Correlation of Meiotic DSB Formation and Transcription Initiation Around Fission Yeast Recombination Hotspots

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ABSTRACT Meiotic homologous recombination, a critical event for ensuring faithful chromosome segregation and creating genetic diversity, is initiated by programmed DNA double-strand breaks (DSBs) formed at recombination hotspots. Meiotic DSB formation is likely to be influenced by other DNA-templated processes including transcription, but how DSB formation and transcription interact with each other has not been understood well. In this study, we used fission yeast to investigate a possible interplay of these two events. A group of hotspots in fission yeast are associated with sequences similar to the cyclic AMP response element and activated by the ATF/CREB family transcription factor dimer Atf1-Pcr1. We first focused on one of those hotspots, *ade6-3049*, and Atf1. Our results showed that multiple transcripts, shorter than the *ade6* full-length messenger RNA, emanate from a region surrounding the *ade6-3049* hotspot. Interestingly, we found that the previously known recombination-activation region of Atf1 is also a transactivation domain, whose deletion affected DSB formation and short transcript production at *ade6-3049*. These results point to a possibility that the two events may be related to each other at *ade6-3049*. In fact, comparison of published maps of meiotic transcripts and hotspots suggested that hotspots are very often located close to meiotically transcribed regions. These observations therefore propose that meiotic DSB formation in fission yeast may be connected to transcription of surrounding regions.

KEYWORDS meiosis; meiotic recombination; chromatin; transcription; DNA double-strand break formation

MEIOTIC recombination ensures proper chromosome segregation during the first meiotic division and creates genetic diversity (Hunter 2015). It is initiated by programmed DNA double-strand breaks (DSBs), introduced predominantly at chromosomal regions called recombination hotspots (de Massy 2013). Since DSBs are potentially lethal DNA insults, the initiation process of meiotic recombination is regulated in many ways (Keeney *et al.* 2014). Factors that directly promote the reaction, such as the topoisomerase-like protein Spo11 and its partners, and the chromatin structure are central players of meiotic DSB regulation. In addition, DSB formation is known to be influenced by other chromosomal events such as DNA replication (Borde *et al.* 2000; Murakami and Keeney 2014; Wu and Nurse 2014) and transcription (de Castro *et al.* 2012; Sun *et al.* 2015).

Possible connection between meiotic recombination and transcription has been reported in many organisms. For example, early studies on budding and fission yeasts have demonstrated that transcription factors bind to hotspots and activate recombination (White *et al.* 1991; Kon *et al.* 1997). It is also shown that locations of hotspots coincide with those of promoters in various species, including budding yeast (Pan *et al.* 2011) and *Arabidopsis thaliana* (Choi *et al.* 2013). While these observations appear to indicate a functional interaction of the two events, the situation is much more enigmatic: recombination activation by hotspot-binding transcription factors in yeasts is not always accompanied by transcription activation, and is rather context dependent (Mieczkowski *et al.* 2006; Pan *et al.* 2011; Zhu and Keeney 2015). In species including fission yeast

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(see below) (Fowler *et al.* 2014) and mice (Brick *et al.* 2012), hotspots are located outside of promoters of protein-coding genes. Therefore, whether and how transcription influences initiation of meiotic recombination is still controversial, and analyzing their interplay may shine a new light on the mechanism of meiotic recombination.

Fission yeast *Schizosaccharomyces pombe* is an excellent model organism for studying meiotic recombination. Its DSB hotspots are preferentially localized in large intergenic regions, but unlike budding yeast they rarely coincide with transcription promoters (Fowler *et al.* 2014). Historically, recombination studies in this organism has been led by a hotspot called *ade6-M26*, which is created by a G/T point mutation within the *ade6* ORF, and is associated with a cyclic AMP response element (CRE)-related *M26*-sequence (ATGACGT; *M26* mutation is underlined.) (Ponticelli *et al.* 1988; Szankasi *et al.* 1988). This hotspot is intriguing because it is bound and activated by Atf1-Pcr1, a heterodimer of sequence-specific DNA-binding transcription factors (Kon *et al.* 1997). Both Atf1 and Pcr1 belong to the well-conserved activating transcription factor/CRE binding (ATF/CREB) transcription factor family, and are transcription regulators that bind to promoters of various stress-responsible genes through CRE sequences (Chen *et al.* 2003). Later analyses have shown that Atf1 and Pcr1 also activate other mutation-created *M26*-sequence-dependent hotspots located in the *ade6* ORF (Fox *et al.* 2000; Steiner and Smith 2005a). More importantly, Atf1, Pcr1, and the *M26* sequence are likely to activate meiotic recombination at authentic hotspots in the fission yeast genome (Steiner and Smith 2005a; Wahls and Davidson 2010; Fowler *et al.* 2014).

We have been interested in how meiotic DSB formation occurs at fission yeast hotspots, including *M26*-sequence-dependent hotspots. Our previous studies have proposed that Atf1-Pcr1 facilitates DSB formation at *ade6-M26* at least partly by modulating the surrounding chromatin structure (Mizuno *et al.* 1997; Yamada *et al.* 2004, 2013). Although recombination at this hotspot is not caused by increased transcription of *ade6* messenger RNA (mRNA) (Grimm *et al.* 1991; Kon *et al.* 1997), a later study found a short transcript specific to and emanating from around *ade6-M26* (Hirota *et al.* 2008). These previous reports led us to wonder whether Atf1-Pcr1 involves transcription to activate meiotic recombination. In this study, based on the analyses of another *M26*-sequence-dependent hotspot, *ade6-3049*, as well as the Atf1 protein, we discuss a possibility that Atf1 promotes DSB formation through inducing transcription from its binding site. Moreover, comparison of previously published genome-wide data sets unveiled proximity of hotspots and meiotically transcribed regions. These observations may suggest that, at least at a subset of fission yeast hotspots, transcription participates in regulation of meiotic recombination initiation.

