The double-strand break landscape of meiotic chromosomes is shaped by the Paf1 transcription elongation complex in *S. cerevisiae*


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Running title: Paf1 complex modulates meiotic DSB distribution

Keyword: Meiosis, recombination, H3K4 methylation, PAF, Rtf1, double-strand breaks

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Abstract

Histone modification is a critical determinant of the frequency and location of meiotic double-strand breaks (DSBs), and thus recombination. Set1-dependent histone H3K4 methylation and Dot1-dependent H3K79 methylation play important roles in this process in budding yeast. Given that RNA polymerase II associated factor 1 complex, Paf1C, promotes both types of methylation, we addressed the role of Paf1C component, Rtf1, in the regulation of meiotic DSB formation. Similar to a set1 mutation, disruption of RTF1 decreased the occurrence of DSBs in the genome. However, the rtf1 set1 double mutant exhibited a larger reduction in the levels of DSBs than either of the single mutants, indicating independent contributions of Rtf1 and Set1 to DSB formation. Importantly, the distribution of DSBs along chromosomes in the rtf1 mutant changed in a manner that was different from the distributions observed in both set1 and set1 dot1 mutants, including enhanced DSB formation at some DSB-cold regions that are occupied by nucleosomes in wild-type cells. These observations suggest that Rtf1, and by extension the Paf1C, modulate the genomic DSB landscape independently of H3K4 methylation.
DNA double-strand breaks (DSBs) are a kind of DNA damage that is deleterious to cells unless it is repaired. Unrepaired DSBs lead to genome instability by forming broken chromosomes. DSBs are repaired by either homologous recombination or non-homologous end joining (KROGH and SYMINGTON 2004; SYMINGTON and GAUTIER 2011). During vegetative growth, cells have to repair DSBs, which are accidentally created by exogenous impacts, such as ionizing radiation, as well as by internal sources, e.g. due to oxidation. Meiotic cells are equipped with a program to introduce DSBs along the genome for the induction of the homologous recombination. Recombination generates a crossover, a reciprocal exchange of parental DNAs, which is essential for the correct segregation of homologous chromosomes during meiosis I (LICHTEN and DE MASSY 2011; PETRONCZKI et al. 2003). Diversity in the gamete genome is also produced by meiotic recombination.

Programmed formation of DSBs on meiotic chromosomes is catalyzed by the topoisomerase II-like protein Spo11 and its binding partner Ski8 (KEENEY et al. 1997; LAM and KEENEY 2015). The activity of Spo11 is also regulated by essential accessory proteins or protein complexes: the Mre11-Rad50-Xrs2 (MRX) complex, the Rec114-Mer2-Mei4 (RMM) complex, Rec102 and Rec104 (DE MASSY 2013). In addition, DSB formation during meiosis is controlled by the chromosomal structure. Three meiosis-specific chromosomal proteins, Hop1, Red1, and Mek1/Mre4, are necessary for efficient DSB formation (Xu et al. 1997). Furthermore, a meiosis-specific cohesin complex containing Rec8 kleisin regulates the distribution of DSBs in the genome (KLEIN et al. 1999; KUGOU et al. 2009; SUN et al. 2015). Interestingly, the majority of proteins required for DSB formation, including components of the RMM complex, are localized on chromosome axes rather than chromatin loops. In meiotic nuclei, chromosome axes with multiple chromatin loops are a basic structural unit of the meiotic chromosome, and are involved in various meiotic chromosomal events including the regulation of DSB formation (BLAT et al. 2002; PANIZZA et al. 2011).

Meiotic DSBs are non-uniformly distributed along the genome (BORDE and DE MASSY 2013; DE MASSY 2013; LAM and KEENEY 2015). In some
chromosomal regions, known as recombination hotspots, DSBs are more frequent than in other parts of the genome. Moreover, the genome contains loci, recombination cold spots, where DSB frequency is lower than in other regions. In the budding yeast, most of the DSBs are introduced in nucleosome-free sites associated with 5′-regulatory regions, which contain the transcription start site of genes (PAN et al. 2011). This observation suggests that an open chromatin structure is essential for DSB formation. In the context of chromatin structure, histone modifications, which modulate the structure and dynamics of chromatin, also play a critical role in Spo11-dependent DSB formation during meiosis. Among these modifications, histone H3K4 trimethylation (H3K4me3) is a key determinant of DSB formation in budding yeast (BORDE et al. 2009; SOLLIER et al. 2004). H3K4me3 is introduced by a protein complex referred to as COMPASS (COMplex ASsociated with Set1) (SHILATIFARD 2012). Among eight subunits of COMPASS, Set1 is the catalytic subunit responsible for mono-, di-, and trimethylation of H3K4 (JAENNING 2010; SHILATIFARD 2012). Deletion of the SET1 gene, as well as point mutations in H3K4, reduce frequency of DSBs and change their distribution in a region-specific manner (BORDE et al. 2009; SOLLIER et al. 2004).

The mechanism of H3K4me3 involvement in the chromatin-based regulatory circuitry for the generation of DSBs is described as the “axis-tethering” model, which was originally proposed by Kleckner and colleagues (ACQUAVIVA et al. 2013; BLAT et al. 2002; PANIZZA et al. 2011; SOMMERMEYER et al. 2013). The H3K4 trimethyl mark is recognized by the PHD finger domain of Spp1, a component of COMPASS. Importantly, Spp1 is localized to chromosome axes while DSB regions are located on chromatin loops (ACQUAVIVA et al. 2013; SOMMERMEYER et al. 2013). Thus, a hotspot region is recruited to chromosome axes through the interaction of H3K4me3 on the loop with Spp1 on the axis, which, in turn, is a region enriched with the RMM complex. Indeed, the RMM component Mer2 binds to Spp1 (ACQUAVIVA et al. 2013; SOMMERMEYER et al. 2013). In this model, COMPASS plays a dual role in regulation of DSB formation: methylation of H3K4 by Set1 and recruitment of DSB sites to chromatin axes by Spp1.

Spo11 is an evolutionarily conserved protein in most organisms with
sexual reproduction (Keeney 2001). The regulatory pathway for recombination hotspots is also highly conserved. H3K4 methylation is associated with recombination hotspot activities in several higher eukaryotes including human and mouse (Pratto et al. 2014; Smagulova et al. 2011). In these two species, H3K4 methylation at hotspots is catalyzed by the meiosis-specific histone methyltransferase PRDM9 (PR domain-containing protein 9) (Baudat et al. 2010; Myers et al. 2010; Parvanov et al. 2010), which specifically recognizes a particular DNA sequence through its multiple zinc finger arrays. The types and numbers of zinc fingers of PRDM9 determine the diversity of recombination hotspots in these organisms.

In budding yeast, Set1-catalyzed H3K4 methylation is promoted by another histone modification, monoubiquitination of histone H2BK129, through trans-histone crosstalk (Shilatifard 2012). H2BK129 ubiquitination is mediated by the coordinated action of the E3 ligase Bre1 and the E2-conjugating enzyme Rad6, as well as by the RNA polymerase II associated factor 1 complex (Paf1C). Paf1C, containing the subunits Rtf1, Cdc73, Paf1, Ctr9, and Leo1, is known to regulate transcription elongation by RNA polymerase II through interaction with Set1/COMPASS as well as with proteins involved in the termination and processing of mRNAs (Jaehning 2010). Deletions of Paf1C components differentially affect H3K4 methylation levels. The rtf1 deletion abolishes H3K4me and severely decreases H3K79me, while the leo1 mutation does not affect either H3K4me or H3K79me (Krogan et al. 2003; Ng et al. 2003). Previously, we showed that Rad6 and Bre1 are critically important for efficient DSB formation and entry of budding yeast into meiosis I (Yamashita et al. 2004). However, the role of Paf1C during meiosis remains largely unknown. Interestingly, human Paf1C contains SKI8 whose yeast orthologue is essential for meiotic DSB formation, in addition to the five conserved components of Paf1C; RTF1, CDC73, PAF1, CTR9, and LEO1 (Kim et al. 2010).

