0.21 μM RPA, ± 0.4 μM Srs2), and the means (ø) ± 1 sd and distributions of filament length classes are shown. Scale bars: 100 nm. White arrows indicate RPA-ssDNA complexes and red arrows Rad51 filaments.

Figure 4. Rad55-Rad57 interact with Srs2 and inhibit Srs2 helicase. a, Pull-down with 4 nM (1 pmol) Rad55-Rad57 and 2.7, 8, or 16 nM Srs2. **b**, Pull-down with 4 nM Rad55-Rad57 and 8 or 16 nM Rad51. **c**, Quantitation of results in (a) and (b) and additional experiments. **d**, Pull-down with 4 nM Rad55-Rad57 and 8 nM Srs2 ± 40 nM Rad51. GST was used as control. S: supernatant, W: wash, E: eluate. **e**, Helicase assay. **f**, 28 nM Rad51 ± 25 nM Rad55-Rad57 were incubated with 1.5 nM 3'-tailed substrate before addition of 120 nM Srs2 protein. Product yields at 20 min. were quantified as shown in **g**. HEAT DEN.: heat denatured substrate, shown are means ± 1sd, n=3.

Methods

Protein purification: Yeast Rad51, RPA, and Srs2 proteins were purified as described [1,2]. The purification of Rad55-Rad57 was adapted from a previously published protocol [3]. Yeast cells overexpressing GST-Rad55-His6-Rad57 were grown and harvested as described [3]. Cells were disrupted in Buffer B containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 1M NaCl, 10 % (v/v) Glycerol, 10 mM β-mercaptoethanol, and protease inhibitor cocktail (1 mM PMSF, 2 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidin) using glass beads (0.5 mm glass beads; BioSpec Products, Inc.). The cell lysate was centrifuged at 40,000 rpm for 45 min using a Ti50.2 rotor. The supernatant was collected and loaded onto a pre-equilibrated Glutathione Sepharose

4B column (GE Healthcare, NJ). After washing with buffer A (20 mM Tris HCl pH 7.5, 1 mM EDTA, 1 M NaCl, 5 mM β-mercaptoethanol, and 10 % glycerol), the GST-tagged proteins were eluted with Buffer A containing 20 mM reduced glutathione plus protease inhibitor cocktail. Fractions containing the GST-Rad55-His6-Rad57 heterodimer, as determined by 10 % SDS-PAGE, were pooled and dialyzed against Buffer C (50 mM NaH_2PO_4 pH 8.0, 1 M NaCl, and 10% glycerol) containing the protease inhibitor cocktail. Then the pool was loaded onto a pre-equilibrated Ni-NTA agarose column and washed with Buffer C plus protease inhibitor cocktail. The bound complexes were eluted with Buffer C containing 0.5 M NaCl, 0.1 mM PMSF, and 250 mM imidazole, and analyzed by 10 % SDS-PAGE. Fractions containing stoichiometric Rad55-Rad57 heterodimer were pooled, concentrated, dialyzed into the storage buffer containing 20 mM Tris HCl pH 7.5, 0.5 M NaCl, 0.1 mM EDTA, 1 mM DTT, and 10 % glycerol, and then stored in aliquots at -80°C. The absence of contaminating enzymatic activities and DNA in protein preparations was verified as described [4].

Purification of 600 nt ssDNA: As published previously[5], 600 bp dsDNA fragments biotinylated at a one 5' end were generated by PCR from *Pst*I-linearized ϕX174 DNA using primers WDHY427 5′-TTATCGAAGCGCGCATAAAT-3′ and 5′ biotinylated WDHY431 5′-GTCTTCATTTCCATGCGGTG-3′. The biotinylated dsDNA was loaded onto a HiTrap Streptavidin HP column (Amersham Biosciences), and non-biotinylated single stranded DNA was eluted with 60 mM NaOH.

