Supplementary Table 1. Yeast strains

Strain	Genotype*	Source or reference
LSY0697	*MAT**a** met17-s ADE2*	[1]
LSY1026	*MAT**a** met17-s ADE2 mus81::KanMX6*	[2]
LSY1801-4C	*MAT**a** met17-s ADE2 mus81:: KanMX6 yen1::HIS3*	This study
LSY2105-5A	*MAT**a** met17-s ADE2 rad1::LEU2 yen1::HIS3*	This study
LSY2105-6D	*MAT**a** met17-s ADE2 rad1::LEU2 mus81::KanMX6*	This study
LSY2105-29B	*MAT**a** met17-s ADE2 mus81:: KanMX6 rad1::LEU2 yen1::HIS3*	This study
LSY2108-3C	*MAT**a** met17-s ADE2 rad1::LEU2*	This study
LSY2109-104C	*MAT**a** yen1::HIS3 met17-s ADE2*	This study
LSY2202-10C	*MATα ade2-n his3::NatMX4 met22::klURA3 rad1::LEU2*	[2]
LSY2202-11A	*MATα ade2-n his3::NatMX4 met22::klURA3 mus81::KanMX6 rad1::LEU2 yen1::HIS3*	[2]
LSY2202-15D	*MAT**a** ade2-n his3::NatMX4 met22::klURA3*	[2]
LSY2202-17B	*MATα ade2-n his3::NatMX4 met22::klURA3 mus81::KanMX6 rad1::LEU2*	[2]
LSY2202-19D	*MATα ade2-n his3::NatMX4 met22::klURA3 mus81::KanMX6 yen1::HIS3*	[2]
LSY2202-24C	*MAT**a** ade2-n his3::NatMX4 met22::KLURA3 rad1::LEU2 yen1::HIS3*	[2]
LSY2202-26A	*MAT**a** ade2-n his3::NatMX4 met22::klURA3 mus81::KanMX6*	[2]
LSY2202-42A	*MAT**a** ade2-n his3::NatMX4 met22::klURA3 yen1::HIS3*	[2]
LSY2205-3A	*MATα ade2-I lys2::GAL-ISCEI his3::HphMX4 rad1::LEU2 yen1::HIS3*	[2]
LSY2205-6A	*MATα ade2-I lys2::GAL-ISCEI his3::HphMX4 mus81::KanMX6*	[2]
LSY2205-7C	*MAT**a** ade2-I lys2::GAL-ISCEI his3::HphMX4 mus81:: KanMX6 rad1::LEU2*	[2]
LSY2205-11C	*MATα ade2-I lys2::GAL-ISCEI his3::HphMX4*	[2]
LSY2205-11D	*MAT**a** ade2-I lys2::GAL-ISCEI his3::HphMX4 rad1::LEU2*	[2]
LSY2205-24D	*MATα ade2-I lys2::GAL-ISCEI his3::HphMX4 yen1::HIS3*	[2]
LSY2205-67B	*MAT**a** ade2-I lys2::GAL-ISCEI his3::HphMX4 mus81::KanMX6 rad1::LEU2 yen1::HIS3*	[2]
LSY2205-77B	*MAT**a** ade2-I lys2::GAL-ISCEI his3::HphMX4 mus81::KanMX6 yen1::HIS3*	[2]
LSY1929	*MAT**a**-inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO*	[3]
LSY2540-8A	*MAT**a**-inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO rad1::LEU2*	This study
LSY2540-12A	*MAT**a**-inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO mus81::KanMX6*	This study

LSY2540-16C	*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO yen1::HIS3*	This study
LSY2540-18A	*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO mus81::KanMX6 yen1::HIS3*	This study
LSY2540-19D	*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO mus81::KanMX6 rad1::LEU2*	This study
LSY2566-3D	*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO rad1::LEU2 yen1::HIS3*	This study
LSY2566-5A	*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs inc* (1.2kb) *ade3::GAL-HO rad1::LEU2 yen1::HIS3 mus81::KanMX6*	This study
LSY2520	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO*	[4]
LSY2521	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO yen1::KanMX6*	[4]
LSY2548-1D	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO rad1::LEU2*	This study
LSY2548-11A	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO rad1::LEU2 mus81::KanMX6*	This study
LSY2548-29C	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO mus81::KanMX6 yen1::HIS3*	This study
LSY2548-39D	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO mus81::KanMX6 rad1::LEU2 yen1::HIS3*	This study
LSY2548-42A	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO rad1::LEU2 yen1::HIS3*	This study
LSY2548-66B	*MAT***a**-*inc ura3::HOcs lys2::ura3-HOcs inc* (5.6 kb) *ade3::GAL-HO mus81::KanMX6*	This study

*All strains are of the W303 genotype (*his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100*), only mating type and differences from the standard genotype are listed.

