K9ac in fission yeast. Identifying factors that function in parallel with H3K9ac will be crucial to understand fission yeast meiotic recombination. Such situation may be similar to the cases in Prdm9/C0 fission yeast meiotic recombination. Such situation may be crucial to understand H3K9ac in fission yeast.

These observations imply that multiple chromatin-related factors are involved in the regulation of meiotic recombination in various ways. At the same time, this study also sheds light on the importance of factors other than H3K9ac or precise roles of Set1. Not only further functional analyses of H3K9ac and Set1 but also identifying other factors will be important to understand meiotic recombination in other species.

The major findings of this study are that (i) H3K9ac is associated with fission yeast hotspots and (ii) the H3K9A mutation and set1 deletion mildly, but significantly, affected Rec12-chromatin binding and DSB formation. These observations suggest that multiple chromatin-related factors are involved in the regulation of meiotic recombination in various ways. Additionally, functional analyses of H3K9ac and Set1 but also identifying other factors will be important to understand the in vivo mechanism of meiotic recombination.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–12, Supplementary Materials and Methods and Supplementary References [54–56].

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