Homologue engagement controls meiotic DNA break number and distribution

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Meiotic recombination promotes genetic diversification as well as pairing and segregation of homologous chromosomes, but the double-strand breaks (DSBs) that initiate recombination are dangerous lesions that can cause mutation or meiotic failure. How cells control DSBs to balance between beneficial and deleterious outcomes is not well understood. Here we test the hypothesis that DSB control involves a network of intersecting negative regulatory circuits. Using multiple complementary methods, we show that DSBs form in greater numbers in Saccharomyces cerevisiae cells lacking ZMM proteins, a suite of recombination-promoting factors traditionally regarded as acting strictly downstream of DSB formation. ZMM-dependent DSB control is genetically distinct from a pathway tying break formation to meiotic progression through the Ndt80 transcription factor. These counterintuitive findings suggest that homologous chromosomes that have successfully engaged one another stop making breaks. Genome-wide DSB maps uncover distinct responses by different subchromosomal domains to the ZMM mutation zip3 (also known as cst9), and show that Zip3 is required for the previously unexplained tendency of DSB density to vary with chromosome size. Thus, feedback tied to ZMM function contributes in unexpected ways to spatial patterning of recombination.
Figure 1 | More DSBs form in ZMM mutants. a, Spo11 generates a covalent protein-linked DSB; endonucleolytic cleavage releases Spo11 bound to a short oligo (detection method on left). Resection is followed by strand invasion and ZMM-dependent stabilization of intermediates fated to become crossovers. b, c, Representative pulsed-field gel Southern blots probed for chromosome IX are shown in b and Poisson-corrected DSB quantification shown in c (mean ± s.d., 3 cultures). P, parental; W, wilds. d, e, Representative Spo11–oligo complex time courses are in d and quantification in e (mean ± s.d. for 3 cultures, except at 10 h for msh5 and zip3 analyses (1 culture)). Radiolabelled Spo11–oligo complexes were detected by autoradiography (top) and total Spo11 was detected by anti-Flag western blot (WB, middle). The main labelled species differ in oligo size10. Nearly all of the western blot signal is Spo11 that is processed by Spo11–oligo complexes; arrowhead represents proteolytic product. Bottom, extract samples run separately and stained with Coomassie control for input to immunoprecipitates. In panels c and e, mutants are plotted with wild-type data collected in parallel.

If ZMM mutants make more breaks, then more repair products should also accumulate. To gauge interhomologue recombination, we used strains heterozygous for different arg4 mutations (Fig. 2a). Prophase cells transferred to rich medium abort meiosis, often completing recombination even if unable while still in meiosis2,2. All ZMM mutants tested except msh5 formed more Arg” prototrophs than wild type (Fig. 2b). Increased recombination has been reported in all ZMM mutants examined (including msh4 and msh5), but was not interpreted as evidence for increased DSB frequency13–19 (Supplementary Table 1). Thus, a context-dependent hyper-rec phenotype is a common but previously unrecognized property of ZMM mutants.

We explored this hyper-rec behaviour by quantifying recombinants at three natural DSB hotspots (Fig. 2c and Extended Data Fig. 4a, b). At each, allelic complexes have different flanking and central restriction sites. Crossovers and parental length fragments are resolved by electrophoresis after digestion with flanking enzymes, then DNA is digested in the gel with the central enzyme before electrophoresis in the orthogonal dimension. Noncrossover gene conversion molecules co-migrate with one parent in the first dimension but have a central restriction site matching the other parent (Fig. 2d and Extended Data Fig. 4c–e). Key features are that the hotspots are high-intensity with few/no other DSB sites nearby, and central polymorphisms are positioned to make incorporation into heteroduplex DNA likely. At CCT6 and ERG1, recombinant molecules were 1.7–2.5-fold more abundant in the ZMM mutants tested, with increased noncrossovers and crossovers at or below wild-type levels (Fig. 2e). At GAT1, zip3 mutants displayed fewer crossovers offset by more noncrossovers, for a net frequency comparable to wild type (Fig. 2e). (Refer to Supplementary Discussion of gene conversion tracts and sister chromatid recombination.) These findings reinforce the conclusion that ZMM mutations cause a hyper-rec phenotype that is variable between loci.

DSBs were present at CCT6 and GAT1 in zip3 mutants at late times, well past the point when most DSBs had disappeared in wild type (Fig. 2f, g and Extended Data Fig. 5a). Recombination intermediates (‘joint molecules’) that are transient in wild type were also detected late in zip3. DSBs and joint molecules at CCT6 were also present in zip1 and msh5 mutants later than in wild type but not as long as in zip3, similar to analysis of breaks on chromosome IX (Extended Data Fig. 5b–e). These results agree with data at artificial hotspots in ZMM mutants (for example, ref. 20), but it was not previously possible to evaluate whether DSB numbers were increased and most previous studies dismissed or did not consider this possibility (Supplementary Table 1). We can now combine DSB data with quantification of recombination intermediates and products (Supplementary Table 2); this bookkeeping reveals that msh5, zip1 and zip3 mutants experience 1.8–2.6-fold more detectable DSB-related events at CCT6. Recombination product overabundance yields the same conclusion for zip3 and msh5 at ERG1 (1.7–1.9-fold), whereas wild type and zip3 mutants had similar totals at GAT1. We conclude that ZMM mutants incur more DSBs, but to varying degrees at different loci.