References

1 Bartsch, S., Kang, L. E. & Symington, L. S. RAD51 is required for the repair of plasmid double-stranded DNA gaps from either plasmid or chromosomal templates. *Mol Cell Biol* **20**, 1194-1205 (2000).

2 Ho, C. K., Mazon, G., Lam, A. F. & Symington, L. S. Mus81 and Yen1 promote reciprocal exchange during mitotic recombination to maintain genome integrity in budding yeast. *Mol Cell* **40**, 988-1000 (2010).

3 Aylon, Y., Liefshitz, B., Bitan-Banin, G. & Kupiec, M. Molecular dissection of mitotic recombination in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* **23**, 1403-1417 (2003).

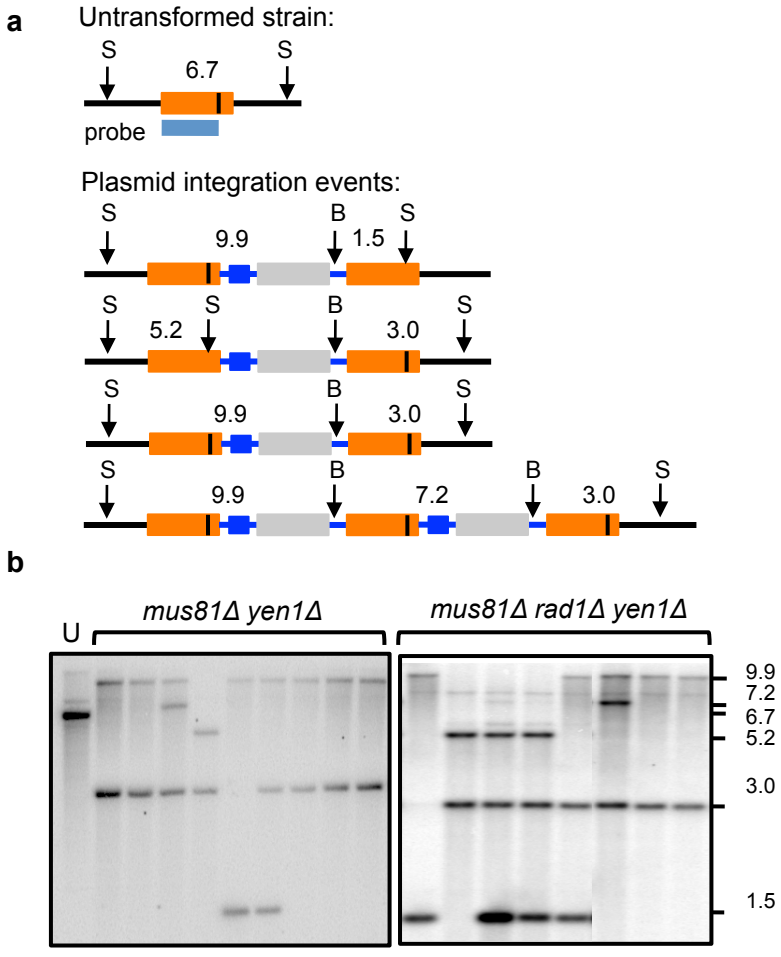
4 Agmon, N., Yovel, M., Harari, Y., Liefshitz, B. & Kupiec, M. The role of Holliday junction resolvases in the repair of spontaneous and induced DNA damage. *Nucleic Acids Res* **39**, 7009-7019 (2011).