In this study, we found that the Paf1C component Rtf1, which is essential for methylation of both H3K4 and H3K79, is also required for efficient DSB formation during meiosis. However, the distribution of meiotic DSBs along chromosomes in the rtf1 mutant was different from that in the set1 and set1 dot1 mutants. In the rtf1 set1 double mutant, the pattern of DSBs was similar to the
one in the $rtf1$ mutant and DSB frequency was less than that in either $set1$ or $rtf1$ single mutants. Location analysis of lost and gained DSB hotspots indicates a role of Rtf1 in promoting DSBs in divergent promoter regions and preventing DSBs in regions that are occupied by nucleosomes in wild-type cells. These observations suggest that Rtf1 and, probably, the Paf1C, modulate the generation of DSBs even in the absence of Set1 and concomitant H3K4 methylation.
Materials and Methods

Yeast strains and strain construction
All strains used in this study were derived from the *Saccharomyces cerevisiae* SK1 diploid strains NKY1551 (MATα/MATa, ho::LYS2/′, lys2/′, ura3/′, leu2::hisG′, his4X-LEU2-URA3/his4B-LEU2, arg4-nsp/arg4-bgl) or MSY832/833 (MATα/MATa, ho::LYS2/′, lys2/′, ura3/′, leu2::hisG′, trp1::hisG′). Genotypes of each strain used in this study are listed in Table S1. Deletion alleles of CDC73, CTR9, LEO1, PAF1, and RTF1 were constructed using PCR-mediated gene deletion (Wach et al. 1994). The set1 and dot1 deletion mutants were described previously (Ban Ismail et al. 2014). The primers used for strain construction are listed in Table S2.

Cytological analysis and antibodies
Chromosome spreads were prepared as described (Shinohara et al. 2000). Immunostaining analysis of Rad51 or Mre11 on meiotic chromosome spreads was conducted as described previously (Shinohara et al. 2008). Briefly, after staining, chromosome spreads were observed using an epifluorescence microscope (BL51; Olympus) with a 100× objective (NA1.4). Images were captured by a CCD camera (Cool Snap; Photometrics) at room temperature, and then processed using iVision (Sillicon) software. Pseudo-coloring was performed using iVision and Photoshop (Adobe) software. At each time point, about 100 spreads were image-captured and analyzed for counting foci. Primary antibodies directed against Rad51 (guinea pig, 1:500 dilution) and Mre11 (rabbit, 1:500 dilution) were used. Secondary antibodies (Alexa-fluor-488 or -594, GE Healthcare) directed against primary antibodies from the different species were used at a 1:2000 dilution.

Western blotting
For western blotting, cell precipitates were washed twice with 20% (w/v) trichloroacetic acid (TCA) and then disrupted with glass beads (1mm in diameter) using a bead beater (Yasui Kikai Co. Ltd.). Precipitated proteins were recovered by centrifugation and then suspended in the sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After adjusting pH to 8.8, samples were heated to 95 °C for 2 min. Protein samples were subjected to SDS-PAGE and transferred to PVDF membranes (Immobilon P, Millipore). Antibodies against Cdc5 (sc-33625, SantaCruz; 1:1000), Clb1 (sc-50440, SantaCruz; 1:1000), Zip1, Red1, and the α-subunit of yeast tubulin (YOL1/34, Serotec; 1:1000) were used. Anti-Red1 and anti-Zip1 antisera were described previously (Zhu et al. 2010). Antibodies against histone H3K4-me3 (ab8580; 1:1000), -me2 (07-030; 1:1000), -me1 (ab8895; 1:1000) and H3K79-me3 (ab2621; 1:1000) were from Abcam or Upstate Biotechnology.

**Pulsed field gel electrophoresis**

For pulsed field gel electrophoresis (PFGE), chromosomal DNA was prepared in agarose plugs as described in (Bani Ismail et al. 2014; Farmer et al. 2011) and run at 14 °C in a CHEF DR-III apparatus (BioRad) using the field 6V/cm at a 120° angle. Switching times followed a ramp from 15.1 to 25.1 seconds. Durations of electrophoresis were 41 h for chromosome III, and 44 h for chromosomes V and XII.

**Southern blotting**

Southern blotting was performed as described previously (Shinohara et al. 2003; Storlazzi et al. 1995). DNA molecules were transferred to a Nylon membrane (Hybond N, GE Healthcare). Restriction enzymes digesting DNA used for Southern blotting were as follows: the HIS4-LEU2 locus, PstI; YCR048W locus, BglII; GAT1 locus, PstI; PES4 locus, Stul; ELO2 locus, PstI. “Probe 291” was used for Southern blotting at the HIS4-LEU2 locus (Storlazzi et al. 1995). Locations of probes for detecting DSBs at other loci are as follows: YCR048W (215426-216686; yeast genome coordinate of chromosome III), GAT1 (97171-97980 on chromosome VI), PES4 (194848 to 196286 on chromosome VI) and ELO2 (186502-187165 on chromosome III). For DSBs located along chromosomes III, V, and XII, CHA1, RMD6, and AYT1 were used as probes, respectively. Image Gauge software (Fujifilm) was used to quantify bands. The background of obtained images was subtracted using the software, and the lane profiles were exported to and analyzed by Excel (Microsoft).
**ssDNA mapping**

The genome-wide analysis of DSB positions using ssDNA enrichments in *dmc1* mutants was performed as described previously (BLITZBLAU et al. 2007; BLITZBLAU and HOCHWAGEN 2011). Total genomic DNA for comparative hybridization was collected from a synchronous meiotic culture at 0 and 5 hours after induction of meiosis. ssDNA was enriched on benzoylated naphthoylated DEAE (BND) cellulose. Approximately 1.5 µg of ssDNA from the two time points were differentially labeled with Cy3-dUTP and Cy5-dUTP (GE Healthcare) and cohybridized to a customized 44K tiled microarray (Agilent). Each experiment was performed in duplicate and with a dye swap between experiments. The datasets are available at the Gene Expression Omnibus (GEO) under accession number GSE72827.

**Microarray data analysis**

Probes were mapped to the annotated S288C_R64-1 reference genome (Saccharomyces Genome Database). Cy3 and Cy5 levels were calculated using Agilent Feature Extractor CGH software. Background normalization, log2 ratios, and scale normalizations across each set of duplicated experiments were calculated with the sma (Statistics for Microarray Analysis) package in R (Blitzblau et al. 2007). Hotspots signals were defined as >3 features within 1kb of each other that all had p values <0.15 (using the pnorm function in R) for both sets of experiments. Hotspots were then defined as the merged region from start to end of all significant data points. Datasets were intersected to obtain hotspots shared and unique of *dmc1* and *rtf1 dmc1*. For shared hotspots, the midpoint between the respective summits was calculated and used for further analysis. To assay hotspot enrichment over background relative to gene bodies, the expected number of peaks was calculated assuming a random distribution of hotspots and compared to the observed peaks numbers. To determine nucleosome occupancy of the three hotspot classes (i.e. peaks maintained, lost, and gained in *rtf1 dmc1* mutants), published high-resolution Spo11 oligonucleotide data of wild-type cells (Pan J. et al., 2011; GEO accession numbers: GSE26449 and GSE26452) was used to identify the expected DSB
summit underlying each ssDNA peak. These data were then used to calculate average nucleosome occupancy as a function of distance from the hotspot summits. Relative replication timing of hotspot classes was determined using published replication timing data of wild-type cells (BLITZBLAU et al. 2012: GEO accession number GSE35667).

