Materials and methods

Strains
The rad51 (YTM134), rad54 (YTM132), and rad55 (YTM167) deletions were introduced into YDB057 by PCR-mediated gene disruption using kanMX4 (Wach et al., 1994) as a template. The rad52::hisG was introduced into YDB057 by crossing with ANY102 (S288C background). Alternatively, a rad52 mutant strain without GFP constructs (YTM171) was used, since a rad52 deletion mutant in the YDB057 background was too sick to analyze (it was easily reverted into DSB-deficient strain). The RAD52-YFP (YTM178; MATα, leu2, rad3, his3, RAD52-YFP-URA3-YFP) was constructed as described (Lisby et al., 2003) using a S288C derivative, YTM98 (MATα, leu2, rad3, his3).

Cytology
Cells were grown to a density of 1X10^7 cells/ml in YP-raffinose medium and HO endonuclease was induced by addition of 2% galactose. Strains repairing a DSB were treated after 60 min, collected and re-suspended with YP medium containing 2% glucose to repress the GAL10::HO gene. Cells were spheroplasted and surface-spread on glass slides in the presence of detergent (lipsol) and fixative (4% paraformaldehyde). After drying, nuclei were immuno-stained as described previously (Shinohara et al., 2000). The slides were incubated with either rabbit anti-Rad51 or rabbit anti-Rad52 and mouse anti-GFP (3E6; Molecular Probes) overnight at 4°C, followed by incubation with secondary antibodies for 2 h at room temperature. For double staining of Rad51 and Rad52, rabbit anti-Rad51 and rat anti-Rad52 were used. Alexa-488, -594 and –647-conjugated anti-IgG antibodies (Molecular Probes) were used as a secondary antibody. Epi-fluorescence microscopy was carried out using an Olympus BX51 microscope. Images were captured with a cooled charge-coupled device digital camera (Cool Snap; Photometrix) and processed using IP lab (Solution systems) and Photoshop (Adobe) software. For triple or quadruple staining, filter exchange was done using an automatic
filter wheel (Photometrix). The absence of offset between each filter was confirmed using 200nm micro-sphere beads with multiple colors (Tetraspeck™, Molecular Probes).

For analysis of DNA damage-induced focus formation, cells were grown to log phase in YPD medium and treated with 10 µg/ml of Zeocin (Invitrogen) for 3 h. Chromosome spreads were prepared and stained as described (Padmore et al., 1991; Shinohara et al., 2000).

For analysis of GFP and CFP foci in intact cells, cells were grown and induced with galactose, as described above. Under these conditions, the efficiency of HO endonuclease cutting in sir3 cells was determined by Southern blotting to be ~75-80% at each HO cut site (data not shown). Cells were fixed by direct addition of paraformaldehyde (2% final concentration) to 1 ml samples of cultures for 10 minutes at room temperature, pelleted, washed for 10 minutes in 0.1 M potassium phosphate (pH=6.6), pelleted again and resuspended in 50 µl of fresh potassium phosphate.

Visualization of LacI-GFP and TetR-GFP or -CFP fusion proteins bound to chromosomal operator arrays was achieved by fluorescence microscopy as follows. At least 100 nuclei per strain for each time-point were scored for GFP or CFP foci using an Olympus BX41 fluorescence microscope with Chroma GFP- and CFP-bandpass filters, respectively. Three-dimensional images were acquired as 16 optical sections spaced 0.2 µm apart along the Z axis using a cooled CCD camera mounted onto a DeltaVision™ fluorescence microscopy system as described (Muhlemann et al., 2001; Rines et al., 2002).

**Cumulative analysis**

Non-cumulative curves for foci and DSBs were converted into cumulative curves as described (Padmore et al., 1991; Shinohara et al., 2000). We assumed 88% of cells passed focus-positive stage, since 88% of cells suffered a DSB. In addition, the assumption that the same percent of cells passed through focus-positive stage was also employed. We assumed that most of DSBs are processed by the homologous recombination pathway (Haber, 1998). And mutants defective in the recombination accumulated up to 80%. Alternatively, we employed 73% for the value, which is
obtained from percent of recombinant molecules in Southern blotting. These two results were essentially similar (T. M. and A. S., unpublished results).

**Physical analysis**

**Southern blotting**

When cells were harvested for immuno-staining at intervals (see above), a second aliquot was collected for DNA analysis. Genomic DNA was prepared as described (White and Haber, 1990). The DNA was digested with Styl and subjected to electrophoresis in a 0.8% agarose gel for 18 h at 10 V/cm. Blots were visualized using a phosphor-imager, BAS2000 (Fuji film Co. Ltd.). For DSBs in the sir3 mutant, DNAs were digested with HindIII and probed with a fragment from X region.