Supplementary Table 2a. Number of crossover (CO), noncrossover (NCO) and break-induced replication (BIR) events in red/white sectored colonies

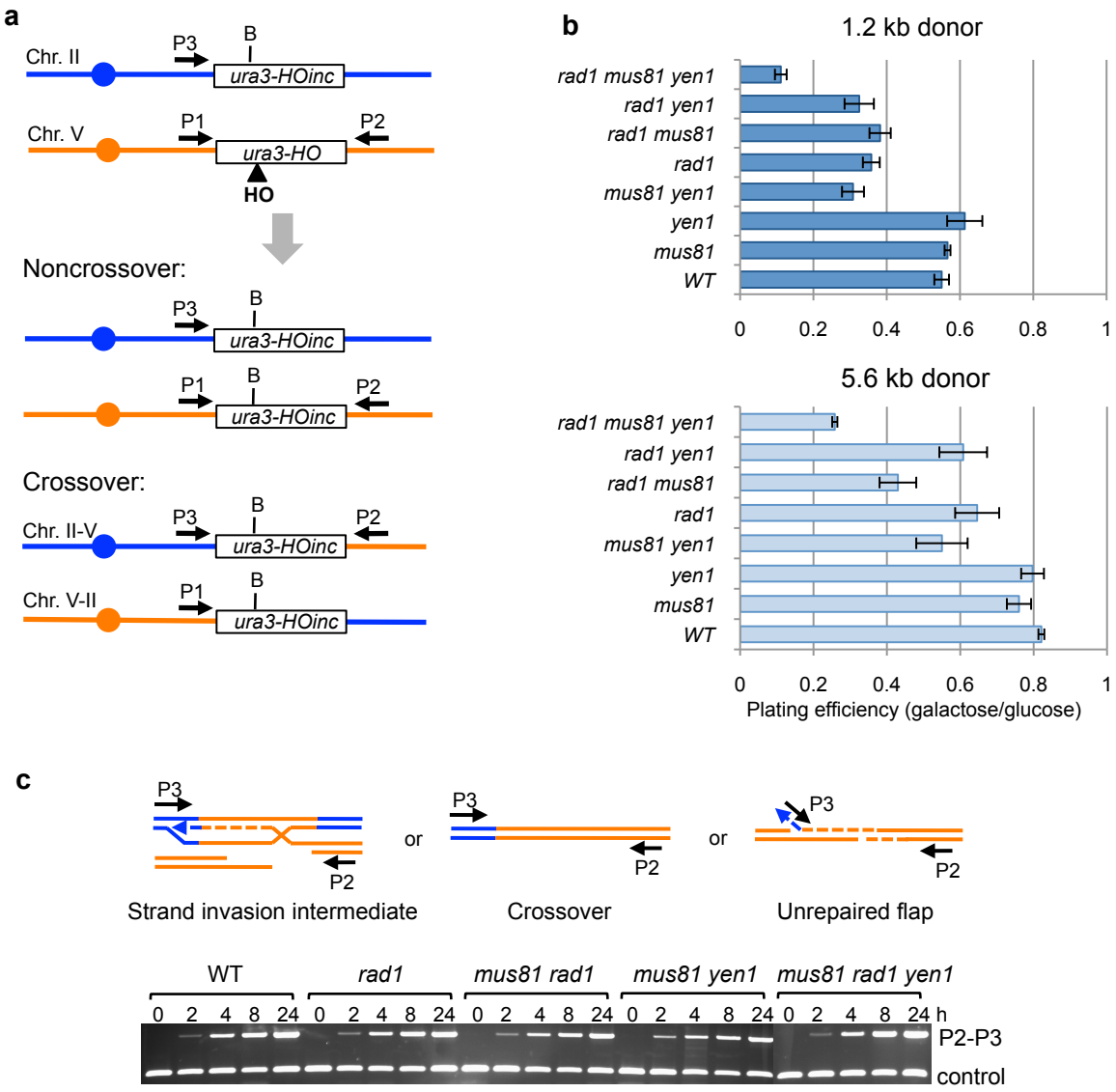
	CO	NCO	BIR	Total
WT	290	939	15	1244
yen1	114	324	4	442
mus81	114	525	15	654
rad1	270	870	26	1166
rad1 yen1	74	243	7	324
mus81 rad1	108	454	24	586
mus81 yen1	6	166	22	194
mus81 rad1 yen1	0	92	20	112

Supplementary Table 2b. Fisher's exact test *P* values between relevant strains for CO events (gray area) or BIR events (white area) in red/white-sectored colonies