Materials and Methods

Yeast strains

Yeast strains used in this study are listed in Supplemental Material, Table S1 in File S1. To construct an *atf1-HRA*Δ strain, *atf1* DNA fragment lacking the homologous recombination activation (HRA) region was transformed into an *atf1*Δ::*ura4*$^+$ cell.

Northern blotting and RACE analyses

h^+/h^- diploid cells were induced to enter meiosis by transferring them into nitrogen-free medium, and collected at indicated times after induction. Total RNAs were analyzed by Northern blotting with ^{32}P-labeled DNA probes. Signals were quantified using a Fuji BAS 2000 Bio-Imaging Analyzer (Fujifilm). RACE was performed with SMARTer RACE 5′/3′ Kit (Clontech Laboratories) according to the manufacturer's instructions.

Tiling array and high throughput sequencing data analysis

The meiotic transcript map and the map of oligonucleotides attached to Rec12 (Rec12-oligo) were previously described in de Castro *et al.* (2012) and Fowler *et al.* (2014), respectively. The fission yeast genome annotation (version of May 12, 2016) was downloaded from PomBase (http://www.pombase.org/). The data were processed using Perl (http://www.perl.org/) and R (http://www.r-project.org/).

Chromatin analyses

The chromatin structure around *ade6-3049/3057* was examined as previously described in Mizuno *et al.* (1997), except for the following modifications. Genomic DNA was treated with 0, 20, or 30 units of micrococcal nuclease (MNase) (Takara), digested with *Sac* I, and separated on a 1.2% agarose gel. The probe was amplified by PCR (see below), and labeled with ^{32}P using the Random Primer DNA Labeling Kit version 2 (Takara).

Other methods and information

Recombination frequency was measured by random-spore analyses. Chromatin immunoprecipitation (ChIP) and budding yeast one-hybrid analyses were performed as described in Yamada *et al.* (2013) and Morita *et al.* (2011), respectively. For ChIP of Rec12-DNA linkage, cross-linking was omitted. Antibodies used were anti-H3K9ac (Millipore, Bedford, MA), M2 anti-FLAG (Sigma Chemical, St. Louis, MO), and anti-Atf1 (Takemata *et al.* 2016). Meiotic DSB fragments were detected as previously described in Steiner *et al.* (2002). For all statistical analyses, the one-tailed Student's *t*-test was used. Primers used for PCR are listed in Table S2 in File S1, or previously described (Yamada *et al.* 2013).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Reagents are available upon request.

Results and Discussion

Short transcripts associated with the ade6-3049 recombination hotspot

We were intrigued by an ectopic transcript starting from around the *ade6-M26* hotspot (Hirota *et al.* 2008). Wondering whether

other *M26*-sequence-dependent hotspots are also associated with similar transcripts, another *M26*-sequence-dependent hotspot, *ade6-3049*, was tested. This hotspot, activated by Atf1-Pcr1, is located near the 3′ end of the *ade6* gene and its activity is stronger than that of *ade6-M26* (Figure 1A) (Steiner *et al.* 2002; Steiner and Smith 2005b). h^+/h^- diploid cells carrying the *ade6-3049* hotspot, along with those carrying the nonhotspot control allele *ade6-3057*, were induced to enter meiosis by nitrogen withdrawal, and their total RNA was analyzed by Northern blotting with a probe recognizing the entire *ade6* ORF. The results revealed dramatically different transcript patterns between *ade6-3049* and *ade6-3057* (Figure 1B), as explained in the next paragraph.

In the *ade6-3049* cells, multiple RNAs [at least five: full length (FL), and S1–S4] containing *ade6* sequence were detected both before and after meiosis induction. In stark contrast to *3049*, *ade6-3057* cells produced only one *ade6* RNA species (FL), which gradually declined through meiosis progression. Therefore, the *M26* sequence at *ade6-3049* was associated with unique transcripts. These results, together with a previous one on *ade6-M26*, prompted us to infer that spurious transcription initiation may be a common feature among at least a part of *M26*-sequence-dependent hotspots.

To gain further insights, the hotspot-associated transcripts around *ade6-3049* were thoroughly analyzed. Northern blot analyses using two probes hybridizing to either side (5′ or 3′) of *3049/3057* mutations suggested that the longest RNA present both in *3049* and *3057* cells would be the full-length *ade6* mRNA (Figure 1, C and D; transcript FL). Also, all shorter RNAs, specifically those detected in the *3049* cells, were hybridized by either upstream (Figure 1C; transcripts S1, S2, and S3) or downstream (transcript S4) probes (Figure 1D). Transcription start and termination sites were subsequently determined by RACE analyses. Remarkably, four shorter transcripts started from a 500-bp region containing the *3049* site, with three of them being transcribed to upstream of the *ade6* gene, and the other one, downstream (Figure 1E and Figure S1A). Moreover, ChIP using anti-Atf1 antibody confirmed that Atf1 is indeed localized around *ade6-3049*, but not *ade6-3057* (Figure S1B). These results may imply that Atf1, which binds to the *M26* sequence at *ade6-3049*, functions as a transcriptional activator to instigate transcription.