Separate pathways controlling DSB number

Recombination products and DSBs accumulate in cells lacking Ndt80, a transcription factor controlling pachytene exit21,22, and therefore it has been suggested that this stage in prophase ends a period permissive for DSB formation2–22, further supported by recent studies5,22,23. Indeed, Spo11–oligo complexes reached 1.2–1.4-fold higher than the wild-type maximum and remained high through late time points in ndt80 mutants (Fig. 3a, b and Extended Data Fig. 6). Heteroallele recombination was also increased (1.6-fold, Fig. 2b). Pachytene delay/ arrest via Ndt80 inhibition is a hallmark of ZMM mutants5,24, suggesting that increased DSBs might be an indirect consequence of arrest23, perhaps analogous to increased DSB numbers when CHK-2 kinase activity is prolonged in Caenorhabditis elegans25,26. If so, then ZMM mutations should cause no change if Ndt80 is absent. However, more Spo11–oligo complexes (Fig. 3a, b and Extended Data Fig. 6) and heteroallele recombinants (Fig. 2b) accrued in zip3 ndt80 and msh5 ndt80 double mutants than in ndt80 single mutants. Furthermore, msh5, zip1 and zip3 had similarly increased Spo11–oligo complexes (Fig. 1e) despite different arrest phenotypes (Fig. 3c). Thus, although the ZMM mutant DSB phenotype is probably influenced by the combined effects of Ndt80 inhibition and a hyperactivated DNA damage response, meiotic arrest per se does not explain ZMM mutant-provoked DSB increase.

Instead, we infer that a ZMM-dependent process(es) is more directly responsible for inhibiting DSB formation. A plausible mechanism is that chromosomes that have engaged their homologues undergo structural changes that render them unlit Spo11 substrates27,28. ‘Homologue engagement’ could mean synaptonemal complex formation and/or progression of crossover-designated recombination intermediates, both promoted by ZMMs. Supporting this model, DSB-promoting factors Hop1 and Red1 accumulate on chromosomes in ZMM mutants28, proteins required for DSBs are displaced from pachytene chromosomes in wild-type yeast (for example, ref. 29), and Hop1 orthologues are displaced after synapsis in yeast and mouse27,28.

Hyper-rec behaviour in ZMM mutants is reconciled with tetrat data demonstrating globally reduced crossing over (for example, ref. 30) by noting that there is a reduced per-DSB likelihood of generating a crossover that offsets increased DSBs (Supplementary Discussion). Our findings
Figure 2 | Hyper-rec phenotype of ZMM mutants. a. Top, schematic of arg4 heteroalleles, showing open reading frames and mutated restriction sites. Bottom, Spo11-oligo profile shows DSB distribution (RPM, reads per million mapped; smoothed with 201-base pair (bp) Hann window). b. Heteroallele recombination frequencies (mean ± s.d.). *Significantly different from wild type (P < 0.02, Student’s t-test); **significantly different from ndt80 (P < 0.006). c. Recombination reporter at the CCT6 hotspot. d. Representative Southern blots of parental and recombinant DNA molecules (crossovers (COs) and noncrossover gene conversions (NCOs)) at CCT6 resolved by two-dimensional gel electrophoresis. e. Recombination frequencies (mean ± s.d.). Crossover frequencies were halved to convert to per-DSB equivalent because each crossover yields two recombinant molecules. *Total recombination significantly different from wild type (P < 0.003); **crossing over significantly different from wild type (P < 0.04). f, g. DSBs at CCT6 and GAT1. A representative Southern blot probed for CCT6 is in f and quantifications for CCT6 and GAT1 are in g (mean ± s.d. for 3 cultures, except 8 h for zip3 mutants at GAT1, analysed twice). JMs, joint molecules.

Shaping the DSB landscape

If ZMM-dependent feedback works via chromosome structure changes linked to homologue engagement, then it should be spatially patterned. We tested this by deep-sequencing Spo11 oligos to map DSBs (Fig. 4a and Supplementary Table 3). Control cultures with a fully functional Spo11–protein A fusion agreed with each other and previous results11 (Extended Data Figs 2, 8 and data not shown). The DSB ‘landscape’ is shaped by combinatorial action of many factors that operate hierarchically11,34. At short scales (sub-kilobase (kb)), the landscape is dominated by hotspots, mostly in nucleosome-depleted promoters. This pattern was unaffected in zip3 mutants, in that DSBs formed in the same hotspots (Fig. 4a, Extended Data Figs 4f, 8c and Supplementary Table 4).