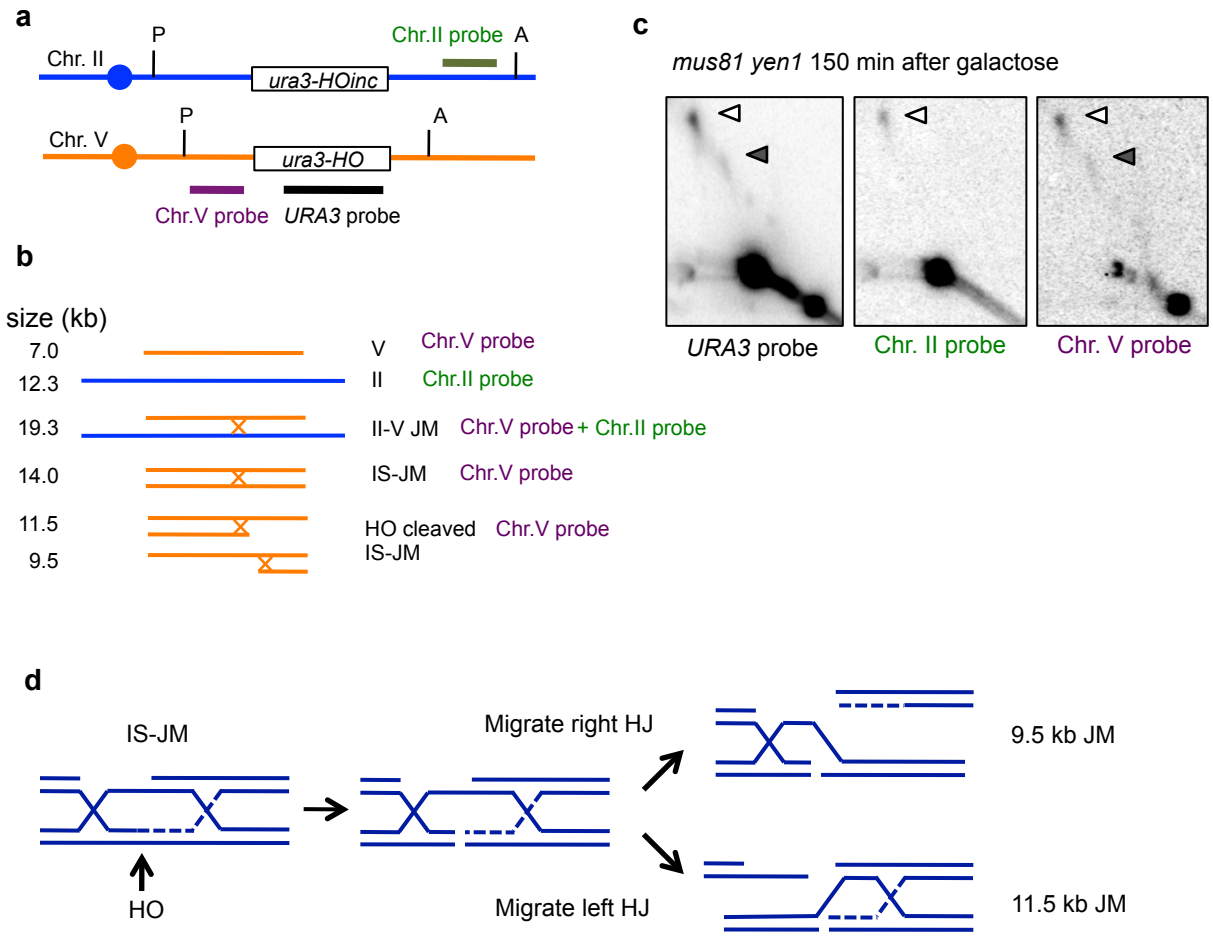
	WT	*mus81*	*rad1*	*mus81 rad1*	*mus81 yen1*	*mus81 yen1 rad1*
WT	--	0.003	0.96	0.018	0.0001	0.0001
mus81	0.19	--	0.004	0.66	0.0001	0.0001
rad1	0.06	0.86	--	0.023	0.0001	0.0001
mus81 rad1	0.0002	0.075	0.033	--	0.0001	0.0001
mus81 yen1	0.0001	0.0001	0.0001	0.0006	--	0.089
mus81 yen1 rad1	0.0001	0.0001	0.0001	0.0001	0.1224	--