Results

_Yeast Paf1C components are necessary for meiosis_

To examine a possible role of Paf1C during meiosis, we constructed deletion mutants for five genes, _PAF1_, _CDC73_, _RTF1_, _LEO1_, and _CTR9_ encoding various components of the complex in the background of the SK1 strain, which enters meiosis in an efficient and synchronous manner. As has been previously demonstrated (KROGAN et al. 2002; MUELLER and JAEHNING 2002; SQUAZZO et al. 2002), all five deletion mutants were viable, but showed different mitotic growth rates (data not shown). Among them, the _paf1_ and _ctr9_ deletion mutants grew poorly in the vegetative phase and entered meiosis inefficiently and asynchronously (HS, unpublished results), consistent with previous reports (FOREMAN and DAVIS 1996; KOCH et al. 1999; SHI et al. 1996). Therefore, we focused our further studies on the three remaining deletion mutants, _rtf1_, _cdc73_, and _leo1_. First, we checked levels of the two types of histone H3 methylation promoted by Paf1C, H3K4me (me1, me2, and me3) and H3K79me (me3), in cell lysates by western blotting (Figure 1A). As a control we also included a _set1_ deletion mutant, which is defective in H3K4 methylation. In wild-type cell lysates, levels of both H3K4me3 and H3K79me3 were almost constant during meiosis with similar levels observed during vegetative growth, which corresponds to 0 h time point of the meiotic time course. On the other hand, levels of H3K4me1 and me2 increased slightly 2 h after induction of meiosis in wild-type cells. The _set1_ deletion completely abolished H3K4 methylation, but did not affect levels of H3K79me3. As reported previously (JAEHNING 2010), the _rtf1_ mutation abolished detectable levels of the H3K4me3/me2/me1 and strongly reduced H3K79me3 compared to the levels detected in wild-type cells. Defective H3K4 methylation in the _rtf1_ mutant might be due to reduced Set1/COMPASS binding to the promoter.
The *cdc73* mutation decreased H3K4me3 to about two-thirds of the wild-type level, while intensities of H3K4me1, H3K4me2, and H3K79me3 were reduced by half (Figure S1). The deletion of the *LEO1* gene did not affect either H3K4 or H3K79 methylation (Figure S1).

We checked spore viability of the above three mutants (Figure 1B). The *rtf1* and *cdc73* mutants had significantly reduced spore viability levels of 83.4% and 87.9%, respectively, relative to the wild-type viability level of 97.8% (chi-square test, $P$ value = $7.6 \times 10^{-13}$ and $2.6 \times 10^{-8}$). At the same time, the *leo1* mutant had spore viability of 95.0%, which was nearly equal to that in the wild type. The distribution of viable spores per tetrad in the *rtf1* and *cdc73* mutants did not show any bias (Figure 1B). Since the dependence of DSB formation on the activity of Set1 is critical for meiosis (Sollier *et al.* 2004), we expected to observe decreased spore viability (89.5%) in the *set1* mutant in accordance with an earlier report (Bani Ismail *et al.* 2014). To ask whether the *paf1C* mutations are epistatic to the *set1* mutation with respect to their effects on spore viability, we constructed several double mutants that combined the *set1* mutation with mutations in the genes encoding Paf1C components. The *set1 cdc73* double mutant exhibited 90.2% spore viability, which was similar to levels seen in the *cdc73* single and the *set1* single mutant. However, the *rtf1 set1* double mutant had a reduced spore viability of 77.5%, which was less than the levels detected in either *set1* or *rtf1* single mutants (chi-square test, $P$ value = $4.5 \times 10^{-5}$ for *set1* and 0.031 for *rtf1*). This observation suggests that Set1 and/or Rtf1 also affect meiosis independently of H3K4 methylation.

To avoid complicated interpretations due to differential contributions of each Paf1C component to histone modification, we focused our further analysis on the *rtf1* mutation, which abolished detectable levels of H3K4me and strongly reduced H3K79me relatively to the levels seen in the wild-type yeast. The entry into meiosis I (MI) division was analyzed by 4′,6-diamidino-2-phenylindole (DAPI) staining. Compared to the time of MI onset in wild-type cells, *rtf1* mutant cells entered MI with an approximately 2.5-h delay. The delay in the *rtf1* mutant was ~2 h shorter than the delay in the *set1* mutant (Figure 1C) (Bani Ismail *et al.* 2014; Sollier *et al.* 2004). Importantly, the *rtf1 set1* double mutant showed similar kinetics of the entry into MI as the *rtf1* single
mutant, suggesting that Rtf1 works upstream of Set1. To examine the progression of meiotic prophase in a different way, we analyzed the timing of expression of meiosis-specific chromosome proteins Red1 and Zip1 as early prophase markers and Cdc5 Polo-like kinase and Clb1 cyclin as markers of pachytene exit (Figure 1D). In the rtf1 mutant, the emergence of Red1 and Zip1 proteins was similar to that in wild-type cells. However, the timing of their disappearance was clearly decelerated compared to that observed in wild-type cells. An even slower timing of Red1 and Zip1 disappearance was noted in the set1 mutant.

An even slower timing of Red1 and Zip1 disappearance was noted in the rtf1 mutant. As for the expression of Clb1 and Cdc5 proteins (CHU and HERSKOWITZ 1998), their appearance in the rtf1 mutant peaked at ~8 h, which is roughly a 2-h delay in comparison to the time of their emergence in wild-type cells. The set1 mutant showed a similar delay in the initial expression of the two pachytene-exit markers, but it also exhibited a retarded disappearance of these proteins in relation to their dynamics in the rtf1 mutant. Total expression levels of the four marker proteins in the rtf1 set1 double mutant were similar to those in the rtf1 mutant. These results showed that, like Set1, Rtf1 is necessary for timely expression of proteins at pachytene exit and support the hypothesis that Rtf1 functions upstream of Set1 during meiosis.

The Paf1C component Rtf1 plays a role in DSB formation

Given the established role of Paf1C-dependent histone H3K4 and H3K79 methylation marks in the formation of meiotic DSBs (BANI ISMAIL et al. 2014; BORDE et al. 2009; SOLLIER et al. 2004), we evaluated the effect of the rtf1 mutation on the formation of meiotic DSBs. First, we studied DSB repair at the recombination hotspot HIS4-LEU2 (Figure 2A). In the wild type, DSBs appear at 3 h, peak at 4 h, and then gradually disappear (Figure 2, B and C). The rtf1 mutant showed a delay of ~1 h in DSB appearance and a peak at 5 h with reduced steady-state levels of DSBs to 18% of the levels seen in the wild-type (at 4 h). This finding shows that Rtf1 regulates the efficiency of DSB formation.

