Molecular Cell, Volume *30*

Supplemental Data

Microarray-Based Genetic Screen Defines *SAW1*, a Gene Required for Rad1/Rad10-Dependent Processing of Recombination Intermediates

Fuyang Li, Junchao Dong, Xuewen Pan, Ji-Hyun Oum, Jef D. Boeke, and Sang Eun Lee

Supplemental Experimental Procedures.

Strains. All single and double gene deletion mutants were constructed by one step gene replacement using a PCR-derived *KANMX* module (Wach et al., 1994). Double mutant strains were constructed by first replacing the *KANMX* selectable marker from the single gene deletion using a DNA fragment containing the *TRP1* marker, and then deleting the second gene by one step gene replacement using the now available *KANMX* module again. Individual tests for SSA mutants using a plasmid-based SSA assay were carried out using the yeast nonessential gene deletion collection (both *MAT***a** and **a**/α; Invitrogen). Yeast strain expressing Rad1-3HA were constructed by replacing the endogenous *RAD1* gene in the tNS1379 strain with the PCR product carrying *RAD1-3HA* and the *KANMX* marker. To construct *rad1-D825A* mutant, *RAD1* gene fused to 3HA was cloned to the *Kpn*I/*Eco*RI site in pUC18 to generate pDJ5, and mutagenized by Quick Change site directed mutagenesis kit using the primer set, DJP26 (AGGATGTCGTCATTGTGGCCACACGTGAGTTTAATGCCTC) and DJP32 (GATGAAGGACAGTCGTTTTC), according to the manufacturer's manual (Stratagene, Inc). After replacing the genomic *RAD1* to the mutant *rad1-3HA*, *rad1* mutation was

verified by the UV sensitivity test, cleavage by *Msc*I and DNA sequencing of the whole *RAD1* gene.

Microarray analysis of mutants. The *Bsu*36I restriction enzyme-linearized or circular pNSU208 was transformed to yeast deletion pools comprising all nonessential gene deletions in haploid or diploid cells by a standard yeast transformation protocol (Gietz and Schiestl, 1991). Transformants were pooled and the genomic DNA was isolated by the glass bead genomic DNA extraction. The UPTAG and DOWNTAG bar codes uniquely assigned to each gene deletion strain were amplified by Cy3 (green)-labeled universal primers for linearized pNSU208 or Cy5 (red)-labeled universal primers for circular plasmid. Primer sequences, PCR and hybridization conditions, and microarray analysis were previously described (Ooi et al., 2001; Yuan et al., 2005). Further analysis was limited to the mutants showing greater than a 3-fold reduction in linear plasmid transformation efficiency vs. that with the circular plasmid.

Ribosomal DNA recombination assay. Wild type and the mutant derivatives carrying an *ADE2* marker integrated into the ribosomal DNA array were grown overnight and then plated onto YEPD plate. Colonies were allowed to grow for 48 h at 30°C, and then placed at 4°C for additional 48 h. The number of half-red half-white colonies was determined; each was assumed to represent a marker loss event during the first cell division after plating. The number of half-sectored colonies divided by the total number of colonies (not including entirely red colonies) was reported as the rate of rDNA recombination.

Chromosomal SSA assay. tNS1379 and derived cells grown in SC-Trp medium to $2x10^7$ cells/ml were induced to HO expression by addition of galactose at the final concentration of 2%. At indicated time points, genomic DNA was isolated, and digested

with *Bgl* II (NEB), subjected to Southern blotting hybridization with a radiolabeled probe shown in Fig. 1A.

Measurement of 3' flap cleavage efficiency during gene conversion. Yeast centromeric plasmids carrying two copies of inverted *lac*Z sequences, one of which contains an HO recognition site and various sizes of sequence deletions (Colaiacovo et al., 1999) were transformed into SLY556 and its derivatives lacking the HO recognition site at the *MAT* locus. Yeast transformants were grown overnight in SC–Ura medium with glycerol as a sole carbon source to ~$3x10^7$ cells per ml. Serial dilutions of cultures were plated onto YEP-dextrose and YEP-galactose media. After 3 days of growth at 30^{o}C, colonies were replica-plated onto SC-Ura plate to measure the percentage of plasmid retention. The percentage of plasmid retention is calculated as the fraction of colonies retaining the repaired plasmid on SC–Ura divided by the total number of colonies on YEPD. A minimum of 1000 colonies per strain was examined.