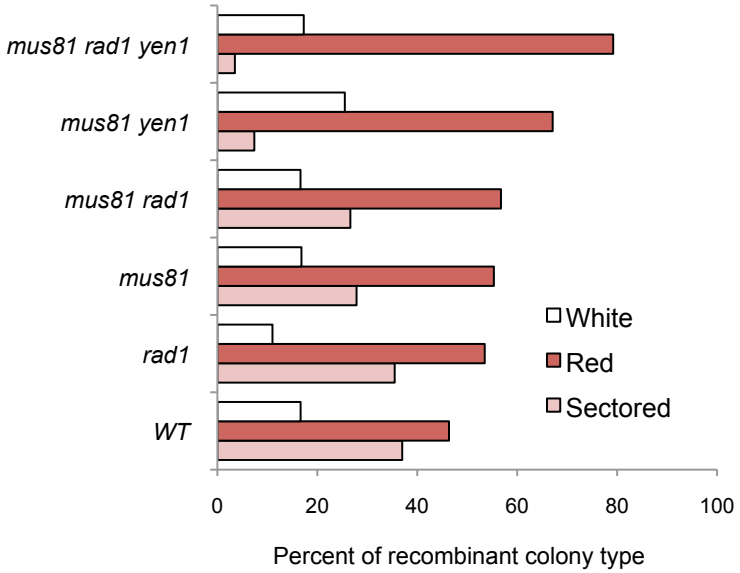
Supplementary Figure 1. Homologous plasmid integration occurs in the absence of Mus81 and Yen1. a Digestion of genomic DNA with SnaBI (S) and BamHI (B) distinguishes between the untransformed strain (U), random integration and homologous integration at the *MET17* locus. Conversion of the chromosomal *met17-s* allele gives rise to the distinct restriction fragments observed from independent transformants. **b** Representative transformants with a stable Ura$^+$ phenotype from the *mus81Δ yen1Δ* and *mus81Δ rad1Δ yen1Δ* mutants.



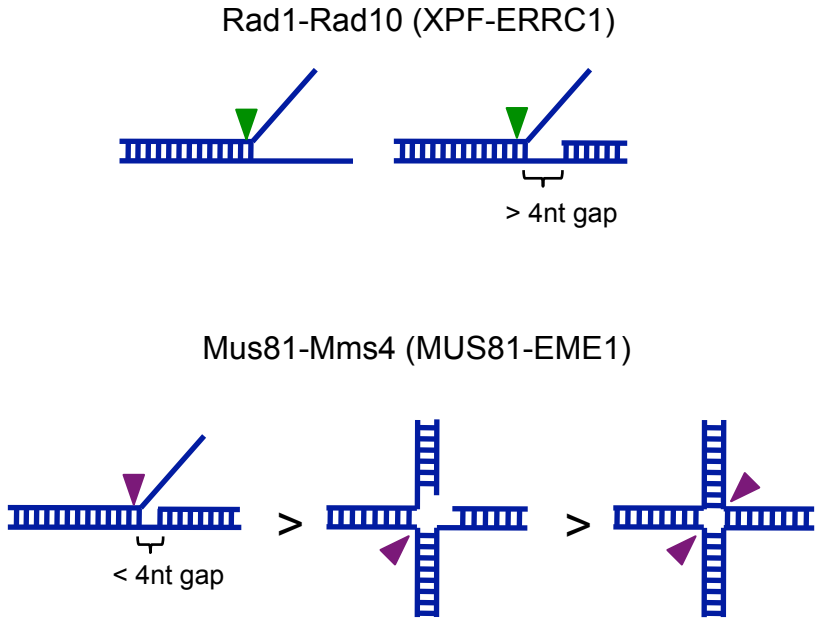
Supplementary Figure 2. Rad1 is required for noncrossover repair between 1.2 kb ectopic repeats. a, Schematic representation of the ectopic recombination assay; 1.2 or 5.6 kb sequences homologous to the *URA3* sequence and containing a mutant HO site (HOinc) are inserted at the *LYS2* locus on Chr. II. Primers P1, P2 and P3 are used to monitor cutting efficiency and repair of the 1.2 kb substrate by PCR. **b**, Repair efficiency was determined by the number of colony-forming units on galactose-containing medium compared with glucose-containing medium using at least three colonies for each strain. Errors bars show standard deviations and significance was determined by the *t* test. **c**, PCR using primers P2 and P3 detects strand invasion intermediates, crossover products and noncrossover products with an unrepaired heterologous flap.