We next analyzed Rad51 foci on meiotic chromosomes (Figure 2D). Immunostaining of the Rad51 protein, which binds single-stranded DNA (ssDNA) of DSB ends for strand exchange in meiotic recombination (SHINOHARA et al. 1992), shows punctate staining as foci on chromosome spreads (Figure
2D) (Bishop 1994). The number of Rad51 foci is a good estimate of DSB numbers in a single nucleus. The rtf1 mutant exhibited a delayed assembly and disassembly of Rad51 foci. The number of Rad51-focus positive cells peaked at 6 h, i.e., with an approximately 2-h delay compared to the peak time in the wild type (Figure 2E). The disappearance of Rad51-focus positive cells was also delayed about by ~2 h in the mutant. At 5 h, the average number of Rad51 foci per nucleus in the rtf1 mutant was 21.6 ± 4.1 [mean ± S.E.] (n= 3; for each repeat more than 20 spreads were counted), which was less than the number seen in the wild-type at 4 h (36.2 ± 5.8, n=3; Figure 2F). Thus, the rtf1 mutation significantly decreased the steady number of Rad51 foci. These data suggest that the Paf1C component Rtf1 is necessary for the timely appearance of the correct number of Rad51 foci. The result that Rtf1 regulates DSB formation is consistent with the notion that Rtf1 is critical for H3K4 methylation. However, as will be described below, we found differential roles of Rtf1 and Set1 in DSB formation by a more detailed comparative analysis of the rtf1 single mutant and the rtf1 set1 double mutant.

**Rtf1 and Set1 play independent roles in regulating meiotic DSBs**

To obtain a better understanding of the effect of the paf1C mutation rtf1 on DSB formation and to determine its relationship to the effects of a set1 mutation, we employed the following three approaches to analyze DSBs: mapping of DSBs along the chromosomes using CHEF (clamped homogeneous electric field) gel electrophoresis (on the background of dmc1 and rad50S mutations, which block DSB turnover), analysis of DSBs at single chromosomal loci by conventional Southern blotting, and Mre11 focus counting on the rad50S background. The combination of these methods revealed a unique role of Rtf1 in meiotic DSB formation that is not shared with Set1 and does not involve H3K4 methylation.

**Mapping of DSBs on chromosomes on the dmc1 background**: We mapped DSBs along single chromosomes by separating chromosomes with CHEF gel electrophoresis followed by Southern blotting using a probe for the end of the chromosome. For accurate quantifications, we used the dmc1 mutant background, in which DSBs are not repaired and accumulate instead (Bishop et
We analyzed DSBs on chromosomes III (0.34 Mb in length), V (0.58 Mb), and XII (1.08 Mb). In the dmc1 mutant, each chromosome shows a unique region-specific capacity for DSB formation with hot and cold regions (Borde and Massy 2013; Massy 2013). As reported previously (Bani Ismail et al. 2014; Borde et al. 2009), the set1 dmc1 double mutation reduces the frequency of DSBs along chromosomes III and XII, particularly on the left arm of chromosome III and in the region just to the right of the CEN12 locus (Figure 3, A and B). Most of DSB-hot regions were downregulated and some regions were more prone for DSB formation in the set1 dmc1 mutant than in the wild type (dmc1), as reported previously (Borde et al. 2009; Sollier et al. 2004). On the dmc1 background, the rtf1 mutation led to a slight, but significant reduction in the total number of DSBs on chromosomes III and XII (Figure 3C). Compared to the dmc1 single mutant, the rtf1 dmc1 double mutant had a decreased frequency of some DSBs in DSB-hot regions on both arms of chromosomes III and XII (shown by blue arrows in Figure 3). Importantly, the rtf1 dmc1 double mutant showed a different distribution of DSBs on these chromosomes in comparison to the set1 dmc1 mutant. DSBs in the rtf1 dmc1 were decreased in some regions that were DSB-hot in the wild-type and also affected by the set1 mutation. However, there were regions where DSB levels were apparently increased in the rtf1 dmc1 double mutant relative to their number in the dmc1 mutant (shown in red arrows). Increased levels of DSBs in these regions were not seen in the set1 dmc1 mutant. Thus, the effect of the rtf1 mutation on the DSB landscape is different from that of the set1 mutation. Moreover, the DSB distribution in the rtf1 set1 dmc1 triple mutant was similar to that in the rtf1 dmc1 double mutant, but different from the distribution in the set1 dmc1 mutant. These findings suggest that Rtf1 may function upstream of Set1 in regulating the DSB landscape, and may function in part independently of H3K4 methylation. The rtf1 set1 dmc1 triple mutant exhibited a greater reduction in total DSBs than the decreases detected in the set1 dmc1 or rtf1 dmc1 double mutants, suggesting distinct contributions of Set1 and Rtf1 to the regulation of DSB frequency. Similar, albeit less drastic, effects of the rtf1 mutation on DSB formation are seen on chromosome V (Figure S2A).

From the results of the DSB mapping, three conclusions may be drawn.
First, Rtf1 and Set1 contribute to DSB patterning on chromosomes differentially, in a region-specific and chromosome-specific manner. Second, Rtf1 functions upstream of Set1 in regulating DSB patterning at least on some chromosomes. Third, Rtf1 and Set1 distinctly contribute to the regulation of DSB frequency in the absence of detectable H3K4 methylation marks.

**Mapping of DSBs at individual chromosomal locus on the dmc1 background:** Using conventional Southern blotting, we also analyzed DSBs at the following representative loci: YCR048W, GAT1, and PES4 (Figure 4A). At the YCR048W and GAT1 loci, similar to our findings in the set1 dmc1 mutant, the number of DSBs at 6 h in the rtf1 dmc1 double mutant decreased to about 30% and 40% of the levels seen in the dmc1 mutant, respectively (Figure 4, B and C). Moreover, the rtf1 set1 dmc1 triple mutant exhibited fewer DSBs at both loci compared to the levels observed in rtf1 dmc1 or set1 dmc1 double mutants. These findings support the notion of independent functions of Rtf1 and Set1 in the regulation of DSB formation. Previous studies indicated that the number of DSBs in some regions, e.g., at the PES4 locus, was increased in the set1 mutant compared to the respective DSB frequency in the wild type (BORDE et al. 2009; SOMMERMEYER et al. 2013). We also quantified DSBs at the PES4 locus on the dmc1 background and confirmed that at this location DSBs were more numerous in the set1 dmc1 double mutant than in the dmc1 mutant, consistent with the previous study (BORDE et al. 2009). The rtf1 dmc1 double mutant exhibited moderately increased levels of DSBs at this locus that were more frequent than the DSBs in the dmc1 mutant, and similar to those in the set1 dmc1 mutant. DSB levels in the rtf1 set1 dmc1 triple mutant were similar to those detected in the rtf1 dmc1 double mutant. These observations provided evidence that Rtf1 and Set1 repress DSB frequency at the PES4 locus through a similar mechanism.

We also looked for a locus with a specific increase in DSBs specifically as a result of the rtf1 mutation and identified the ELO2 locus on chromosome III as a candidate. At this locus, the wild-type (dmc1) cells formed very few DSBs (bottom panels of Figure 4, B and C). On the other hand, DSBs were upregulated in the rtf1 dmc1 mutant. This increase in the number of DSBs was less pronounced in the set1 dmc1 mutant. These observations again support the
hypothesis that Rtf1 is of critical importance in regulating DSB formation, and that Rtf1 has a repressive role. Moreover, at least at the ELO2 locus, this repressive role is unlikely to be shared with Set1.

H3K4me marks are recognized by the PHD finger of the Spp1 protein, a constituent of the COMPASS complex. Spp1 binds to chromosome axes and also plays an additional role in the generation of DSBs (Acquaviva et al. 2013; Sommermeyer et al. 2013). We investigated the relationship between Rtf1 and Spp1 in regulating DSB formation along chromosomes (Figure S3). CHEF-Southern for chromosomes III and XII revealed that the rtf1 mutation affected the distribution of DSBs in the absence of Spp1 in a similar way as in the absence of Set1. We also analyzed spore viability of spp1 rtf1 double mutants. spp1 rtf1 double mutants exhibited a frequency of survival of 87.9%, which is reduced relative to spp1 single mutant (Figure S4A). These observations suggest that the Set1-independent role of Rtf1 in DSB control is not mediated by the axis component Spp1. Like the rtf1 and set1 mutants (Figure 1C), the spp1 single mutant showed a delay in the entry into MI (Figure S4B).