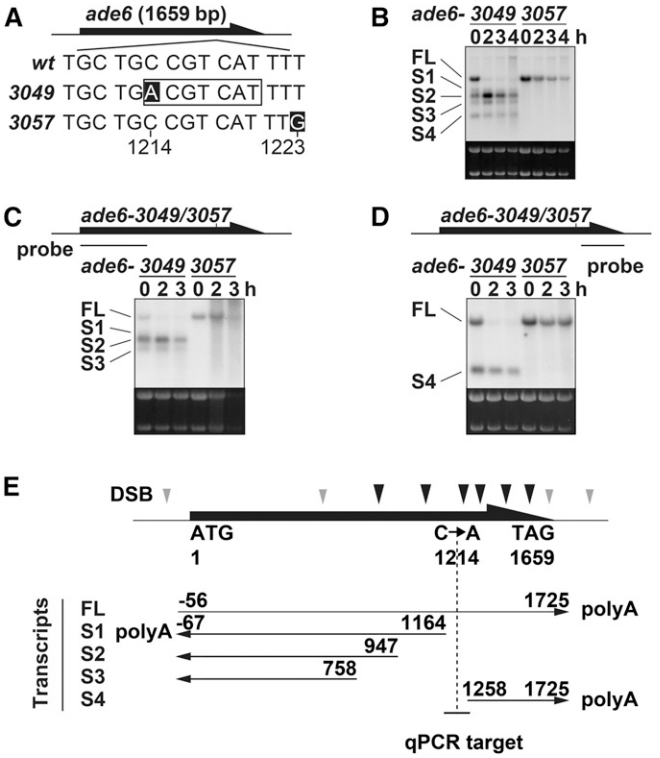

Figure 1 *ade6-3049* mutation induces ectopic transcripts at the *ade6-3049* meiotic recombination hotspot. (A) Positions of *ade6-3049* and *ade6-3057* mutations (white letters). The numbers below the sequences indicate the positions relative to the first A of the *ade6* ORF. (B) Transcripts around the *ade6-3049/3057*. Cells were induced to meiosis by nitrogen depletion and harvested at the indicated time after meiosis induction. Their total RNA was analyzed by Northern blotting using the probe recognizing the full-length *ade6* ORF. Ribosomal RNA stained by ethidium bromide is shown as a loading control. (C) Same analyses as (B), but using a probe recognizing 5′ end of the *ade6* ORF. (D) Same analyses as (B), but using a probe recognizing 3′ end of the *ade6* ORF. (E) Estimated 5′ and 3′ end of *ade6* mRNA based on Northern blotting and RACE analyses. Previously identified break sites are indicated by arrowheads, with stronger break sites by black ones (Steiner *et al.* 2002). The vertical dotted line and the horizontal short line indicate the position of the *3049* mutation and of the quantitative PCR (qPCR) fragment analyzed in Figure 2C, Figure 5B, and Figure S1, respectively. wt, wild type.

Transactivation domain of Atf1 is the HRA region

We then wished to understand how Atf1 is involved in generation of short transcripts at hotspots. In terms of the protein structure, a transcription factor can be often divided into functional domains such as a DNA-binding domain and a transactivation domain. This is also the case with Atf1, as its C-terminal bZIP domain serves as a DNA-binding/protein-dimerization domain (Takeda *et al.* 1995; Kanoh *et al.* 1996; Shiozaki and Russell 1996; Wilkinson *et al.* 1996). Gao *et al.* (2008) later used plasmid-borne, truncated-*atf1* genes to identify additional functional domains: the HRA region, which is necessary and sufficient for recombination activation at *ade6-M26*; the homologous recombination repression region, which represses recombination at the same hotspot; and the osmotic stress activation (OSA) region, which is essential for cell growth in a hyperosmotic environment (Figure 2A). It has not been known, however, whether Atf1 possesses a transactivation domain, and to address this point we performed budding yeast one-hybrid system experiments (Horie *et al.* 1998; Morita *et al.* 2011). In this assay, a protein of interest is fused to the DNA-binding domain of LexA (LexA BD) and expressed in the budding yeast L40 strain, carrying a *lacZ* reporter gene under control of a LexA operator. The fusion protein with transactivation activity can drive transcription of *lacZ* to produce β-galactosidase, an enzyme hydrolyzing *o*-Nitrophenyl-β-galactoside to galactose and *o*-nitrophenol. Colorimetric quantification of *o*-nitrophenol allows us to estimate transactivation activity of the protein fused

to LexA BD. As expected, full-length Atf1 fused to LexA BD, but not LexA BD alone, could activate transcription of *lacZ* (Figure 2B), which suggested that Atf1 is indeed a transcription activator.

Three Atf1 fragments depicted in Figure 2A were similarly analyzed. Among them, the HRA region *per se* was able to activate transcription of the reporter gene, while fragments lacking the HRA region were not. This finding shows that the transactivation domain of Atf1 coincides with the previously identified HRA region. In other words, Atf1 can activate meiotic recombination at *ade6-M26* and transcription through the same domain. In contrast to HRA, the OSA region did not confer transactivation activity to Atf1 fragments. This observation may mean that the OSA region functions independently of transcription activation or specifically in response to osmotic stress, or may be obtained because our assay just failed to detect its transcription-stimulating function. In any case, the HRA region is likely to be a key domain responsible for transactivation activity of Atf1.