On larger scales, however, zip3 showed substantial alterations. Smaller chromosomes form more crossovers per unit length than larger ones35 because of variation in DSB levels11, but what controls DSB differences has been unclear. Remarkably, zip3 mutation eliminated the normal inverse correlation between Spo11-oligo density and chromosome length (Fig. 4b). If the zip3 map is scaled by 1.8-fold (on the basis of peak Spo11-oligo levels, Fig. 1e), all chromosomes had more DSBs but larger ones went up disproportionately (Fig. 4c). Thus, ZMM-dependent feedback is necessary for length-dependent recombination variation in wild type. Perhaps the number (not density) of DSBs governs speed or efficiency of homologue engagement: if so, smaller chromosomes might tend to have more time to accumulate DSBs. A nonexclusive possibility is that can also be reconciled with studies that attributed persistent DSBs in ZMM mutants solely to increased DSB lifespan because, where tested, ZMM mutant and wild-type phenotypes were similar in rad50 separation-of-function (rad50S) or dmc1 backgrounds19,20,31–33 (Extended Data Fig. 7a, b). Dmc1 is an essential strand exchange protein and rad50S mutants cannot remove Spo11 from DSB ends. As these mutations block recombination before ZMM proteins act, they are uninformative for querying ZMM mutant effects. This caveat may also apply to recombination-defective mutants in other organisms.
DSB suppression spreads far relative to chromosome length, with longer chromosomes providing more spreading room.

Subchromosomal domains differed in response to zip3 mutation: Spo11-oligo frequencies increased less than average in 20-kb zones at telomeres and centromeres (where few DSBs form in wild type\(^{1,36,37}\)), and were unchanged or reduced near the ribosomal DNA (rDNA), causing chromosome 12 to be an outlier in whole-chromosome analysis (Fig. 4c, d and Extended Data Fig. 9a). The remaining interstitial regions varied widely, with local regression along chromosomes suggesting alternating domains of greater or lesser change (Fig. 4d). Supporting this conclusion, the change in each hotspot correlated with the change in hotspots located nearby, with correlation strength decaying with distance (Fig. 4e).

To better understand these domains, we compared Spo11-oligo maps to chromosomal features including the distribution in wild type of Zip3 protein, chromosome structure proteins needed for normal DSBs (Hop1, Red1, Rec8) and proteins essential for Spo11 activity (Mei4, Mer2, Rec102, Rec104, Rec114), previously defined by chromatin immunoprecipitation (ChIP)\(^{38,39}\). The magnitude of change in Spo11-oligo density in zip3 correlated with enrichment of Hop1, Rec114, Mei4, Mer2 and Red1, with highest correlation for binning windows \(\geq 20\) kb (Fig. 4f and Extended Data Fig. 9b, c, e). The distributions of these proteins are themselves correlated\(^{30}\) (Extended Data Fig. 9d). We infer that large domains (tens of kb wide) enriched for these proteins tend to be more responsive to ZMM-dependent feedback. G+C content, Spo11-oligo density in wild type, and distributions of Rec8, Rec102 and Rec104 were uncorrelated or weakly anti-correlated when considered individually (Fig. 4f and Extended Data Fig. 9b, c, e, f). However, we observed a strong scale-dependent correlation with the distribution Zip3 displays when most DSBs have formed and homologues are engaging\(^{39}\) (Fig. 4f and Supplementary Table 5). Our findings elucidate the locus-to-locus variability of ZMM mutant hyper-rec behaviour and reveal that ZMM-dependent feedback shapes the DSB landscape in wild type.
Conclusions

We propose that the logic of DSB control involves a drive towards DSB formation that is restrained quantitatively, spatially and temporally by distinct but intersecting negative influences (Fig. 4h). We note several implications. First, Spo11 catalytic potential exceeds what is realized in any one meiosis. Thus, DSB numbers may underestimate the severity of biochemical defects in mutants22. Second, counterintuitive effects arise when feedback loops are severed or hyperactivated, for example, in dmc1 or rad50S backgrounds. The ZMM mutations likely impinge on multiple circuits simultaneously, removing restraints on Spo11 activity by disrupting homolog engagement and inhibiting Ndt80 activation, but also hyperactivating negative regulatory circuits via the DNA damage responsive kinase Tel1 (and possibly Mec1). This ‘push-me–pull-you’ interplay undoubtedly affects the final number and distribution of DSBs. Our results support the conclusion that crossovers in ZMM mutants are not identical in number and provenance to crossovers that form without ZMM intervention in wild type. Third, our findings explain puzzling aspects of set11 mutant yeast and Prdm9 mutants. If DSB number control is separate from Spo11 targeting (which requires Set1 or PRDM9 (ref. 40)), then the default for Spo11 to make breaks until restrained by feedback explains why DSBs form in relatively normal numbers but different locations in these mutants. This also undermines more extreme versions of the ‘hotspot paradox’ in which biased gene conversion is predicted to eliminate all hotspots over time and thereby prevent DSB formation (for example, ref. 41): the logic of DSB control makes it impossible for inactivation of individual hotspots to render chromosomes immune to Spo11. Fourth, our findings support the hypothesis that altered DSB distributions tie feedback control to the source of altered recombination distributions caused by certain mutations or heterozygosity for large-scale chromosome structure variants in other species69. Finally, we speculate that organisms such as mouse readily form synaptonemal complexes and purification, amplification and sequencing of Spo11 oligos in meiotic cultures were prepared according to standard methods. Labelling of Spo11–Oligo complexes and purification, amplification and sequencing of Spo11 oligos provides a link between recombination enzymes and synaptonemal complex proteins. Cells 102, 245–255 (2000).