**PCR analysis**

The above DNA was also used for PCR to analyze strand invasion/primer extension. Primers used here are forward (Ya, TTTTCTTTTAGTTTCAGCTTTCCGC) and reverse (MAT distal, TGAACCGCATGGGCAGTTTAC). Quantified analysis was carried out using a Real time PCR machine (iCycler™, Bio-Rad) using Syber-Green I (iQ™ Syber; Bio-Rad) as an indicator dye. Briefly, DNA samples were diluted in series and triplicates for each dilution were analyzed to obtain a standard curve; curves with more than collection-coefficient=0.997 were used for the standard. DNA samples at each time point were analyzed for triplicates. As an internal reference, primers for the ARS305 locus are used; forward, TGCAACAGTATTCCGAC; reverse, ACACGATCCACGCTGTCCCA.

**Formation of ssDNA complexes with Rad51 and Rad52**

13 µl of reaction mixture contains 20 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 1 mM DTT, 0.05% NP-40, 50 µg/ml BSA and 3 µM biotinated-dT₆₀ (or dT₃₀) in the presence of 2 mM ATP or its absence. 1 µl of Rad52 protein was added to the mixture and incubated for 5 min at 25 °C, followed by the addition of 1 µl of Rad51 protein and further 10-min incubation. The biotinated-DNA was captured by the addition of 5 µl of Dynabeads M-280 Streptavidin (Dynal) followed by further incubation for 15 min. The supernatants
were removed and proteins were eluted from the beads with 0.1% SDS. The eluates were analyzed by 10% SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R-250 or further visualized with Western blotting. Quantification of gels was carried out using an imager analyzer, LAS1,000 (Fuji Film Co.Ltd).
References

Supplementary Figure 1. Recombination intermediates and product during mating-type switching

(A) Southern blotting analysis. Wild type cells (YDB057) were incubated with medium containing galactose for one-hour, collected, and resuspended with medium containing glucose. The DNA was analyzed by Southern blotting using a probe described in Figure 1B. The blots (A) were analyzed, and amounts of each product were quantified and plotted (B). DSBs, closed triangles; products, closed diamonds. Lane 1, treated before galactose; Lanes 2-10, treated with galactose; Lane 11-19, treated without galactose.

(B) Quantification of DSBs and products as well as SIE intermediates. DNA at each time-point was quantified for SIE using PCR as described in Experimental Procedures.

(C) Southern analysis of DSB in sir3 cells. The sir3 mutant (YDB236) was incubated with medium containing galactose. The DNAs were analyzed as shown in Materials and methods.

Supplementary Figure 2. Rad51 and Rad52 focus formation in various mutants. The rad51 (YTM134), rad52 (YTM171), rad55 (YTM167) and rad54 (YTM132) mutants were analyzed for HO-induced Rad51 and Rad52 focus formation (both shown in red). The DSB was induced transiently by incubating each cell with medium containing galactose. Typical images of each focus at early (at t=90 min) and late times (at t=180 min) in various mutants are shown. The rad52 mutant lacks a GFP-binding site. Some spreads do not show a GFP spot (green), possibly due to the disappearance of the GFP-binding site by hyper-resection of the DSB ends in the mutants. Bars indicate 2 µm.

Supplementary Figure 3. DNA damage-induced Rad51 and Rad52 focus formation in Rad52-YFP cells. (A) Wild type (YTM98) and RAD52-YFP (YTM178) cells were treated with 40 µg/ml of Zeocin (Invitrogen) for 2 h. Chromosome spreads were prepared and stained with anti-Rad52 (green) together with anti-GFP (red) or anti-Rad51 (green) antibodies. Wild type shows punctate staining of Rad52 as well as Rad51. RAD52-YFP
YFP cells shows a few large focus containing both Rad52-YFP and Rad51. A bar indicates 2 μm. (B) Percentages of cells positive for one Rad51 or Rad52 focus before and after the treatment of Zeocin were measured. Two classes of number of the focus are shown; 1-3 foci or more than 4 foci per nuclei.
Supplementary Figure 1. Miyazaki et. al.

A

![Image of gel electrophoresis with markers for parental MAT, α, and Product.]

B

![Graph showing DSB, SIE, and Product with time in minutes on the x-axis and percentage of products on the y-axis.]

C

![Image of gel electrophoresis with markers for HML, HMR, DSB-HML, MAT, DSB-HMR, and DSB-MAT.]
Supplementary Figure 2
Miyazaki et. al.

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2 μm
Supplemental Figure 3. Miyazaki et. al.

A

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B