**DSB frequency differs between rtf1 and dot1 set1 mutants**

It is known that Paf1C promotes H3K79 methylation through its action on H2BK123 ubiquitination (Jaehning 2010). Our previous study showed that the Dot1 histone H3K79 methyltransferase regulates DSB formation, particularly in the absence of SET1 (Bani Ismail et al. 2014). Therefore, we hypothesized that some of the effects of Rtf1 on the emergence of DSBs may be mediated by the action of Dot1 in addition to that of Set1. To explore this possibility, we compared DSBs along chromosomes III and XII in rtf1 dmc1, rtf1 set1 dmc1 and dot1 set1 dmc1 mutants (Figure 3). As reported previously (Bani Ismail et al. 2014), the dot1 set1 dmc1 triple mutant had fewer DSBs on both chromosome III and chromosome XII than the set1 dmc1 double mutant. The right arm of chromosome III and the left arm of XII were particularly affected in the triple mutant. The pattern seen in the set1 dot1 dmc1 triple mutant is clearly different from that observed in the rtf1 dmc1 double mutant. Furthermore, there were fewer DSBs in the set1 dot1 dmc1 triple mutant than in the rtf1 dmc1 double mutant. The difference was clearly seen in the right arm of chromosome III and
in the left arm of chromosome XII. We conclude that Rtf1 affects the DSB landscape irrespectively of the Dot1 function, i.e., independently of H3K79 methylation. Moreover, Rtf1 may have a negative (repressive) role on DSB formation in the absence of two histone methylation marks, H3K4me and H3K79me. Previous results showed that the combination of the set1 and dot1 mutations can suppress dmc1-arrest in prophase-I (Bani Ismail et al. 2014). On the other hand, the rtf1 mutation did not suppress the arrest (Figure 2B).

**Mapping of DSBs in the rad50S background:** To more precisely estimate changes in DSB levels, we also mapped DSBs on the background of the rad50S mutation, which accumulates unresected DSBs and thus yields a discrete Southern signal (Alani et al. 1990) (Figure 5, A and B). On chromosome III, the rtf1 rad50S mutant exhibited a reduced DSB frequency of 35.2% compared to the 47.2% of wild type (Figure 5C). DSB frequencies were more strongly reduced in the set1 rad50S mutant (22.1%) and the rtf1 set1 rad50S triple mutant showed more reduced DSB frequency than either double mutant, suggesting non-redundant roles of Rtf1 and Set1 in DSB frequency. This conclusion was confirmed using conventional Southern blotting to analyze DSBs at the YCR048W locus on chromosome III (Figure 5D). At this locus, rtf1 rad50S and set1 rad50S double mutants exhibited similarly reduced amounts of DSBs compared to the numbers detected in the wild type (28 and 13%, respectively at YCR048W; 8 h; Figure 5E). Moreover, the rtf1 set1 rad50S triple mutant exhibited an even larger reduction of DSBs in comparison to the numbers seen in the rad50S mutant (4.3% at YCR048W; 8 h time point). Unlike on the dmc1 background, the DSB pattern on chromosome III was very similar between rtf1 rad50S and set1 rad50S double mutants, as well as the rtf1 set1 rad50S triple mutant (Figure 5B). This observation indicates similar effects of rtf1 and set1 on DSB patterning on chromosome III in the rad50S background. Notably, the DSB pattern on chromosome XII is different between rtf1 rad50S and set1 rad50S mutants, and the rtf1 set1 rad50S mutant exhibited a similar pattern to the rtf1 rad50S mutant. Thus, the contributions of Rtf1 and Set1 with respect to DSB pattern and frequency are different between in the background of dmc1 and rad50S, and also between chromosomes (See Discussion).
Counting Mre11 foci on the rad50S background: Finally, as an independent measure of overall DSB levels, we performed immunostaining analysis of Mre11, which binds to DSB ends and shows focal staining on meiotic chromosomes of the rad50S background. The number of Mre11 foci is proportional to that of DSBs in the genome (MS, unpublished results). We determined the average number of Mre11 foci in the rft1 rad50S and set1 rad50S double mutants (Figure 5F). In the rad50S single mutant, the average number of Mre11 foci comprised 24.3 ± 4.6 (mean ± S.E.; n=3) at 8 h (Figure 5G). In the rft1 rad50S and set1 rad50S double mutants that number was reduced to 10.2 ± 0.78 and 12.2 ± 0.32, respectively. This finding was another confirmation of the hypothesis that Rtf1 and Set1 have a comparable contribution towards DSB formation. The rft1 set1 rad50S triple mutant exhibited an even larger reduction of Mre11 foci with the average number of 4.2 ± 0.7, i.e., much lower than the levels detected in the double mutants. These findings indicate that Rtf1 and Set1 independently contribute to the formation of Mre11 foci, further supporting the notion that Rtf1 and Set1 have different functions in the regulation of DSB formation.

Genomic features of DSB redistribution in rtf1 mutants.

To specifically investigate the genomic features associated with the altered hotspot distribution in rtf1 mutants, we determined hotspot patterns across the genome using a microarray-based approach (BLITZBLAU et al. 2007). ssDNA arrays measure the relative levels of ssDNA, which transiently accumulates at DSB sites during meiotic recombination. In the absence of DMC1, ssDNA is not turned over (BISHOP et al. 1992), allowing for a cumulative measurement of meiotic DSB activity (BLITZBLAU et al. 2007; Bühlér et al. 2007). Analysis of ssDNA profiles of dmc1 and rtf1 dmc1 mutants confirmed the redistribution of the DSB hotspots along chromosomes III, V and XII (Figure 6A) seen in the Southern blot analysis (Figure 3 and Figure S2), and revealed similar changes across the entire genome (Figure S5A and File S1). Inspection of individual ssDNA peaks further indicated that the redistribution is largely a consequence of differential DSB activity at existing hotspots rather than the appearance of de novo hotspots.
To quantify these changes, we compiled a list of the strongest ssDNA peaks, representative of the strongest DSB hotspots, using a stringent significance cutoff. This analysis yielded 476 significant peaks in the dmc1 mutant and 413 peaks in the rtf1 dmc1 mutant (Figure 6B). The majority of the wild-type (dmc1) hotspots (279) were maintained in the absence of RTF1. In the rtf1 dmc1 mutant, 197 peaks fell below the significance cutoff when compared to the dmc1 mutant, while 134 new hotspot peaks became significant. Thus, the activity of a large fraction of hotspots is differentially affected by the loss of RTF1.

Based on these broad changes, we investigated whether specific chromosomal features are associated with these altered DSB patterns. No clear associations with larger scale chromosomal features, such as proximity to telomeres or centromeres, were observed (data not shown). In addition, we observed that hotspots gained in the absence of RTF1 were somewhat enriched in regions far from replication origins (Figure 6C). This bias is statistically significant (Wilcoxon-Mann-Whitney test), but the functional importance of this effect is unclear, as we failed to observe a significant difference with respect to average replication timing (Figure S5B).

In wild type, the strongest hotspots preferentially occur in the promoter regions of divergent gene pairs (BLITZBLAU et al. 2007). As RTF1 function is linked to transcriptional elongation (MUELLER and JAEHNING 2002; SQUAZZO et al. 2002), we wondered whether this promoter bias is altered in the rtf1 dmc1 mutants. We classified hotspot peaks based on their location with respect to gene bodies and whether they occurred in the intergenic regions between divergent, convergent or tandem gene pairs (Figure 6D). These analyses revealed a significant redistribution of hotspots in the rtf1 dmc1 mutants (Chi-square test; p=0.007), and showed that the hotspots most affected by the loss of RTF1 are those occurring in promoters at divergent genes. By contrast, hotspots between tandem or convergent gene pairs and in gene bodies remained largely unaffected. These data indicate that RTF1 is not required for the overall promoter bias of meiotic DSB hotspots but that it plays a role in increasing DSB activity in the promoters of divergent gene pairs.