Deletion of the HRA region decreases 3049-bound Atf1, but does not affect protein stability

To understand how the HRA region functions in fission yeast cells, we constructed a strain in which HRA-coding sequence was deleted from the native *atf1*$^+$ locus (*atf1-HRA*Δ), and compared *atf1-HRA*Δ cells to *atf1*$^+$ cells. Before analyzing recombination and transcription, binding to the *ade6-3049* hotspot and protein stability of Atf1 were tested. To achieve highly synchronous meiosis progression, *pat1-114* haploid cells of both *atf1* backgrounds were induced to enter meiosis by the temperature-shift method. ChIP using anti-Atf1 antibody was performed to compare Atf1 levels at the *ade6-3049* hotspot and at the control site *prp3*$^+$. As shown in Figure 2C, although both wild-type Atf1 and HRA-lacking Atf1 (Atf1-HRAΔ) were substantially enriched more at *3049* than at *prp3*, the enrichment of Atf1-HRAΔ was reduced to about a fourth to fifth of that of wild-type Atf1. However, by Western blotting using the same antibody, we observed that Atf1 protein levels in *atf1*$^+$ and *atf1-HRA*Δ cells were comparable to each other as far as we tested (Figure 2D).

The reduced binding of Atf1-HRAΔ to *ade6-3049* can be interpreted in several ways. First, HRA deletion may perturb the protein structure of Atf1 to interfere with binding to the *3049* hotspot. A second interpretation is related to a recent report on transcription activation of the *fbp1*$^+$ gene, whose promoter is bound by Atf1: binding of Atf1 to the *fbp1*$^+$ promoter is strengthened through downstream (*i.e.*, *fbp1*$^+$) transcription by Atf1 (Takemata *et al.* 2016). If this is the case with *ade6-3049*, Atf1 may self-enforce its *3049* binding by producing *3049*-associated short transcripts; while Atf1-HRAΔ may not generate the short transcripts well enough to enhance its *3049* binding. Third, there is a caveat that the antibody could not equally recognize Atf1 and Atf1-HRAΔ unless they are denatured by SDS and/or not cross-linked with formaldehyde. Therefore at this stage, we cannot determine what is attributable to decreased ChIP signals of Atf1-HRAΔ at *ade6-3049*. Nevertheless,

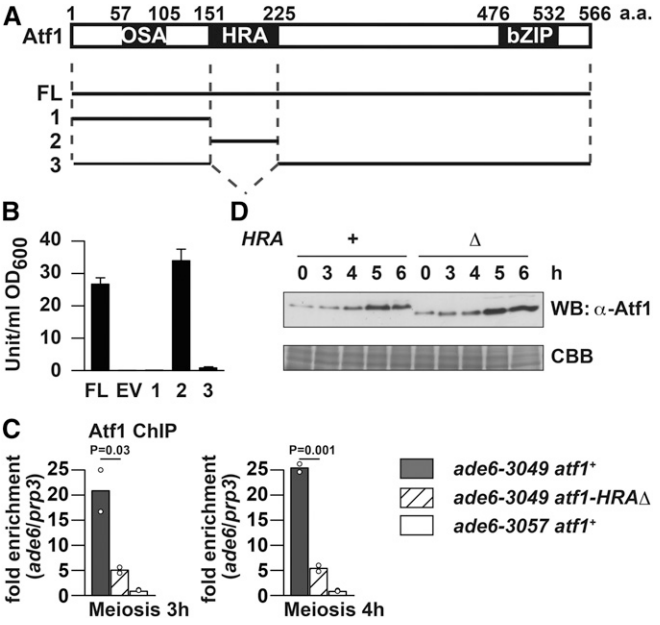

Figure 2 Transactivation domain of Atf1 is the previously identified HRA region, which is dispensable for Atf1 protein stability. (A) Previously known functional domains of Atf1 (Gao *et al.* 2008) and Atf1 fragments tested for transactivation activity. FL, full length (of Atf1). (B) Quantification of β-galactosidase activity detected in cells expressing LexA BD-Atf1 fusion protein. Results of three independent experiments with SDs are shown. EV, empty vector. (C) ChIP of Atf1 and Atf1-HRAΔ. Indicated cells of *pat1-114* background were induced to enter meiosis by temperature-shift method, and harvested at indicated hours after the induction. ChIP using anti-Atf1 antibody was performed, and DNA was isolated from immunoprecipitates as well as whole-cell extracts. Obtained DNA was analyzed by real-time qPCR, where fragments corresponding to *ade6-3049/3057* and the *prp3*$^+$ promoter were amplified. Relative enrichment at *ade6-3049/3057* over *prp3* is shown. Bar graphs are created based on mean values of two independent experiments (shown by ○). (D) Western blotting of Atf1 and Atf1-HRAΔ during meiosis. *pat1-114 atf1*$^+$ and *pat1-114 atf1-HRA*Δ cells were induced to enter meiosis by temperature-shift method, and harvested at indicated hours after the induction. Whole-cell extract was analyzed by Western blotting using anti-Atf1 antibody (WB: α-Atf1). An SDS-PAGE gel in which the same amount of whole-cell extract was fractionated was stained by Coomassie brilliant blue and is shown as a loading control (CBB).

as HRA deletion appeared not to compromise protein abundance of Atf1, we decided to explore its effects on recombination and transcription.