Monitoring the kinetics of 3' flap appearance and removal during SSA. Logarithmic cultures of yeast tNS1379 and its mutant derivatives in pre-induction glycerol medium were induced to create a DSB in chromosome V flanked by *ura3* direct repeat sequences by addition of 2% (final conc. w/v) galactose to the medium. Aliquots of yeast cultures were removed at one-hour intervals before and after HO expression, washed with ice-cold water and genomic DNA was isolated by standard glass bead and phenol/chloroform extraction. Genomic DNA was digested by *Sau*3AI for 16 h. The heat inactivated genomic DNA digests were subjected to real time PCR using DJP5 (GAGCACAGACTTAGATTGGT) and DJP6 (CCCGACTATGCTATTTTAATCA) primers to amplify the 3' flap sequence, 5'ACT1

(GTTCCCAGGTATTGCCGAAAGAA) and 3'ACT1 (TGGTGAACGATAGATGGACCAC) primers to monitor *Sau*3AI cutting efficiency, and 5'-PRE1 (CCCACAAGTCCTCTGATTTACATTCG) and 3'-PRE1 (ATTCGATTGACAGGTGCTCCCTTTTC) primers as control. The percentage of *Sau*3AI resistant DNA was calculated as the 2 X PCR values of DJP5/DJP6 primer from each time point divided by corresponding *PRE1* PCR values and the PCR efficiency of DJP5/DJP6. The PCR efficiency of DJP5/DJP6 is obtained by dividing PCR value of DJP5/DJP6 with 5'-PRE1/3'-PRE1 before induction (t=0) sample. Only the samples with >95% *Sau3*AI cutting efficiency in ACT1 PCR were used to calculate the percentage of *Sau3*AI resistant DNA at the HO–induced DSB.

Yeast two-hybrid assay. The SSA proteins (Rad1, Rad10, Msh2, Msh3, Srs2, Slx1, Slx4, Saw1, Rad52, Rad59) fused to the DNA binding domain or the transcription activation domain of Gal4 were expressed in two different haploid yeast strains of opposite mating type (*MAT*α and *MAT***a**). The strains were then mated and serial dilutions of the resulting diploid strains were plated on yeast synthetic media with or without histidine and 20 mM 3-AT. Plates were incubated at 30^{o}C for 3-4 days; activation of the reporter gene (*HIS3*) in the diploid strain indicated an interaction between protein pairs.

Pull-down assay for detecting protein complexes in extracts. GST-Saw1 and GST affinity purified from *E.coli* BL21 (LysS) cells harboring either pGEX4T-1 or pGEX4T-1-Saw1 were immobilized to glutathione Sepharose 4B and pre-incubated with Phosphate Buffered Saline (PBS) containing 5% BSA for 1 h. Yeast extracts expressing Rad1-TAP, Msh2-TAP, Msh3-TAP, Rad51-TAP, Rad52-TAP, Rad14-TAP, and yKu70-TAP were incubated with beads for 2 h. After extensive washing with 20 volumes of PBS

containing 0.2% Triton X-100, beads were collected by centrifugation and the bound proteins were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane. The TAP fusion proteins were detected by peroxidase-antiperoxidase soluble complex (Sigma).

Pull-down assay involving ^{35}S-labeled proteins. Purified GST-Saw1 and GST were immobilized to glutathione Sepharose 4B and incubated with ^{35}S-labeled Rad1, carboxy terminal truncated Rad1, Msh2, Rad52 synthesized by TNT-coupled reticulocyte lysate systems (Promega) using pRS316-Rad1, pRS316-rad1ΔC, pRS316-Msh2, pRS316-Rad52 in buffer A (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM dithiothreitol, 1 % Triton X-100). The beads were collected by centrifugation and washed extensively with buffer A. Proteins released from the beads were separated by SDS-PAGE and detected by phosphorimaging analysis of the dried gels (Amersham).

Table SI. Genotypes of strains used in this work.