Given that RTF1 regulates histone modifications (Figure 1A), we asked
whether the changed hotspot activity observed in the rtf1 dmc1 mutants was reflected in the local chromatin structure. Genome-wide meiotic nucleosome occupancy has been determined for wild-type cells (Pan et al. 2011). Therefore, we investigated whether hotspots with changed activity upon loss of RTF1 differed in their wild-type nucleosome occupancy from hotspots that were unaffected (Figure 6E). To obtain the necessary DSB resolution for this analysis, we identified the precise sites of DSB formation underlying the ssDNA peaks by correlating them with available Spo11 oligonucleotide data (Pan et al. 2011). This analysis revealed that hotspots that become more active in the rtf1 dmc1 mutants have a higher chance to be occupied by nucleosomes in wild type. By contrast, DSBs maintained between the dmc1 and rtf1 dmc1 mutants or lost in the rtf1 dmc1 mutant are largely depleted of nucleosomes in wild type. These observations suggest that suppression of DSB formation by RTF1 is associated with increased local nucleosome occupancy, raising the intriguing possibility that Rtf1, and perhaps Paf1C, modulates DSB activity by controlling chromatin accessibility.

Discussion

Paf1C promotes DSB formation

Previous studies in the budding yeast showed that methylation of two histone H3 lysines, H3K4 and H3K79, has an important role during meiotic DSB formation (Bani Ismail et al. 2014; Börde et al. 2009; Sollier et al. 2004). In particular, histone H3K4 methylation is a critical determinant of efficient DSB formation as well as selection of DSB sites (Acquaviva et al. 2013; Börde et al. 2009; Sommermeyer et al. 2013). However, in the absence of H3K4 methylation, DSB levels in the genome are only moderately reduced, while the overall distribution of DSBs along chromosomes, with some exceptions, remains largely unaffected (Börde et al. 2009). The complex effect of the H3K4-methylation deficit on DSB formation is apparent at the PES4 locus that exhibits increased levels of DSBs in the absence of this methylation mark. Similar to the H3K4 methylation mutant, the set1 mutant still shows a reasonable number of DSBs, harboring ~60% of DSBs detected in the wild type (Bani Ismail et al. 2014; Börde et al. 2009).
Consistent with this mild DSB defect, the \textit{set1} mutant and H3K4 methylation defective mutants showed high spore viability. This implies that H3K4 is not essential of DSB formation \textit{per se}, but rather plays a regulatory role in efficient DSB formation, as proposed (LAM and KEENEY 2015). Moreover, in the \textit{set1} mutant, residual DSBs were non-randomly distributed along the genome, suggesting the presence of additional determinants that shape the DSB landscape. Indeed, we recently found that Dot1 controls DSB frequency only in the absence of the Set1-dependent H3K4 methylation through the methylation of H3K79. However, the \textit{set1 dot1} double mutant maintained a reasonable number of DSBs (40-50\% of the levels seen in wild type) (BAI ISMAIL \textit{et al.} 2014), indicating the existence of other determinants for DSB formation.

Initially, we were interested in the role of Paf1C in DSB formation since Paf1C is required for histone H2B ubiquitination, which, in turn, promotes both Set1-dependent H3K4 methylation and Dot1-dependent H3K79 methylation (JAEHNING 2010). This functional link predicts that DSB defects seen in \textit{paf1c} mutants should be very similar to those in the \textit{set1 dot1} double mutant. However, although H3K4 methylation was abolished and H3K79 methylation strongly reduced in a mutant of the Paf1C component, \textit{RTF1}, this mutant exhibited changes in DSB distribution and frequency that were different from those in the \textit{set1 dot1} double mutant. These data suggest a role for Rtf1, and possibly for the whole Paf1C, in DSB formation that is distinct from the roles of Set1 and Dot1. Moreover, the \textit{set1 rtf1} double mutant had fewer DSBs than the \textit{set1} or \textit{rtf1} single mutants, consistent with a scenario whereby Rtf1 regulates DSB formation in the absence of Set1, and, conversely, Set1 promotes DSB formation in the absence of Rtf1. These roles of Set1 and Rtf1 are apparently independent of H3K4 methylation. The H3K4me-independent role of Set1 can be explained by the recent finding that Spp1, a component of the Set1-containing complex, directly binds to Mer2 (ACQUAVIVA \textit{et al.} 2013; SOMMERMEYER \textit{et al.} 2013).

\textit{Paf1C shapes the DSB landscape}

Importantly, the distribution of DSB along a single chromosome in the \textit{set1 dot1} double mutant is almost identical to that in the \textit{set1} single mutant. This observation suggests the existence of additional factors determining DSB
landscape on various chromosomes, particularly in some DSB-hot and DSB-cold regions in the absence of H3K4 and H3K79 methylation marks. In this study, we demonstrated that the DSB landscape in the *rtf1* mutant was altered in a different way compared to changes in DSB distribution in the *set1* and the *set1 dot1* mutants. Although overall DSB levels were reduced in the *rtf1* mutant, regions that displayed few DSBs in the wild type as well as in *set1* mutant cells, showed an increased capacity for DSB formation. Genome-wide ssDNA mapping identified 134 hotspots that show significantly increased DSB activities in the absence of Rtf1. This finding suggests a repressive role of Rtf1, and thus of the whole Paf1C, on the generation of DSBs in a region-specific manner. The formation of DSBs in these regions is independent of H3K4me. Therefore, Rtf1 may play distinct roles in the control of DSB formation, positively affecting it through H3K4 methylation, as well as negatively regulating it. In some regions, Paf1C apparently promotes DSB formation through its role in H3K4 methylation. In other regions, however, this protein complex represses the emergence of DSBs, possibly through mechanisms other than histone modification.

A possible scenario is that Paf1C modulates the dynamics or distribution of nucleosomes in promoter regions, as has been described for the *SER3* promoter (Pruneski *et al.* 2011). A redistribution of nucleosomes upon loss of Paf1C function may explain why hotspots that become more active in the *rtf1* mutant in fact appear occupied by nucleosomes in wild type. If this model applies, one would expect that nucleosome occupancy would be specifically be reduced at these hotspots in the *rtf1* mutant. Conversely, the apparent loss of hotspots from divergent promoter regions may be the result of increased nucleosome occupancy at these loci in the *rtf1* mutant. A detailed analysis of nucleosome occupancy and dynamics in the *rtf1* mutant will help address this possibility. Moreover, any effects on nucleosome dynamics need not be direct, as Paf1C is mainly known for its function in mediating nucleosome re-deposition and histone modification during transcriptional elongation (Mueller and Jaehning 2002; Pruneski *et al.* 2011; Squazzo *et al.* 2002). Indeed, stronger DSB sites in the *set1* mutant were enriched in promoters of genes that were more highly transcribed in the *set1* mutant (Borde *et al.* 2009). A similar situation may apply for *rtf1* mutants, although at least in mitotic cells, *rtf1* deletion does not lead to
strong changes in promoter usage (Penheiter et al. 2005).