Deletion of the HRA region reduces meiotic DSB formation and recombination at ade6-3049

DSB formation at the *ade6-3049* hotspot was examined by monitoring two products: DSB fragments and Rec12 (the fission yeast homolog of Spo11) covalently attached to broken ends (Rec12-DNA linkage). For effective detection of these two molecules, which are transient and otherwise difficult to observe, analyses were performed on *pat1-114 rad50S* haploid cells, as the *rad50S* mutation prevents DSB repair and accumulates Rec12-attached DSB ends. Also, the *rec12*$^+$ locus was engineered to express C-terminally FLAG-tagged Rec12 in the analyzed cells, which are fully adept at DSB formation (Cromie *et al.* 2007; Yamada *et al.* 2013).

To observe DSB fragments at *ade6-3049*, genomic DNA; isolated from *atf1*$^+$ *ade6-3049*, *atf1*$^+$ *ade6-3057*, and *atf1-HRA*Δ *ade6-3049* cells; was digested with the restriction enzyme *Afl* II and analyzed by Southern blotting, as described previously (Figure 3A) (Steiner *et al.* 2002). The results showed that *3049*-associated DSB signals, which are at the background level around *ade6-3057*, are weaker in *atf1-HRA*Δ mutants than in *atf1*$^+$ cells. Although distribution of break sites was not dramatically altered, quantification of two independent experiments showed that DSB frequency at 5 hr is 6.7 and 4.2% in *atf1*$^+$ and *atf1-HRA*Δ (*P*-value = 0.01), respectively (Figure 3B). Therefore, DSB formation at *3049* was compromised by deletion of the HRA region.

The above observation was further verified by measuring Rec12 covalently attached to DSB ends. Cells 5 hr after meiosis induction were harvested without formaldehyde-cross-linking and analyzed by ChIP of Rec12-FLAG (Figure 3C). At *ade6-3049*, Rec12-DNA linkage was less precipitated in *atf1-HRA*Δ cells than in *atf1*$^+$ cells, which suggests that HRA deletion impaired DSB formation at this hotspot. In contrast, at the *sat1* hotspot (Miyoshi *et al.* 2012), which does not harbor an *M26* sequence, *atf1*$^+$ cells and *atf1-HRA*Δ cells produced similar amounts of Rec12-DNA linkage. Based on these results, we concluded that HRA would promote DSB formation at the *ade6-3049* hotspot. It should be pointed out that effects of HRA deletion were less overtly manifested by Southern blotting than by Rec12-DNA linkage ChIP. The reason for this difference is currently unknown, but we emphasize that a same conclusion was drawn from two distinct procedures.

Consistently, lack of HRA reduced recombination frequency between *ade6-3049* and *ade6-M375*, but not between *ade6-3057* and *ade6-M375* (Figure 3D). These experiments suggest that HRA is specifically involved in DSB formation as well as subsequent recombination at *ade6-3049*, and probably other *M26*-sequence-dependent hotspots. They were in agreement with a previous observation that expression of HRA-lacking Atf1 from a multi-copy plasmid does not fully restore meiotic recombination defects at *M26* in *atf1*Δ cells (Gao *et al.* 2008).

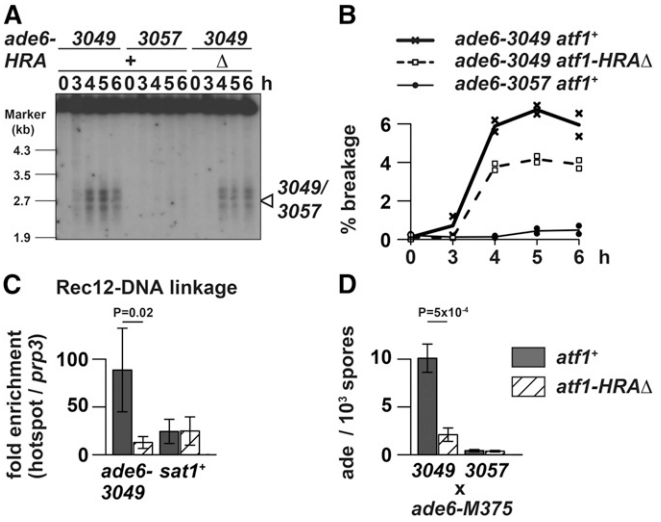

Figure 3 Deletion of the Atf1 HRA region affected meiotic recombination at *ade6-3049*. (A) Effects of HRA deletion on DSB formation at the *ade6-3049* hotspot. Indicated cells of the *pat1-114 rad50S* background were induced to enter meiosis by temperature-shift method, and harvested at the indicated hours after the induction. Genomic DNA was isolated, digested with *Afl* II, and analyzed by Southern blotting as previously described (Steiner *et al.* 2002). The position of the *3049* or *3057* mutations is marked by the arrowhead. (B) Quantitative analyses of DSB formation presented in (A). The DSB (%) values were obtained by dividing the signal intensity of broken DNA fragments over that of the unbroken parental fragment. Mean values of two independent experiments are plotted. (C) Effects of HRA deletion on Rec12-DNA linkage production at the *ade6-3049* hotspot. Rec12-FLAG-expressing *pat1-114 rad50S* strains with or without HRA were induced to enter meiosis by temperature-shift method, and harvested 5 hr after the induction. Rec12-FLAG-DNA linkage was chromatin immunoprecipitated by anti-FLAG antibody. DNA isolated from immunoprecipitates and whole-cell extracts was analyzed by real-time qPCR, where fragments corresponding to the hotspots (*ade6-3049* and *sat1*) and the *prp3*$^+$ promoter (*prp3*) were amplified. Relative enrichment at hotspots over *prp3* was calculated and mean values of three independent experiments as well as their SD are shown. (D) Effects of HRA deletion on meiotic gene conversion at the *ade6-3049* hotspot. Recombination frequency was measured between *ade6-M375* and either *ade6-3049* or *ade6-3057* by counting the number of ade$^+$ spores. Mean values of three independent experiments and their SD are shown.