METHODS SUMMARY

Yeast strains are of the SK1 background (Supplementary Table 6). Synchronized meiotic cultures were prepared according to standard methods. Labelling of Spo11–Oligo complexes and purification, amplification and sequencing of Spo11 oligos were carried out using methods adapted from previous studies89,91.

Online Content

Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions D.T., N.M. and X.Z. performed experiments and analysed the data. N.M. optimized purification of the Spo11–protein A fusion, prepared the sequencing libraries to map DSBs and performed preliminary data analysis on sequencing reads. S.K. analysed sequencing data. D.T. and S.K. wrote the paper.

Author Information Sequencing data were deposited at GEO under accession number GSE48299. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.K. (s-keeney@ski.mskcc.org).
METHODS

Yeast strains and plasmids. Strains were of the SK1 background (Supplementary Table 6). The zip1 deletion and one of the ndt80 deletions (ndt80A::LEU2) were provided by N. Kleckner, the mut5 and zip3 deletions were provided by N. Hunter, the spo16 deletion was provided by A. Shinohara, and a second ndt80 deletion (ndt80A::KanMX4) was provided by S. Burgess. The dmc1, pm3 and zip4 deletions were made by replacing the coding sequence with the hygromycin B phosphotransferase gene (hphMX4). Gene disruption was verified by PCR. All mutants were analysed into the desired strain backgrounds by crossing and tetracy dissection. The Spo11-Flag strain was provided by K. Ohta and the protein A tagging construct was provided by M. Roux. The constructs for two-dimensional gel electrophoresis analysis of crossovers and noncrossovers at recombinants at CECT6, ERG1 and GAT1 were engineered by a series of two-step gene replacements. For CECT6 on chromosome IV, Sall sites were introduced in intergenic regions at Saccharomyces Genome Database (SGD) coordinates 832534 and 838251 in one strain; Sall sites were introduced separately at coordinates 833537 (in YDR186c) and 837893 (in CECT6) in another strain along with a Small site between YDR186c and CECT6 at coordinates 838002 and 838503. For ERG1 on chromosome VII, SacI sites were introduced in intergenic regions at coordinates 844276 (in RBG2) and 845464 (in ORP1) with a Sall site at coordinate 845874 (between ERG1 and ATC2). In a separate strain, SacI sites were introduced at coordinate 845470 (in CECT6) and coordinate 852145 (in ORP1). For GAT1 on chromosome VI, KpnI sites were introduced at coordinates 90967 and 100083 (both intergenic) along with a BamHI site between FRS2 and GAT1 at coordinates 95715 and 95717. Separately, KpnI sites were introduced at coordinates 92986 (BUD27) and coordinate 99889 (intergenic). Further details are in ref. 42 and available on request.

Culture methods. With the exception of Spo11-oligo mapping, synchronous meiotic cultures were prepared as described46–48. In brief, cells were grown in YPA (1% yeast extract, 2% Bacto Peptone, 1% potassium acetate) for 13.5–14 h at 30 °C. Yeast strains and plasmids. For the direct DSB measurements and heteroallele recombination analysis. For the analysis as described46. Immunoprecipitation of Spo11–oligo complexes was performed using 5 μg of mouse monoclonal anti-Flag M2 antibody (Sigma). Precipitated Spo11–oligo complexes were end-labelled in NEBuffer 4 (New England Biolabs) containing 3–10 μl of [α-32P]dCTP and terminal deoxynucleotidyl transferase (TdT). 25 μl of reaction mixture was added to the beads, mixed, and incubated at 37 °C for 1–2 h. Spo11-oligo complexes were eluted by adding 25 μl of NUPAGE loading buffer (diluted 2× and supplemented with 83.3 mM dithiothreitol (Invitrogen)) and boiling for 5 min. End-labelled Spo11-oligo complexes were separated on a Novex 4–12% gradient denaturing polyacrylamide gel (Invitrogen) then transferred to PVDF membrane using the iBlot membrane (Invitrogen) and visualized by phosphorimager. Blots were probed with mouse monoclonal anti-Flag M2 conjugated to horseradish peroxidase (Sigma). Chemiluminescence detection was performed according to the manufacturer’s instructions (ECL or ECL Prime, Amersham). Protein quantity was estimated by separating 1 μl of extract on a Novex 4–12% gradient denaturing polyacrylamide gel and staining with Coomassie blue.

Spo11-oligo purification for mapping. Spo11 oligos were prepared for sequencing similar to methods described previously47, with modifications. Hayplolds strains with Spo11 C-terminally tagged with five copies of the protein A tag were patched from a frozen stock onto a YP-glycerol plate and grown at 30 °C overnight to select for respiration competence. Cells were mated on YPD (1% yeast extract, 2% Bacto Peptone, 2% Bacto dextrose) plates then streaked for single colonies and grown for 48 h at 30 °C. A single colony was inoculated into 5 ml liquid YPD medium and grown overnight at 30 °C. The saturated YPD culture was used to inoculate 25 ml liquid SPS medium (0.5% yeast SPS medium in 0.5 ml liquid YPD medium and grown overnight at 30 °C. The saturated YPD culture used to inoculate 25 ml liquid SPS medium (0.5% yeast extract, 1% peptone, 0.67% yeast nitrogen base without amino acids (Difco), 1% potassium acetate, 0.05 M potassium biphosphate, pH 5.5) to OD400 0.8 and grown for 7 h at 30 °C. This culture was used to inoculate 1 l SPS medium in a 2.18 fl ground Fernbach flask to OD600 0.05. Flasks were incubated at 30 °C for 12–16 h, to OD600 4.5–6. Cells were collected by centrifugation, washed once in deionized water, re-suspended in 0.6 l sporulation medium (2% potassium acetate and 0.001% antifoam 204) and incubated 2.81 baffled flasks (0.61 per flask) at 30 °C for 4 h (wild type) or 5 h (zip3) to approximate times of peak Spo11-oligo levels (Fig. 1e).