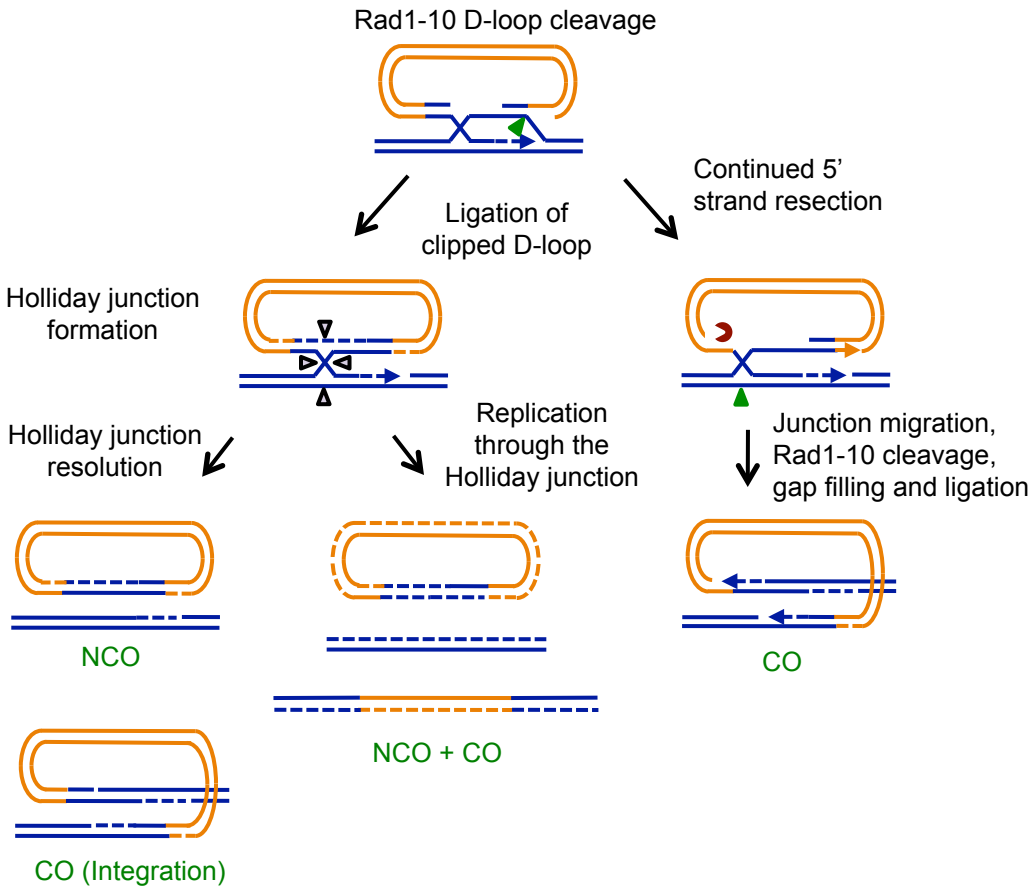
Supplementary Figure 3. Identification of joint molecules using chromosome-specific probes. a, Cartoon shown the locations of the *URA3* probe, Chr.II and Chr.V probes used for hybridization of the 2D gel membrane. **b**, Depiction of all classes of JMs detected and their expected hybridization to chromosome-specific probes. **c**, membrane from the *mus81 yen1* mutant, 150 min time point hybridized with each of the probes. **d**, Model to explain formation of 11.5 and 9.5 JM by HO cleavage of the IS-JM. An IS-JM forms by invasion of an uncut sister chromatid by the HO cut sister. If the IS-JM is not fully gap filled and ligated then the HO would cleave only the newly synthesized strand and its complement and not the D-loop. Branch migration of one of the two junctions towards the DSB would release the junction resulting in a Y-shaped molecule of the same size predicted for a single-end invasion intermediate.



Supplementary Figure 4. Percent of each recombinant colony type from the diploid recombination assay. Colonies formed after 1-3 h I-SceI induction were scored as solid red, solid white or red/white sectored in each strain (>500 colonies were scored for each strain from at least three independent trials).



Supplementary Figure 5. Preferred substrates for Rad1-Rad10 and Mus81-Mms4 cleavage.



Supplementary Figure 6. Model for plasmid integration by replication of a sHJ intermediate or Rad1 cleavage. We suggest Rad1-Rad10 clips the D-loop after second end capture if there is a single-stranded DNA gap adjacent to the branch point. Following gap filling and ligation a sHJ intermediate is formed that can be resolved by endonucleolytic cleavage or by replication through the junction at the next S-phase. Alternatively, after the first Rad1-Rad10 cleavage and migration of the crossed stranded structure to the other heterology boundary, if resection continued beyond the branch point the structure could be cut by Rad1-Rad10.