Strain no.	Genotype	Reference
tNS1379	*Δho HML MATΔ::leu2::hisG hmrΔ3 leu2-3,112 ura3-52 trpl thr4 (ura3 [205 bp]-cut site-URA3) pFH800*	(Sugawara et al., 2000)
FLT34	tNS1379 *rad1Δ::KAN*	This study
FLT35	tNS1379 *msh3Δ::KAN*	This study
FLT37	tNS1379 *msh2Δ::KAN*	This study
FLT42	tNS1379 *saw1Δ::KAN*	This study
FLT44	tNS1379 *slx4Δ::KAN*	This study
FLT76	tNS1379 *rad59Δ::KAN*	This study
FLT79	tNS1379 *rad52 Δ::KAN*	This study
FLT80	tNS1379 *RAD1-3xHA::KAN*	This study
SLY1881	tNS1379 *rad1-D825A-3xHA::KAN*	This study
SLY1882	tNS1379 *rad1-D825A-3xHA::KAN saw1Δ::HYG*	This study
YMV80	*hmlΔ::ADE1 MATαΔ::hisG hmrΔ::ADE1 leu2-cs ade3::GAL::HO ade1 lys5 ura3-5, leu2::CS*	(Vaze et al., 2002)
FLY34	YMV80 *rad1Δ::KAN*	This study
FLY42	YMV80 *saw1Δ::KAN*	This study
FLY44	YMV80 *slx4Δ::KAN*	This study
FLY111	YMV80 *leu2::csΔ*	This study
FLY341	YMV80 *leu2::csΔ rad1Δ::KAN*	This study
FLY421	YMV80 *leu2::csΔ saw1Δ::KAN*	This study
FLY441	YMV80 *leu2::csΔ slx4Δ::KAN*	This study
SLY556	*hoΔ hmlΔ hmrΔ MATα-inc ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO*	This study
FLS1	SLY556 *saw1Δ::KAN*	This study
FLS2	SLY556 *slx4Δ::KAN*	This study
FLS3	SLY556 *rad1Δ::TRP1 saw1Δ::KAN*	This study
FLS4	SLY556 *rad1Δ:: TRP1 slx4Δ::KAN*	This study
FLS5	SLY556 *saw1Δ:: TRP1 slx4Δ::KAN*	This study
FLB1	BY4741 *SAW1-TAP*	This study
FLB2	BY4741 *SAW1-TAP::HIS3 rad1Δ::KAN*	This study
FLB3	BY4741 *RAD1-TAP::HIS3*	This study
FLB4	BY4741 *RAD52- TAP::HIS3*	This study
FLB5	BY4741 *MSH2-TAP::HIS3*	This study
FLB6	BY4741 *MSH3-TAP::HIS3*	This study
FLB7	BY4741 *RAD14-TAP::HIS3*	This study
FLB8	BY4741 *KU70-TAP::HIS3*	This study
FLB9	BY4741 *SAW1-TAP RAD52-13MYC::KAN*	This study
SLY1809	YMV80 *mre11Δ::KAN*	This study
SLY1821	*W303 MATa ade2-1 leu2-3112 can1-100 trp1-1 ura3-52 his3-11, 15 RDN1::ADE2 RAD5*	(Bitterman et al., 2002)
SLY1822	SLY1821 *rad1Δ::KAN*	This study
SLY1824	SLY1821 *saw1Δ::KAN*	This study
SLY1854	SLY1821 *slx4Δ::KAN*	This study
SLY1855	SLY1821 *rad52Δ::KAN*	This study
SLY1856	SLY1821 *sir2Δ::TRP*	This study
SLY1829	SLY1856 *slx4Δ::KAN*	This study
SLY1839	SLY1856 *saw1Δ::KAN*	This study
SLY1857	SLY1856 *rad1Δ::KAN*	This study

SLY1858	SLY1856 *rad51Δ::KAN*	This study
SLY1859	SLY1856 *rad52Δ::KAN*	This study
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	Clontech
Y187	*MATa, ura3-52, his3-200, ade2-101, trp1-901,leu2-3,112, gal4Δ, met–, gal80Δ, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	Clontech
SLY1980	AH109 pGAD	This study
SLY1981	AH109 pGAD-Rad1	This study
SLY1982	Y187 pGBD	This study
SLY1983	Y187 pGBD-Slx4	This study

Figure S1. Plasmid based SSA assay. A. Schematic diagram of pNSU208. **B.** SSA repair efficiency from various repair mutants. Repair of pNSU208 linearized by *Bsu*36I restriction enzyme cleavage by annealing of 240 bp of *LacZ* direct repeat sequences on either side of the break in a yeast strain lacking *LEU2* were determined by scoring the number of yeast colonies transformed by linearized pNSU2108 divided by the number of colonies transformed by circular pNSU208. Data represent means $\pm$ s.d. from three independent experiments.