Deletion of the HRA region reduces several short transcripts at ade6-3049

We next compared *3049*-associated short transcripts in *atf1*$^+$ and *atf1-HRA*Δ cells. Northern hybridization probing the entire *ade6* ORF demonstrated that the transcripts' pattern is disturbed in the *atf1-HRA*Δ mutant (Figure 4A). In particular, detailed quantification of blots showed that HRA deletion severely decreases the transcripts S2 and S3, both of which were antisense transcripts starting from around the *M26* sequence (Figure 4, B and C). These results would indicate that the lack of HRA not only impairs recombination, as was originally proposed, but also affects transcription of *3049*-associated short transcripts.

It is intriguing that small amounts of short transcripts were still present even in the absence of HRA. This may be because HRA-lacking Atf1 can, albeit at a reduced level, bind to a*de6-3049* and modify surrounding chromatin to produce short transcripts, as the Atf1 bZIP domain itself has been recently reported to influence nucleosome phasing around its binding site (Garcia *et al.* 2014).

ade6-3049, but not ade6-3057, is associated with open chromatin structure, which is at least partly mediated by HRA

The impact of HRA deletion on both transcription and recombination around *ade6-3049* may suggest that lack of HRA affects the local chromatin environment, since chromatin influences all DNA-templated events. To address this point, chromatin structure around *ade6-3049* was compared between *atf1*$^+$ and *atf1-HRA*Δ cells by a method using MNase. The crude chromatin fraction, isolated from meiotic *pat1-114* haploid cells, was treated with varying amounts of the nuclease, and genomic DNA was subsequently purified.

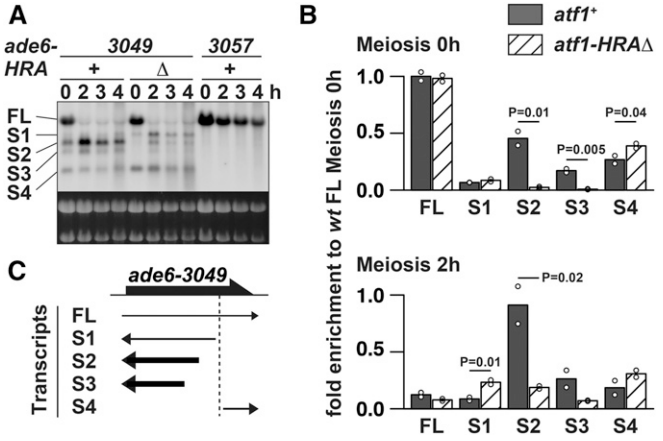

Figure 4 Deletion of the Atf1 HRA region affected transcription at *ade6-3049*. (A) Effects of *HRA* deletion on transcripts production around *ade6-3049*. Total RNA was analyzed by Northern blotting as explained in Figure 1B. (B) Quantitative analyses of *ade6* transcripts shown in (A). Abundance of transcripts, detected in *atf1*$^+$ (filled bars) and *atf1-HRA*Δ (hatched bars) cells at 0 h (top) and 2 hr (bottom) after meiosis induction, were estimated by quantifying signal intensities of each transcript and normalizing them to the mean value of full-length *ade6* mRNA present in *atf1*$^+$ cells at 0 hr. The numbers of transcripts are the same as those indicated in (A). Bar graphs are created based on mean values of two independent experiments (shown by ○). (C) Graphic summary of results shown in (A) and (B). RNAs strongly affected (more than twofold for both time points) by the *atf1-HRA*Δ mutation were shown as bold black arrows. The vertical dotted line indicates the position of the *3049* mutation.