Rad51-ssDNA filament assembly assay: In Figure 1b, Rad51 (0.67 μM) was incubated with 4 μM (nt) ssDNA, in the presence or absence of 0.11 μM Rad55-Rad57, in buffer R containing 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 2.5 mM ATP, 25 μg/mL BSA, 1 mM DTT, 90 mM NaCl, and 5 % glycerol for 10 min. Then 0.25 % glutaraldehyde was used to crosslink the protein-DNA complexes for 15 min. The complexes were separated on a 0.5 % agarose gel, stained with SYBR Gold, transferred to nitrocellulose membrane, and blotted with rabbit polyclonal anti–Rad51 or anti–Rad55 antibodies.

Rad51-ssDNA filament salt challenge assay: In Supplementary Figure 3, Rad51 (0.267 μM) was incubated with 4 μM (nt) ssDNA, in the presence or absence of 0.067 μM Rad55-Rad57, in buffer R containing 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 2.5 mM ATP, 25 μg/mL BSA, 1 mM DTT, and 5 % glycerol for 10 min. Then 5 M stock NaCl solution was added to the reaction to reach a final concentration of 500 mM for a further incubation of 30 min. Glutaraldehyde (0.25 %) was used to crosslink the protein-DNA complexes for 15 min. Complexes were separated on a 0.5 % agarose gel and stained with SYBR Gold. Proteins were transferred to nitrocellulose membrane and blotted with anti–Rad51 antibodies. All DNA concentrations refer to nucleotides (ssDNA) or base pairs (dsDNA).

Protein binding to ssDNA immobilized on magnetic beads: In Figure 2b, a 5′-biotinylated oligonucleotide was immobilized onto magnetic streptavidin beads as previously described [6]. The oligo sequence is 5′-CCCCCCCCCCCCCAAGATAATTTTTCGACTCATCAGAAATATCCGA AAGTGTTAACTTCTGC GTCATGGAAGCGATAAAACTC-3′. In experiments containing Srs2, 10

μL slurry of beads containing 3 μM (nt concentration) ssDNA were incubated with 1 μM Rad51 in the presence and absence of 0.1 μM Rad55-Rad57 in buffer containing 20 mM trienthanolamine, 5 mM $Mg(OAc)_2$, 4 mM ATP, 25 μg/mL BSA, 1 mM DTT, 5 % glycerol, and 25 mM NaCl for 10 min at 22°C. Then 0.1 or 0.33 μM Srs2 protein was added and further incubated for 10 min. The beads were washed, and bound proteins were eluted and quantified as described [7]. Background protein binding was typically less than 3 %.

Topology-based assay for Rad51 dissociation: A published protocol was modified slightly for this assay using M13mp18 ssDNA [8]. In Supplementary Figure 4, 375 nM Rad51, with 0, 80, and 120 nM Rad55-Rad57, were incubated with 9 μM (in nucleotides) circular M13mp18 ssDNA in 25 μL of buffer containing 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 25 μg/mL BSA, 1 mM DTT, and an ATP-regenerating system consisting of 2.5 mM ATP, 20 units/ml creatine kinase, and 20 mM creatine phosphate for 10 min at 30 °C. Then, 100 nM Srs2 and 150 nM RPA were added and incubated for 10 min, before the addition of topologically relaxed pUC19 dsDNA (7 μM in bp) and wheat germ DNA topoisomerase I (3 units). After another 10 min incubation, reactions were stopped by addition of 4 μL stop solution consisting of 1% (w/v) SDS, 75 mM EDTA, 10 mg/mL protease K and further 30 min incuation at 37 °C. DNA species were resolved by electrophoresis on a 1 % TBE-agarose gel and visualized using UV transillumination after ethidium bromide staining. The results were quantitated using ImageQuant.