An alternative, and not necessarily exclusive, model is that Paf1C may contribute to the formation of a particular chromosomal structure. During DSB formation, tethering of recombination hotspots through the 5'-region of genes is critical for the access of the hotspot to the Mer2 protein, a Spo11-activating protein on the axes (Acquaviva et al. 2013; Sommermeyer et al. 2013). One possibility is that Paf1C regulates tethering of DSB sites to the axes by changing the axis or loop structure (Kleckner 2006). Meiotic chromosome axes contain a number of proteins, including Hop1, Red1, Mek1, and the cohesion protein Rec8, whose mutations affect DSB landscape. Interestingly, the positioning of axis protein binding sites is strongly dependent on local transcription (Sun et al. 2015). One possibility is that Paf1C may control the assembly of these axis proteins on DNA molecules through its role in transcription, and, as a result, regulate the structure of the axis and tethered loops. To test this idea, we need to obtain more information on the regions exhibiting high levels of DSBs in the rtf1 mutant on a genome-wide level and their relationships with other chromosomal proteins during meiosis.

In human, in addition to five conserved components, CDC73, CTR9, LEO1, PAF1, and RTF1, PAF1C contains one additional component, SKI8 (Kim et al. 2010). Interestingly, Ski8 in yeast is an essential factor for Spo11-mediated DSB formation (Arora et al. 2004). One possibility that needs to be investigated is that Paf1C may regulate the Ski8-dependent activity of Spo11. This is still an open question since there have been no reports about any functional relationship between Ski8 and Paf1C in yeast. Paf1C also promotes Set2-dependent H3K36 methylation (Jaehning 2010). It is previously shown that the set2 mutant increased DSBs at the HIS4 locus in the rad50S background (Merkel et al. 2008). However, the role of this methylation mark in genome-wide DSB formation also remains to be elucidated.

**Paf1C contributes to DSB formation differentially in rad50S and dmc1 mutants**

One of the surprising results of our study was a drastic difference of the effect of rtf1 mutation on DSB frequency in the rad50S and dmc1 mutants, which tend to
accumulate unresected, protein-linked DSB ends and ssDNA molecules, respectively. During meiosis, DSB ends and ssDNA molecules activate checkpoint kinases Tel1\(^{\text{ATM}}\) and Mec1\(^{\text{ATR}}\), correspondingly. It is well established that these two checkpoint effector kinases control DSB formation (Farmer et al. 2012; Garcia et al. 2015; Gray et al. 2013; LANGE et al. 2011; Zhang et al. 2011). Particularly, it is known that Tel1\(^{\text{ATM}}\) promotes the repression of DSB formation through the phosphorylation of Rec114 (Carballo et al. 2013). We found the rtf1 mutation reduced DSB levels much more on the rad50S background than on the dmc1 background. This difference indicates a positive role of Rtf1 in DSB formation in conditions when Tel1\(^{\text{ATM}}\) is activated. It is possible that Paf1C mediates the Tel1\(^{\text{ATM}}\)-dependent feedback control of DSBs. Given that Dot1 is involved in Tel1\(^{\text{ATM}}\) signaling during meiosis (Ontoso et al. 2013), we propose a simple mechanism whereby Paf1C controls the Tel1\(^{\text{ATM}}\)-dependent regulation of DSBs indirectly through the Dot1-dependent H3K79 methylation. Alternatively, in the rtf1 mutant, the normal DSB formation pathway is compromised and compensatory DSB formation depends on a Mec1-dependent pathway that is not activated in the rad50S mutant.

Since Tel1\(^{\text{ATM}}\) and Mec1\(^{\text{ATR}}\) are sequentially activated during meiotic recombination from unresected, protein-linked DSBs to resected DSBs, we hypothesize that the formation of early DSBs is likely to proceed initially under the control of Tel1\(^{\text{ATM}}\), predominantly involving H3K4 methylation. In contrast, the formation of late DSBs, apparently under the principal control of Mec1\(^{\text{ATR}}\), may use alternative cues for DSB generation, in addition to H3K4me. Collectively, these processes may affect the outcome of recombination as has been reported recently (Joshi et al. 2015).
Acknowledgements
We thank Drs. Doug Bishop, Susan Gasser and Neil Hunter for discussions. We are also indebted to members of the Shinohara lab, especially Ms. Hisako Matsumoto and Ayaka Tokumura for their technical assistance. This work was supported by JSPS KAKENHI Grant Number 22125001 and 22125002 to AS, as well as by grants from the Takeda Science Foundation to AS. MS was supported by the Japanese Society for the Promotion of Science through the Next Generation World-Leading Researchers program (NEXT). This work was supported in part by NIH grant GM088248 and March Dimes Research Grant 6-FY13-105 to AH. SG was supported by the Indian Government as well as by IPR.

Author contributions
SG, HS, MS, AH and AS conceived and designed the experiments. SG, HS, and MS performed the experiments. SG, MS, and AS analyzed the data. NJP and MMC carried out the microarray experiments and analyzed the data. SG, MS, NJP, AH, and AS wrote the manuscript.
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Figure legends

Figure 1. Paf1C components are necessary for meiosis.
(A) Detection of several histone methylation marks during meiosis. Levels of histone H3K4-me1, -me2 and -me3, H3K79-me3, and α-tubulin as a loading control were verified by western blotting. At each time point, cells were fixed with TCA and cell lysates were subjected to the experimental analysis. Representative images are shown.
(B) Spore viability of various strains was measured after dissecting tetrads. Spores were incubated at 30°C for 3 days. Each bar indicates the percentage of spore viability and total number of dissected tetrads (parentheses). Wild type, NKY1303/1543; rtf1 mutant, SGY3/4; leo1 mutant, SGY7/8; cdc73 mutant, SGY5/6; set1 mutant, SGY45/46; set1 rtf1 mutant, SGY47/48; set1 cdc73 mutant, SGY51/52.
(C) First meiotic cell division was analyzed by DAPI staining in wild type (blue circles; NKY1303/1543), rtf1 (red circles; SGY3/4), set1 (green circles; SGY45/46) and rtf1 set1 (purple circles; SGY47/48) cells. At least 100 cells were counted for each time point. Each plotted value represents the mean values ± standard deviation (S.D.) from three independent time courses.
(D) Expression of various meiotic proteins was verified by western blotting. Representative images are shown. Phosphorylated species of Zip1, Red1, Cdc5, and Clb1 are indicated by arrows. Wild type, NKY1303/1543; rtf1, SGY3/4; set1 mutant, SGY45/46; set1 rtf1 mutant, SGY47/48.

Figure 2. Rtf1 promotes DSB formation
(A) Schematic representation of the HIS4-LEU2 locus. Sizes of fragments for DSB and recombinant analysis are shown with lines below.
(B) DSB formation and repair at the HIS4-LEU2 locus in the wild type and the rtf1 mutant strains were analyzed by Southern blotting. The experiments were independently performed in triplicate and representative blots are shown. Genomic DNA was digested with PstI.
(C) The bands of DSB I were quantified. The symbols represent the wild type (blue circles; NKY1303/1543) and rtf1 mutant (red circles; SGY3/4). Each
plotted value represents the mean values ± standard deviation (S.D.) from three independent time courses.

(D) Immunostaining analysis of Rad51 (green) for the wild type (NKY1303/1543) and rtf1 (SGY3/4) mutant strains was carried out. Representative images are shown; wild type, 4 h; rtf1, 4 and 8 h. The bar indicates 2 µm.

(E) Kinetics of Rad51-focus positive cells in various strains. A focus-positive cell was defined as a cell with more than 5 foci. At least 100 nuclei were counted at each time point. The symbols represent the wild type (blue circles; NKY1303/1543) and rtf1 mutant (red circles; SGY3/4) strains.