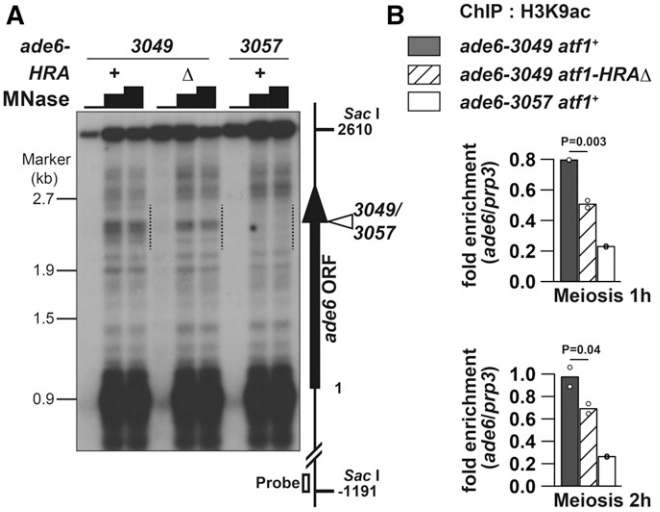

Figure 5 Chromatin structure around *ade6-3049* is more open than around *ade6-3057*, and HRA is involved in the chromatin regulation. (A) MNase sensitivity of chromatin around *ade6-3049/3057*. Indicated cells of the *pat1-114* background were induced to enter meiosis by temperature-shift method, and harvested 3 hr after the induction. MNase-digested DNA was prepared by MNase (0, 20, or 30 units) treatment of chromatin fraction and subsequent purification. DNA was further cut with *Sac* I, fractionated on 1.2% agaraose gel, and analyzed by Southern blotting using the probe indicated by the open box. The vertical arrow and the open arrowhead show the *ade6* ORF and the *3049/3057* site, respectively. The numbers on the left and the right side of the panel indicate the positions of λ/*Eco*T14 I fragments used for electrophoresis marker and the positions from the first A of the *ade6* ORF, respectively. The dotted lines indicate a region in which MNase-sensitive sites are clustered around *ade6-3049*. Presented is an example from two independent experiments, whose results were similar to each other. (B) HRA deletion reduced acetylation level of H3K9 around *ade6-3049*. Indicated cells of the *pat1-114* background were induced to enter meiosis by temperature-shift method, and harvested 1 or 2 hr after the induction. ChIP using anti-H3K9ac antibody was performed as described previously (Yamada *et al.* 2013). DNA isolated from immunoprecipitates and whole-cell extracts was analyzed by real-time qPCR, where fragments corresponding to *ade6-3049/3057* and the *prp3*$^+$ promoter were amplified. Relative enrichment at *ade6-3049/3057* over *prp3* is shown. Bar graphs are created based on mean values of two independent experiments (shown by ○).

The obtained DNA was digested with the restriction endonuclease *Sac* I and analyzed by Southern blotting using a probe hybridizing to one end of the *3049*-containing *Sac* I fragment (Figure 5A).

The analyses on *atf1*$^+$ cells revealed that an ~600-bp region (Figure 5A, dotted lines) surrounding *ade6-3049* contains multiple MNase-hypersensitive sites, while the corresponding region around *ade6-3057* does not; suggesting that chromatin is more accessible around *3049* than *3057*. This difference, similar to the case of the *ade6-M26* hotspot and its negative control locus *ade6-M375* (Mizuno *et al.* 1997), is consistent with the idea that hotspots are located in open chromatin to help DSB machinery function at hotspots.

Remarkably, deletion of the HRA region eliminated several but not all of the *3049*-surrounding MNase-hypersensitive sites (Figure 5A). Therefore, this domain is involved in formation of an accessible chromatin environment around *ade6-3049*, and by doing so, would promote transcription and recombination.

HRA deletion partially reduces hotspot-associated H3Lys9 acetylation

Open chromatin is associated with acetylated histones and we have previously reported that H3Lys9 acetylation (H3K9ac) is a major mark of hotspots in fission yeast (Yamada *et al.* 2013). We hence asked whether deletion of the HRA region may influence the high level of H3K9ac at *ade6-3049*. Since we have observed that histone-acetylation levels decline through meiosis progression (Yamada *et al.* 2004), cells 1 and 2 hr after meiosis induction were examined. As shown in Figure 5B, HRA deletion decreased this modification to an intermediate level between wild type (*atf1*$^+$ *ade6-3049*) and its negative control (*atf1*$^+$ *ade6-3057*) strains. This result indicates that HRA partly but significantly promotes H3K9ac at the *ade6-3049* hotspot, and the histone modification may be involved in transcription, recombination, or both at *3049*.

Possibility of functional connection between 3049-associated transcription and recombination

How are our present results integrated to describe what occurs around *ade6-3049* in meiosis? Several clues are available from this and previous studies. First, transcription preceded DSB formation; short transcripts were already present in premeiotic cells, and in particular, transcript S2 was induced 2 hr after meiosis induction, when proteins involved in DSB

formation are barely expressed (Mata *et al.* 2002). Second, in *atf1-HRA*Δ mutants, recombination frequency was reduced with concomitant diminishment of open chromatin hallmarks, namely MNase sensitivity and H3K9ac, around the hotspot. Third, at a CRE site in the promoter of *fbp1*$^{+}$, transcription activated by Atf1 increases H3K9ac to further stimulate downstream transcription (Takemata *et al.* 2016).

One of possible models deduced from the above three points is as follows. Atf1 that is bound to *3049* stimulates transcription (of short transcripts) to promote H3K9ac and locally decondense surrounding chromatin, and thereby would facilitate DSB formation near the transcription sites. This scenario may be related to a previous report that artificial activation of transcription creates a DSB hotspot by reducing nucleosome occupancy (de Castro *et al.* 2012), in that transcription activates DSB formation. Since other possibilities are also possible and we cannot exclude a possibility that correlation of the two events is merely a coincidence, further analyses are necessary.