Figure S2. Schematic illustration of the genome-wide screen for mutants defective in SSA. Yeast deletion pools were transformed with either circular or *Bsu*36I-digested pNSU208 and then plated onto synthetic complete medium lacking leucine (SC-Leu). After incubating at 30°C for 2-3 days, colonies from circular or linear plasmid transformations were collected and the genomic DNA was extracted by the glass bead-DNA extraction method. Genomic DNA from the circular pNSU208-transformed cells was amplified with Cy5 (red)-labeled universal primers, whereas an equal amount of genomic DNA from *Bsu*36I-digested pNSU208-transformed cells was amplified with Cy3 (green)-labeled universal primers. Fluorescence-labeled PCR products were then mixed and hybridized in duplicate to a oligonucleotide microarray bearing each tag unique to the individual deletion mutants. SSA-defective mutants would be underrepresented in the *Bsu*36I-linearized plasmid-transformed pool only and would have a reduced signal in the Cy3 (green) channel.

Figure S3. Lack of Slx4 or Saw1 did not delay single stranded DNA production next to DNA break. A. HO cleavage and the location of primers (arrows) used in the ChIP assay with anti-Rpa1 antibody. **B.** The levels of RPA in *slx4Δ* or *saw1Δ* at the DSB were detected by ChIP assay using an anti-RPA antibody. Chromatin was isolated at the indicated time after galactose addition, crosslinked with formaldehyde, and fragmented by sonication. After immunoprecipitation and reverse crosslinking, purified DNA was analyzed by qPCR using five sets of primers that anneal 0.1 kb (D), 0.5 kb (C), 1.5 kb (E), 10 kb (B), and 20 kb (A) to the DSB, as well as primers specific for the *PRE1* gene situated on chromosome V as a control. PCR signals from each primer set at different durations of HO expression were quantified and plotted as a graph. IP represents the ratio of the RPA PCR signal before and after HO induction, normalized by the PCR signal of the *PRE1* control. Each point is the average of two separate experiments.

Figure S4. Schematic illustration of the 3' flap removal substrates. Yeast centromeric plasmids carrying two copies of inverted *Lac*Z sequences, one of which contains HO recognition sites and various sizes of deletions were used to determine the frequency of 3' flap cleavage during gene conversion.

Figure S5. Rad1 is required for the stability of Saw1 protein. Cell extracts prepared from wild type and *rad1*Δ strain was separated by SDS-PAGE and the Saw1-TAP fusion protein was detected by PAP reagent (Sigma).

Figure S6. Sucrose gradient sedimentation analysis of Saw1-TAP and Rad1 in response to MMS-induced DNA damage. Position of S value standard (blue dextran, 200 kd) are indicated. Crude cell extracts were prepared from logarithmic yeast culture with or without treatment by 0.02% MMS. The extracts were loaded onto a 5-40% sucrose gradient and after centrifugation at 37,500 rpm for 16.5 h, fractions were collected and separated on a SDS-PAGE and transferred to PVDF membrane for Western blot analysis. TAP-tagged Saw1 was detected by PAP (Sigma). Rad1 was detected using anti-Rad1 antibody (a kind gift from Dr. Alan Tomkinson).

Figure S7. Expression of rad1-D825A mutant sensitizes to UV irradiation. To measure UV sensitivity, strains expressing wild type or mutant rad1 were diluted on YEPD plates, irradiated by 80 Jm^{-2} UV using a stratalinker UV source, and incubated for 3 days. Shown are the two different isolates of *rad1-D825A* mutants.

Figure S8. *rad1*-D825A mutant is defective in flap removal during DSB repair by gene conversion. The effect of *rad1-D825A* mutation on 3' flap removal was tested using a plasmid based flap removal assay with pFP120 carrying 308 and 610 bp of 3' flaps. The percent plasmid retention was plotted for *RAD1* deletion cells carrying either vector only, wild type *RAD1*, or *rad1-D825A* mutation. Data represent means $\pm$ s.d. from at least three independent experiments.

Supplemental References

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem *277*, 45099-45107.

Colaiacovo, M.P., Paques, F., and Haber, J.E. (1999). Removal of one nonhomologous DNA end during gene conversion by a RAD1- and MSH2-independent pathway. Genetics *151*, 1409-1423.

Gietz, R.D., and Schiestl, R.H. (1991). Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast *7*, 253-263.