Cells were centrifuged and washed with 50 mM EDTA, transferred to a 30-mL syringe, extruded into liquid nitrogen, and stored at −80 °C. Yeast cell powder was prepared by placing the frozen paste into canisters of a Retch MCM31 mill (pre-cooled to −30 °C) and grinding five times for 3 min at 30 Hz. Yeast powder was transferred to a pre-chilled 50-ml tube and stored at −80 °C. Extract was prepared by transferring the yeast powder to a pre-chilled 40-mL glass Dounce homogenizer and homogenizing in two volumes of cold 10% trichloroacetic acid. Lysate was centrifuged at 14,000 r.p.m. in an SS-34 rotor (Sorvall) for 20 min. The supernatant was removed and cell pellet was re-suspended in SDS extraction buffer (2% SDS, 0.5 M Tris-HCl, pH 8.1, 10 mM EDTA, 0.05% bromophenol blue). β-mercaptoethanol was added to 0.288 M, the extract boiled in a water bath for 5 min, then centrifuged at 14,000 r.p.m. in an SS-34 rotor for 40 min. The supernatant was poured into fresh tubes, diluted with an equal volume of 2× NuPAGE sample buffer (Triton X-100, 3% glycerol, 60 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA) and incubated with CLB-Sepharose beads (GE) for mock IP (4 h at 4 °C mixing end-over-end, 1.5 ml extract per 200 μl beads). Supernatant was removed into fresh tubes and mock beads were stored on ice. The supernatant was incubated with 200 μl IgG Sepharose Fast Flow beads (GE) per 1 ml of extract for 4 h at 4 °C mixing end-over-end, then beads were recovered. Mock and IP beads were washed three times with 10 mL cold 1× IP buffer. Protein was eluted from mock or IP beads with 350 μl 2× NuPAGE LDS buffer (Invitrogen) by boiling for 5 min, followed by a second elution with 350 μl 0.5× NuPAGE LDS buffer. The eluates were combined and diluted with 700 μl of 2× IP buffer, then incubated with 30 μl CLB-Sepharose for 16 h at 4 °C. Beads were washed five times with 100 μl 2× IP buffer (IP), 4 °C overnight with end-over-end rotation. The beads were recovered and subsequently washed with 1 ml Proteinase K buffer (100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% SDS, 1 mM CaCl₂) lacking SDS, then re-suspended in 600 μl Proteinase K buffer and 100 μg purified Proteinase K, and incubated overnight.
at 50°C with end-over-end rotation. The supernatant was collected using a SPIN-X tube (Corning) and ethanol precipitated with 0.3 volume of 9 M ammonium acetate, 10 μg of DNA-free glycogen and 2.5 volumes of 100% ethanol. Spool1 oligos were quantified by end labelling with [α-32P]GTP and TdT (Fermentas) and comparing to a known quantity of similarly labelled 30-nucleotide synthetic oligo.