Pervasive correlation between transcription and recombination initiation sites

Having shown that transcribed regions of short transcripts and meiotic DSB sites are closely located to each other at the *ade6-3049* hotspot, we wondered whether this possible connection could be extended to other hotspots. This notion appears to be incompatible with a known feature that promoters of protein-coding genes are not major sites for DSB hotspots in fission yeast. However, high-resolution mapping of Rec12-oligo, byproducts of DSB processing, proved that hotspots often encompass a large region with multiple Rec12-oligo sites scattered (Fowler *et al.* 2014). In addition, hotspots are directed to loci expressing noncoding RNAs (Wahls *et al.* 2008). These observations led us to consider that at least a part (or "edge") of hotspots may be adjoined to transcribed regions. We therefore compared meiotic transcriptome data at 3 hr after meiosis induction (de Castro *et al.* 2012), instead of gene positions, and a Rec12-oligo hotspot map (Fowler *et al.* 2014). As shown in Figure 6A, most hotspots are found in intergenic regions, as previously reported (compare rows "Rec12-oligo" and "gene") (Cromie *et al.* 2007; Fowler *et al.* 2014). Remarkably, regions associated with Rec12-oligo are in many cases located near those where at least one strand is transcribed (compare rows "Rec12-oligo" and "mRNA"), suggesting that a part of a hotspot is frequently juxtaposed to meiotically transcribed regions.

To gain more comprehensive insights, the same data sets were exploited to map meiotic transcripts around all hotspots, which were aligned across the *y*-axis according to the hotspot width (Figure 6B). The comparison showed that most hotspots are juxtaposed to transcribed regions (Figure 6B), reminiscent of the cases explained in Figure 1E (*ade6-3049*) and Figure 6A. Therefore, spatial proximity between transcription and DSB formation can be widely observed in fission yeast.

Finally, we also asked how chromatin structure is related to the above relationship by comparing a heat map of nucleosome depletion to those presented in Figure 6B (Figure S2). Consistent with previous reports that fission yeast hotspots,

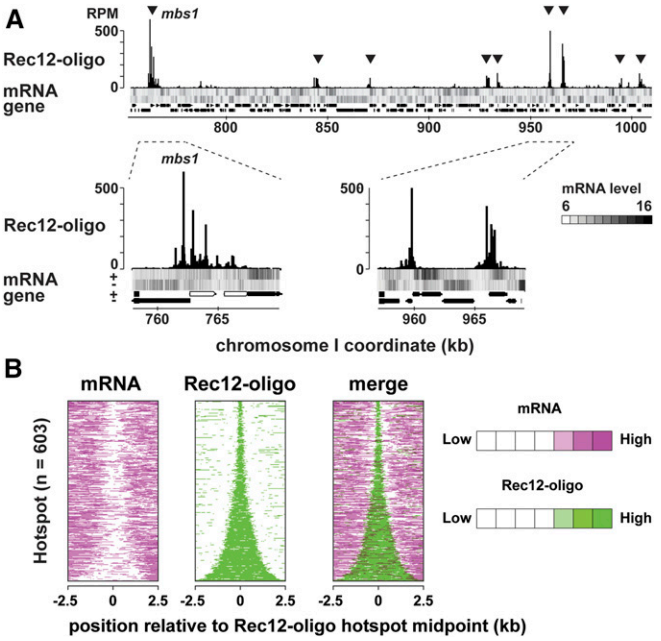

Figure 6 Correlation between transcription and recombination initiation sites around fission yeast hotspots. (A) An example of uniquely mapped Rec12-oligo and complementary DNA of meiotic transcripts hybridized to genome tiling array. The *x*-axis shows the chromosomal coordinates in base pairs, and the *y*-axis shows reads per million base pairs (RPM). The heat map shows the $\log_2$ signal strength of forward and reverse transcripts. Black and white represent up- or downregulation of gene expression. Protein-coding genes and RNA genes are shown as filled black and gray boxes at the bottom of the figure, respectively. Two regions are zoomed to show the locations of Rec12-oligo and transcripts within hotspots. (B) Heat maps of transcripts (left) and Rec12-oligo (middle) around hotspots. All hotspots on the genome (n = 603) were ranked by width and their midpoints were aligned. Data of transcripts (magenta) and Rec12-oligo (green) were overlaid to show on a color scale (right). Magenta and green represent high signals, while white represents low signals.

including *ade6-M26* and *ade6-3049*, generally have low nucleosome occupancy (de Castro *et al.* 2012; Yamada *et al.* 2013), our analysis found that hotspots can be generally superimposed on low-level nucleosome regions. Furthermore, it is noteworthy that the width of hotspots and NDRs correlated with each other, implying functional relation between the two regions. It should be noted, however, that a more recent and higher resolution analysis unveiled that the extent of nucleosome depletion is much milder than budding yeast hotspots (Fowler *et al.* 2014). One possibility to account for all these observations would be that transcription adjacent to hotspots set up a moderately accessible chromatin region, which would be sufficient for fission yeast DSB machinery to initiate meiotic recombination.

Conclusion

Contribution of transcription (and transcription factors) to meiotic DSB formation has been less appreciated partly due to the fact that its (their) roles are context dependent. In this study, however, we demonstrate that the HRA region of Atf1,

which had been shown to activate recombination at *ade6-M26*, is also a transactivation domain and that it directly or indirectly promotes both production of short transcripts and meiotic recombination at the *ade6-3049* hotspot. In addition, revisiting genome-wide data of meiotic transcripts and Rec12-oligo raise a possibility that DSB formation occurs in close proximity to transcribed regions.

Recent studies on various organisms reveal that mechanisms of meiotic DSB formation vary among species, and that, even in a single species, there are several ways to accomplish this event (Brick *et al.* 2012; Borde and de Massy 2013; Ismail *et al.* 2014). Considering such diversity, exploring possible connection between transcription and meiotic recombination may be necessary to thoroughly understand the initiation mechanism of meiotic recombination.

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