Protein interaction assays: GST-Rad55-His6-Rad57 (4 nM) or 30 nM GST (GE Healthcare) were incubated with increasing amounts of either Srs2 or Rad51 in buffer P containing 25 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 50 mM NaCl, 1 mM DTT, 10 % glycerol, and 0.05 % NP-40 for an hour at room temperature (Fig. 4a-d). Equilibrated and BSA-treated Glutathione-Sepharose 4B beads were added to the mixture and incubated for another hour. The beads and supernatant were separated through centrifugation and the beads were washed twice with binding buffer P. The pulled-down protein complexes were eluted by boiling at 95°C for 3 min in 10 μL SDS-PAGE loading buffer, separated through a 10 % SDS-PAGE gel, and the protein bands were visualized through immunoblots and quantitfied by ImageQuant. In Figure 4a-b, 1/16th of the supernatant and wash were loaded. In Fig. 4d, 1/7th of the supernatant and wash were loaded. For the competition protein binding assay (Supplementary Figs. 7, 8), the two proteins were incubated for 30 min before the addition of an increasing amount of the third challenging protein, as specified in the diagrams. After another 30-min incubation, equilibrated and BSA-treated Glutathione-Sepharose 4B beads were added to the mixture and incubated for another hour. Analysis and quantitation was performed as described above. The anti-Rad51, Rad55, Rad57 antibodies were generated in rabbits, the anti-Srs2 antibody was purchased from Santa Cruz Biotechnology, Inc.

Helicase assay: The assay followed a published protocol and the substrates were prepared exactly as described before [9]. In Figure 4f, 28 nM Rad51 with 0 or 25 nM Rad55-Rad57 were incubated with 1.5 nM oligo substrate with 3′ tail in buffer containing 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 25 μg/mL BSA, an ATP-regenerating system consisting of 2.5 mM ATP, 20 units/ml creatine kinase, and 20 mM creatine phosphate, as well as either 1 mM DTT

and 40 mM NaCl (Fig. 4e-g, Fig. S10c, d) or 5 mM DTT and 10 mM NaCl (Fig. S10a) for 10 min at 30 °C. Then 120 nM Srs2 protein was added to initiate the helicase reaction. After 20 min incubation, the reactions were stopped by adding 4.5 μL stop buffer containing 150 mM EDTA, 2 % SDS, 163 nM unlabeled oligo, and 4.3 mg/mL protease K into 9 μL reaction sample. The DNA species were separated through electrophoresis on a 10 % TBE-PAGE gel, which was dried and analyzed by a Storm phosphoimager. The bands were quantified by densitometry using ImageQuant.

DNA strand exchange assay: In Supplementary Figure 11, Rad51 (3.3 μM) was incubated with 0.3 μM Rad55-Rad57 or the corresponding amount of Rad55-Rad57 storage buffer and 10 μM ϕX174 ssDNA for 15 min at 30 °C in buffer containing 30 mM Tris-Acetate (pH 7.5), 4 mM $Mg(OAc)_2$, 75 mM NaCl, 1 mM DTT, 2.5 mM ATP, 50 μg/mL BSA, 20 mM phosphocreatine, and 80 ng/μL creatine kinase. 0.56 μM RPA and 0, 333, 222, 167, 125 nM of Srs2 were added, and incubated for another 30 min. Then 10 μM (bp) *Pst*I-linearized ϕX174 dsDNA and 4.8 mM spermidine were added and further incubated for 120 min. Samples were deproteinized and separated by electrophoresis on a 0.8 % TBE-agarose gel. Images were recorded using a FluorChem8900 imaging system (Alpha Innotech) after staining with SYBR-Gold (Invitrogen), and quantified with ImageQuant. Percentage of joint molecule (JM) was calculated according to the equation JM%=(JM/1.5)/(JM/1.5+NC+dsDNA). Percentage of product formation was calculated according to the equation product%=(JM/1.5+NC)/(JM/1.5+NC+dsDNA).