**Library preparation for sequencing.** Approximately 300 fmol of Spool1 oligos were subjected to TdT tailing at their 3’ ends. Material eluted from mock beads was processed in parallel to determine specificity of the IP (data not shown). Tailing was carried out in a total volume of 40 μl containing 1 × NEBuffer 4, 20 U TdT and 13.8 μM GTP at 37°C for 5 h, followed by heat inactivation of TdT at 75°C for 10 min. The tailed oligos were ligated to a double-stranded DNA adaptor optimized for the Illumina HiSeq platform as follows: the tailing reaction was supplemented by 10 × T4 RNA ligase 2 buffer (500 mM Tris-HCl, pH 7.6, 50 mM MgCl2, 50 mM β-mercaptoethanol) to 1 ×, 25 mM ATP to 0.5 μM, 5 pmol double-stranded customized P7 adaptor, 300 fmol T4 RNA ligase 2 (gift from Stewart Shuman, MSKCC), and dH2O to a final volume of 50 μl. P7 adaptor sequences are 5’-GTGACTGTAGTGATCGATCGCTTCCTCCGATCTCC and 5’-AATGTAGAAGACGACGCTGATCGATCGCTCC and 5’-AGATCGAAGACGACGCTGATCGATCGCTCC and 5’-AATGTAGAAGACGACGCTGATCGATCGCTCC and 5’-AATGTAGAAGACGACGCTGATCGATCGCTCC as the P7 primer: 5’-CAAGATCGTTPY (gift from Stewart Shuman, MSKCC) and dH2O to a final volume of 50 μl. P7 adaptor sequences were annealed and purified by non-denaturing polyacrylamide gel electrophoresis before use in the ligation reaction. Ligation was carried out overnight at room temperature. Complementary strands were synthesized as follows: the ligation reaction was supplemented with 2 mM dNTP to a final concentration of 30 μM and 10 U Klenow polymerase (New England Biolabs), and incubated at 25°C for 15 min. After Klenow inactivation (75°C, 10 min), extension reactions were supplemented with 0.3 volume of 9 M ammonium acetate, 10 μg of DNA-free glycogen and 2.5 volumes of 100% ethanol. DNA was precipitated at −20°C overnight and centrifuged at 16,000g. The pellet was rinsed with 70% ethanol, air dried, and dissolved in a mixture of 9 μl water and 15 μl formamide loading buffer. Extension products and 100 bp ladder (radialabelled with T4 polynucleotide kinase and [γ-32P]ATP) were separated on a 10% denaturing polyacrylamide gel. The region between −50 to 200 nt (equivalent to −10 to 50-nucleotide Spool1 oligos with (rG)3 tails plus ligated adaptor) was excised, crushed, and eluted in 400 μl 10 mM Tris-HCl, pH 8.0 at 37°C overnight with mixing. Elution mixture was spin through a SPIN-X tube, then 0.3 volume of 9 M ammonium acetate, 10 μg DNA-free glycogen, and 2.5 volume of 100% ethanol. DNA was precipitated on dry ice at −20°C overnight and centrifuged at 16,000g. The pellet was rinsed with 70% ethanol and air dried. The 3’ ends of gel-purified, denatured DNA strands were tagged with T6P by destroying the dried pellet in 40 μl tailing reaction containing 1 × NEBuffer 4, 30 U TdT and 50 μM GTP, then incubating at 37°C for 5 h. The tailed oligos were ligated to a second set of customized double-stranded DNA adaptors (P5) and complementary strands were synthesized as above. The P5 adaptor is a mixture of four duplexes. The oligos for one duplex are 5’-CACTCCTTTCCCTACACGACGCTCTTCCGATCTC and 3’-GGACTAGATCAGATCGCTCC and 5’-AGATCGAAGACGACGCTGATCGATCGCTCC and 3’-AATGTAGAAGACGACGCTGATCGATCGCTCC. After annealing, the four duplexes were mixed in approximately equimolar ratio. This provides diversity of base composition at the beginning of the sequencing reaction. If this diversifier region were not present, the sequencer would encounter ambiguity in defining the precise start and end positions for reads that map to positions starting with one or more C residues or ending in one or more G residues. In such cases, the 5’ and 3’ ends of each read were defined so as to provide the longest contiguous sequence match with the genome.

## Bioinformatic analysis

Statistical analyses were performed using R version 2.15.3 (http://www.r-project.org/) or GraphPad Prism 6.0. Mapping of Illumina reads to the target genome was performed according to a pipeline essentially as described in previous studies. Adaptor sequences were removed from both the 5’ and 3’ ends, then reads were mapped to the S. cerevisiae genome (SGD version June 2008, that is, sacCer2) using gmapper-b (2_1_1b) from the SHRMp mapping package. The specific sequencing parameters used were: -u g -1000 -q 1000 -m 10 -i 10 -h 0.5 -r 0.5 -g 1000, which forces ungapped alignments (-u by itself does not suppress all gapped alignments but sets an effectively infinite gap opening penalty). To increase sensitivity for short reads we set the seeds for the following Pearson r = 0.4 - 1.0, 1000 - 10 000, which forces ungapped alignments (-u by itself does not suppress all gapped alignments but sets an effectively infinite gap opening penalty).

After mapping the reads were separated into unique and multiple mapping sets, but only uniquely mapped reads were analysed in this study (multiple mapping reads constituted a small minority of the total). A full copy of the source code is available online at http://bio.mskscc.org/public/Thacker_ZMM_feedbac.

Because the tRNA array is represented in the S. cerevisiae genome by only 1.9 copies of the repeat unit, oligos that span the boundary repeats map to a single position even though they come from a repetitive sequence. Therefore, reads of this type were moved to the multiple mapping set. The wild-type data sets contained a small number of spurious reads (<0.4% of total) from contamination of the Spool1-oligo sequencing libraries with PCR primers from the TELI locus; these reads were deleted from the maps. Because of the variable number of g residues added by terminal transferase to the 3′ end of Spool1 oligos and to the 3′ end of the reverse complementary strands, there is ambiguity in defining the precise start and end positions for reads that map to positions starting with one or more C residues or ending in one or more G residues. In such cases, the 5’ and 3’ ends of each read were defined so as to provide the longest contiguous sequence match with the genome. Raw and processed sequence read data have been deposited in the Gene Expression Omnibus (GEO) database [http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE48299)]. This accession also contains the curated maps (unique mapping reads only) in wiggle format to allow direct visualization in appropriate genome browsers, for example, the UCSC browser [http://genome.ucsc.edu/ (using genome version sacCer2).