(F) Kinetics of the number of Rad51 foci was analyzed in different strains. The symbols represent the wild type (blue circles; NKY1303/1543) and rtf1 mutant (red circles; SGY3/4) strains. The number of Rad51 foci in Rad51-positive nuclei (with more than 5 foci) was counted at each time point. The average number of foci per positive nucleus (± S.D. from three independent experiments) is shown on the top.

Figure 3. Rtf1 and Set1 differently promote DSB formation
Distribution of DSBs along chromosomes XII (A) and III (B) was analyzed by PFGE followed by indirect labeling of one chromosome end using probes for CHA1 and AYT1, respectively. Samples from meiotic time courses of the dmc1 single (blue, MBY009/010), rtf1 dmc1 double (red, SGY859/860), set1 dmc1 double (green, SGY862/863), set1 dot1 dmc1 triple (orange, SGY545/546), and set1 rtf1 dmc1 triple (purple, SGY870/871) mutants were analyzed. On the left of the chromosome III panels, the approximate sizes of the chromosomes and the positions of the recombination hotspots, HIS4-LEU2, ELO2, and THR4 are indicated. Blue arrows on the right side indicate DSBs, which are reduced by the rtf1 mutation. Red arrows on the right side indicate DSB bands, which are increased by the rtf1 mutation. Graphs below show the traces of the phosphor-imager signal at 8 h. Pairwise comparison of traces are shown as indicated. The dmc1 single, blue; rtf1 dmc1, red; set1 dmc1, green; set1 dot1 dmc1, orange; set1 rtf1 dmc1, purple. Quantification of total DSBs for each chromosome by CHEF analysis of various strains (C) was carried out as described in Materials and Methods. Each plotted value represents the mean
values ± standard deviation (S.D.) from three independent time courses

**Figure 4. Rtf1 promotes the formation of DSBs in the absence of Set1.**
(A) Schematic representations of the YCR048W, GAT1, PES4, and ELO2 loci. Sizes of DSB fragments are shown with lines below.
(B) DSB formation at the above loci was determined in different strains by Southern blotting. The experiments were independently performed in triplicate and representative blots are shown.
(C) The major DSB bands (DSB I) at the YCR048W, GAT1, PES4 and ELO2 loci were quantified. The symbols represent the dmc1 mutant (blue circles; MSY9/10), rtf1 dmc1 mutant (red circles; SGY859/860), set1 dmc1 mutant (green circles; SGY862/863) and dmc1 set1 rtf1 mutant (purple circles; SGY870/871). Each plotted value represents the mean values ± standard deviation (S.D.) from three independent time courses.

**Figure 5. Mre11-focus formation in the rad50S mutant is reduced by the introduction of the rtf1 mutation.**
(A) Distribution of DSBs along chromosomes III and XII was analyzed by PFGE followed by indirect labeling of one chromosome end using probes against CHA1 and AYT1, respectively. Samples from meiotic time courses of strains described above were analyzed.
(B) DSB profiles are shown as pairwise comparisons of the four strains.
(C) Quantification of total DSBs by CHEF analysis of each strain in (C). Details are described in Materials and Methods. Each plotted value represents the mean values from two independent time courses.
(D) DSB formation at the YCR048W locus in different strains was determined by Southern blotting. Genomic DNA was digested with BglII.
(E) DSB frequency was quantified by Southern blotting in the following strains: the rad50S mutant (blue, SGY83/84), rtf1 rad50S mutant (red, SGY107/108), set1 rad50S mutant (green, SGY89/90), and set1 rtf1 rad50S mutant (purple, SGY118/120). Each plotted value represents the mean values ± standard deviation (S.D.) from three independent time courses.
(F) Immunostaining analysis of Mre11 (green) in the rad50S mutant (SGY83/84),
rtf1 rad50S mutant (SGY107/108), set1 rad50S mutant (SGY89/90), and set1 rtf1 rad50S mutant (SGY118/120) strains was carried out. The bar indicates 2 µm.

(G) Quantification of Mre11 foci in various mutants. For each focus-positive chromosome spread, the numbers of Mre11 foci at 8 h were counted and plotted as shown. Error bars represent standard error of the mean (S.E.M.; n=3).

Figure 6. Genome-wide mapping of ssDNAs in the rtf1 mutant.

(A) ssDNA distribution on chromosome III, V and XII in the dmc1 single (blue, MBY009/010), rtf1 dmc1 double (red, SGY859/860). For chromosome V and XII, only ~300 kb region of the left end of the chromosome is shown. Triangles indicate significant hotspots of ssDNA enrichment.

(B) Venn diagram representing the overlap between significant hotspots in the dmc1 single and rtf1 dmc1 double mutants. Blue, the dmc1 only; red rtf1 only; purple common in dmc1 and rtf1 dmc1.

(C) Distribution of distances between DSB peaks and replication origins in different DSB classes. Distance between a DSB peak and the nearest origin was measured and plotted for each class.

(D) Distribution of all significant hotspots in dmc1 and rtf1 dmc1 mutants relative to the position of transcripts for four classes: in genes or between tandem, divergent, or convergent transcripts.

(E) Relationship of average nucleosome occupancy in each class was mapped relative to the distance from the DSB summits (bp).
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A

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<td></td>
</tr>
<tr>
<td>H3K79me3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tubulin</td>
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<td></td>
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</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Wild type</th>
<th>leo1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.V. 97.8% (n=104)</td>
<td>S.V. 95.0% (n=120)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rtf1</th>
<th>set1</th>
<th>rtf1 set1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.V. 83.4% (n=110)</td>
<td>S.V. 89.5% (n=102)</td>
<td>S.V. 77.5% (n=100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>cdc73</th>
<th>cdc73 set1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.V. 87.9% (n=101)</td>
<td>S.V. 90.2% (n=112)</td>
</tr>
</tbody>
</table>

C

Meiosis I

Wild type
rtf1
rtf1 set1
set1
Gothwal et al. Figure 2

A

B

[Image of a gel pattern with bands labeled as DSB II and DSB I]

C

[Graph showing percentage of DSBs over time in meiosis (h)]

D

[Images of fluorescence microscopy showing Rad51 localization in wild type and rtf1背景下。]

E

[Graph showing percentage of Rad51-positive foci over time in meiosis (h)]

F

[Graph showing number of Rad51 foci over time in meiosis (h)]
Gothwal et al. Figure 4

A

BglII

(kb)
P (11)

DSB I (5)

Site I

probe

PstI

PES4

(kb)
P (6.8)

DSB I (2.7)

Site I

probe

StuI

LSB3

(kb)
P (12.4)

DSB I (9)

DSB II (6)

Site II

probe

PstI

ELO2

(kb)
P (12.4)

DSB I (5)

Site I

probe

B

YCR048W

dmc1

rtf1 dmc1

set1 dmc1

rtf1 set1 dmc1

% of DSBs

Time in meiosis (h)

C

GAT1

dmc1

rtf1 dmc1

set1 dmc1

rtf1 set1 dmc1

% of DSBs

Time in meiosis (h)

PES4

set1 dmc1

rtf1 dmc1

rtf1 set1 dmc1

dmc1

% of DSBs

Time in meiosis (h)

ELO2

rtf1 dmc1

set1 dmc1

rtf1 set1 dmc1

dmc1

% of DSBs

Time in meiosis (h)
Gothwal et al. Figure 5

A. Chromosome III and Chromosome XII

B. Graphs showing Mre11 foci and DAPI signals for different genotypes.

C. Bar graphs showing percent of total DSBs in Chromosome III and Chromosome XII.

D. Graph showing DSB I formation over time in meiosis for different genotypes.

E. Graph showing percent of DSBs over time in meiosis for different genotypes.

F. Images of YCR048W with Mre11 foci and DAPI signals.

G. Bar graph showing number of Mre11 foci for different genotypes.