Electron microscopy: To assemble the protein-DNA filament, 2.34 μM Rad51 protein, in the presence or absence of 0.43 μM Rad55-Rad57, was incubated with 7 μM 600 nt ssDNA (+) strand for 10 min at 30°C in 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 1 mM DTT, and 3 mM ATP. RPA (0.21 μM) was added and incubated for another 10 min. Lastly, 0.4 μM Srs2 or buffer control was added and incubated for 10 min. The reaction mixtures were diluted 20-fold in 10 mM Tris-HCl pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$ without chemical fixation. The samples were adsorbed onto 400 mesh carbon-coated copper grids (Ted Pella, Inc., Redding CA), negatively stained with 2 % (w/v) uranyl acetate, blotted, and air-dried. Grids were imaged in a JEOL JEM-1230 transmission electron microscope (JEOL Ltd, Japan). Images of Rad51-filaments were randomly collected from different areas on the grid. 6-10 grids were used for each condition. Images were recorded at a nominal magnification of 40,000× under minimum dose procedures on a Tietz 2048 × 2048 pixel CCD camera (TVIPS, Germany). Immunoaffinity gold labeling of GST-Rad55, as shown in Figure 1d, was adapted from a published protocol [10]. In brief, Rad51-Rad55-Rad57-ssDNA complexes were assembled as described above and crosslinked with 0.25 % glutaraldehyde for 20 min, before deposition on grids. Grids were blocked in 50 μg/mL BSA in TBST for 30 min, and then incubated with goat anti-GST antibody (GE Healthcare) for 30 min. After three 5 min washes with 50 μg/mL BSA in TBST, the grids were incubated in TBST plus a 1:5 dilution of gold particles dressed with rabbit anti-goat antibody (BioAssay Works). After two five min washes in 50 μg/mL BSA in TBST and one five min wash in 5 mM $Mg(OAc)_2$, grids were stained with 2 % (w/v) uranyl acetate before imaging.

***Saccharomyces cerevisiae* strains** are listed in Supplementary Table 2.

Recombination assay: Spontaneous recombination rates between direct repeats were determined following a published fluctuation analysis protocol using the method of the median [11,12]. The direct-repeat recombination substrate has two different *ade2* alleles separated by plasmid sequences and the *URA3* gene [13]. Yeast strains were grown on YPD plates for 2 days at 30°C for single colonies. For each strain, nine independent single colonies were randomly chosen and the entire colony was used to inoculate 4 mL YPD liquid culture. Liquid cultures were grown for 2-3 days at 30°C to reach stationary phase. Cells were collected, washed with sterile H_2O, and suspended into 1 mL sterile H_2O. 100 μL of appropriate dilutions of each culture were spread on two plates each of SD-ADE-URA. Cells were incubated for 2 days at 30°C. For each culture, the number of colonies on YPD were counted and totaled to determine the total cell number. The number of colonies on SD-ADE-URA were counted to determine the median number of recombinants. For each strain, recombination rates were measured independently three times and the mean values with standard deviations are shown.

MMS sensitivity assay: Yeast strains were grown overnight in liquid YPD to mid-log phase at 30°C, and then diluted to OD_{600} = 1. Serial dilutions of these cell cultures were made with sterile H_2O and spotted onto YPD plates with or without methyl methanesulfonate (MMS). Plates were incubated for three days at 30°C or five days at 22°C before photographing using a FluorChem8900 imaging system (Alpha Innotech).

IR survival assay: Exponentially growing cells ($1x10^7$ to $2x10^7$/ml) in YPD medium at 28°C were collected by centrifugation, washed in cold saline (0.9 % NaCl) , sonicated and resuspended in saline at the desired concentration. The cell suspension was γ-irradiated in a ^{137}Cs irradiator delivering 20 Gy/min. Aliquots of appropriate dilutions were spread on YPD-containing plates prewarmed at either 23°C or 34°C. The plates were incubated at the corresponding temperature for 4 days (34°C) or 6 days (23°C) before counting the colonies. Platings were done in duplicate. The experiments were repeated at least 3 times, and the result of one typical assay is shown.

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