For the studies here, our focus is on the number and position of DSBRs rather than characteristics of the Spool1 oligos themselves, so maps were distilled to record just the positions of 5’ ends of oligos. Each map was normalized to the total number of reads that mapped uniquely to a chromosome (PRM; excluding reads mapping to mitochondrial DNA or the 2μ plasmid), then wild-type and zip3 maps were averaged. Normalized Spool1-oligo counts within the 3.600 previously identified hotspots1 are compiled for each data set in Supplementary Table 4. In analyses evaluating the fold change (that is, Fig. 4c, d, and Extended Data Fig. 9a, d–f), we assumed a global increase in Spool1-oligo number of 1.8-fold based on the difference in peak steady-state levels (Fig. 1e). To prevent dividing by zero and to minimize variability of ratios caused by small changes in denominators, we added a small constant to numerator and denominator before taking the ratio (20 RPM for hotspot-based ratios (approximately 15% of median hotspot Spool1-oligo count), or 0.1-RPM per kb for bin-based ratios (0.13% of median Spool1-oligo density per bin)). Where indicated, Spool1-oligo maps were smoothed with a 201-nt Hann window.

For the correlation analysis in Fig. 4f and Extended Data Fig. 9b, chromosomes were divided into non-overlapping bins of the indicated sizes. Bins that overlapped cenosomal regions (within 20 kb of telomeres, within ±10 kb of centromeres, or in the region from 60 kb leftward to 30 kb rightward of the rDNA) were discarded. The published ChIP enrichment data (log; of ChIP/input; from GEO accession GSE29860 (ref. 38) or Supplementary Table 3 from ref. 39) were averaged within each bin, then compared to the mean log fold-change in Spool1-oligo density in zip3 and correlation coefficients were calculated. The log-transformed data were set approximately normally distributed so that the results of similar overall patterns were obtained if we used Kendall’s τ (data not shown).

Multiple linear regression was performed using the ‘lm’ function in R. Data were averaged in non-overlapping bins of 35 kb, censored for subtelomeric, pericentric and rDNA-proximal regions as described above. Principal component analysis was performed on the correlation matrix of the Rec114, Me4, Mer2, Hop1 and Red1 ChIP data using the ‘prcomp’ function in R. The first principal component accounted for 92.7% of the variance in this data set; the remaining principal components were discarded as they accounted for only 4.1%, 2.2%, 0.8% and 0.3% of the variance, respectively.

To assess spatial correlations for the change in Spool1-oligo density (Fig. 4e), we calculated the correlation coefficient (Pearson’s r) between the log-fold change at DSBR hotspots and the log-fold change for hotspots located within a set of 5-kb windows centred a distance D to the right of each hotspot centre. We varied D from 5 to 200 kb in steps of 2.5 kb and calculated the correlation coefficient separately for...
For comparison, we performed the same analysis to evaluate the correlation between absolute heat (log of the Spo11-oligo count) in hotspots and the heat in 5-kb windows at varying distances. To generate randomized controls for this analysis, we randomly reassigned the heats or log-fold change between hotspots within a chromosome. This randomization strategy preserves the non-random placement of hotspots relative to one another and preserves the correlated behavior (if any) across whole chromosomes. Randomization was repeated 100 times to provide the estimates of the 95% confidence intervals shown in the Fig. 4e.

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Extended Data Figure 1 | Chromosomal breaks in msh5 and zip1 mutants.
Representative pulsed-field gel Southern blots probed for chromosome IX are shown, labelled as in Fig. 1b.
Extended Data Figure 2 | DSB formation appears normal in SPO11-Flag and SPO11-PrA strains. a, Southern blots probed for chromosome III. High molecular weight chromosomal DNA was purified 6 h after transfer to sporulation medium from meiotic rad50S cultures carrying the indicated SPO11 alleles (in spo11-yf the catalytic tyrosine 135 is mutated to phenylalanine), then separated on pulsed-field electrophoresis gels. Samples from a rad50S spo11-HA strain are shown for comparison; haemagglutinin-tagged Spo11 has reduced DSB frequency. Each lane represents an independent culture (SPO11+ samples from the same cultures were run on both gels). PrA, protein A. b, Quantification of blots in panel a and separate blots (not shown) probed for chromosomes VII or VIII. Break frequencies are per cent of DNA in lane (mean ± s.d. of 3–4 cultures). Numbers in parentheses indicate values from each tagged strain relative to SPO11+ for the same chromosome. Relative DSB frequencies at the bottom are averages across the three chromosomes assayed.
Extended Data Figure 3 | Spo11–oligo complexes in msh5 and zip1 mutants. Representative time courses are shown.
Extended Data Figure 4 | Analysis of recombination at three natural DSB hotspots. a, b, Recombination reporters at the ERG1 (a) and GAT1 (b) hotspots. c–e, Representative Southern blots of parental and recombinant DNA molecules at CCT6 (c), ERG1 (d) and GAT1 (e). The arrowhead in e indicates a non-reproducible radiolabelled species. f, Local distribution of DSBs around recombination reporter locations is not altered in zip3 mutants. Spo11-oligo profiles (averages for wild type and zip3 mutant) are smoothed with 201-bp Hann window; zip3 values are offset to separate profiles.
Extended Data Figure 5 | Direct analysis of DSB formation at natural hotspots. a–d, Representative Southern blots of DNA separated on a conventional agarose gel and probed for GAT1 (a), CCT6 (b, c) and ERG1 (d).

The arrowhead in a indicates signal from the CCT6 parental band that remained after stripping and reprobing for GAT1. e, Quantifications for b–d (mean ± s.d. for 3 cultures).
Extended Data Figure 6 | Spo11–oligo complexes in msh5 ndt80 double mutant. Representative time courses are shown.
Extended Data Figure 7 | Effects of dmc1 deletion or spo11 hypomorphic mutation on ZMM mutant phenotypes. a, b, ZMM status is irrelevant in a dmc1 background. Broken chromosomes accumulate to similar levels in a dmc1 single mutant and dmc1 zmm double mutants. Representative pulsed-field gel Southern blots probed for chromosome IX are in a and Poisson-corrected quantification of DSBs is in b (mean ± s.d., 3 cultures). c, Reducing Spo11 activity in a zip3 mutant partially alleviates the prophase I delay/arrest. Meiotic progression was assessed by staining with DAPI (4’,6-diamidino-2-phenylindole) and measuring the percentage of cells that had completed meiosis I (MI) with or without completing meiosis II (± MII). Data are means ± s.d. for 3 cultures, except wild type and spo11-HA, each analysed once.
Extended Data Figure 8 | Spo11-oligo mapping in wild type and zip3 mutant. a, b, Quantitative reproducibility of Spo11-oligo maps. In a, comparisons are shown for individual wild type (WT) or zip3 data sets from the present study, or the previously published spo11-HA data (from ref. 11). Uniquely mapped Spo11 oligos were summed in non-overlapping 5-kb bins and expressed as RPM per kb (plotted on a log scale). In b, pairwise correlation coefficients for the data sets from the current study are shown (Pearson’s r, box colours scaled from blue to red proportional to strength of correlation). For the comparison of this study’s wild-type average with data from Pan et al., \( r = 0.949 \). Note that Pan et al. used a different strain background with different auxotrophies, which may alter DSB distributions51,52, and a hypomorphic spo11 allele (spo11-HA), which may affect DSBs to different extents at different locations53. Note that biological replicates (WT-1 versus WT-2 or zip3-1 versus zip3-2) agreed better than comparisons between cultures of different genotype. c, DSBs form at the same hotspots and with similar distribution within and between hotspots in wild type and zip3. Unsmoothed Spo11-oligo maps are shown in the vicinity of the well-characterized ARE1 (YCR048w) hotspot.
Extended Data Figure 9 | Changes in the DSB landscape in zip3 mutant.

a, Change in Spo11-oligo counts in hotspots grouped by chromosomal context. Tel, within 20 kb of telomeres; Cen, within ±10 kb of centromeres; rDNA, from 60 kb leftward to 30 kb rightward of rDNA; Interstitial, all others. Dashed lines mark values assumed as no change and average change (1.8-fold). Boxes indicate median and interquartile range; whiskers indicate the most extreme data points which are ±1.5 times the interquartile range from the box; individual points are outliers. Subtelomeric and pericentric zones show less increase in zip3 on average, thus, ZMM-dependent feedback contributes less than other, unknown factors to suppressing DSBs in these regions. The zone near the rDNA showed no increase or was even decreased; thus, zip3 mutants are competent for this region’s DSB suppression, which is dependent on the ATPase Pch2 and the replication factor Orc1 (ref. 54). Note that the remaining interstitial hotspots showed highly variable response to zip3 mutation (≥20-fold). b, Correlation between log-fold change in Spo11-oligo counts in zip3 and the binding of the indicated proteins, binned in non-overlapping windows of varying size. Closed symbols, $P < 0.05$. ChIP data are from ref. 38. c, Average ChIP profiles around interstitial hotspots divided into three equal-sized groups according to the average fold change in zip3. Top, the box and whisker plot (as described for a) shows the distribution of fold changes for the three groups. Bottom, ChIP profiles for each of the indicated proteins. Note that the profiles lie atop one another for Rec102 and Rec104. Dashed arrows indicate direction of the change in the average profiles with increasing fold change in zip3. ChIP data are from refs 38 and 39. d, High degree of colinearity of log$_2$-transformed ChIP data$^{38}$ for Rec114, Mei4 and Mer2 (which are essential for DSB formation) and Hop1 and Red1 (axis proteins that promote normal DSB formation). More than 90% of the variance for this combination of ChIP data is captured in the first principal component (PC1). The high degree of correlation between these proteins was described previously$^{38}$. e, Correlations between the fold change in zip3 (zip3 FC, log$_2$ and assuming 1.8-fold increase genome-wide) and various chromosomal features: principal component 1 for Rec114, Mei4, Mer2, Hop1 and Red1 ChIP data (same as in d); chromosome size (log$_2$(bp)); G+C content (%); and ChIP data for the indicated proteins (log$_2$). In d and e, top right panels show pairwise scatter plots and bottom left panels show corresponding correlation coefficients (Pearson’s $r$) for data for interstitial regions binned in 35-kb non-overlapping windows. Essentially identical results were obtained with different window sizes (20–40 kb) or with varying placement of windows (data not shown). f, Essentially no correlation between DSB activity in wild type and change in zip3, whether considering interstitial regions divided into non-overlapping 35-kb bins (upper panel) or interstitial hotspots (lower panel). A 1.8-fold increase genome-wide in zip3 is assumed. Note: fold change is labelled according to a linear scale but plotted in a log scale in panels a, c, f.