Ooi, S.L., Shoemaker, D.D., and Boeke, J.D. (2001). A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae. Science *294*, 2552-2556.

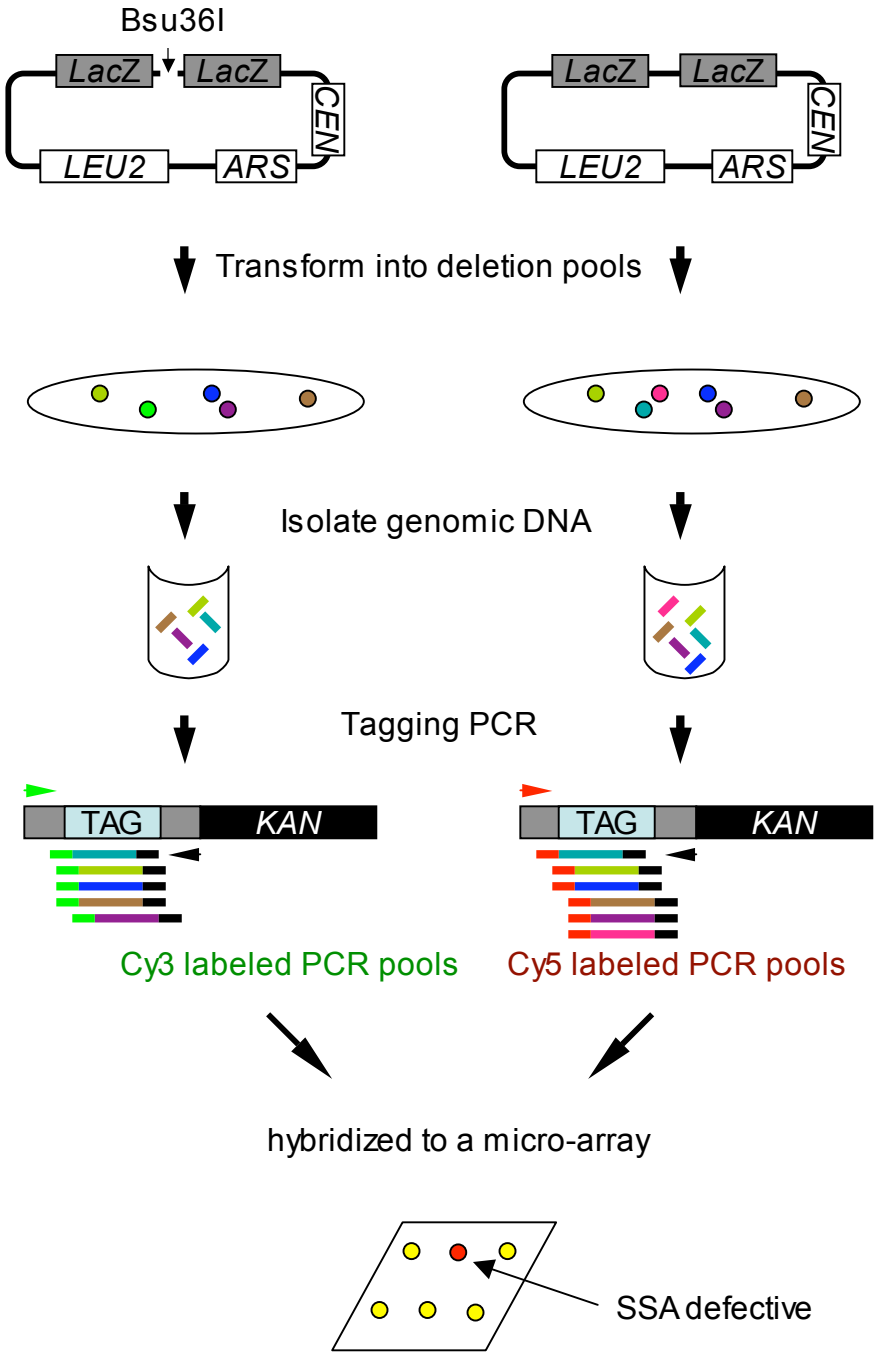
Sugawara, N., Ira, G., and Haber, J.E. (2000). DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair. Mol Cell Biol *20*, 5300-5309.

Vaze, M.B., Pellicioli, A., Lee, S.E., Ira, G., Liberi, G., Arbel-Eden, A., Foiani, M., and Haber, J.E. (2002). Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell *10*, 373-385.

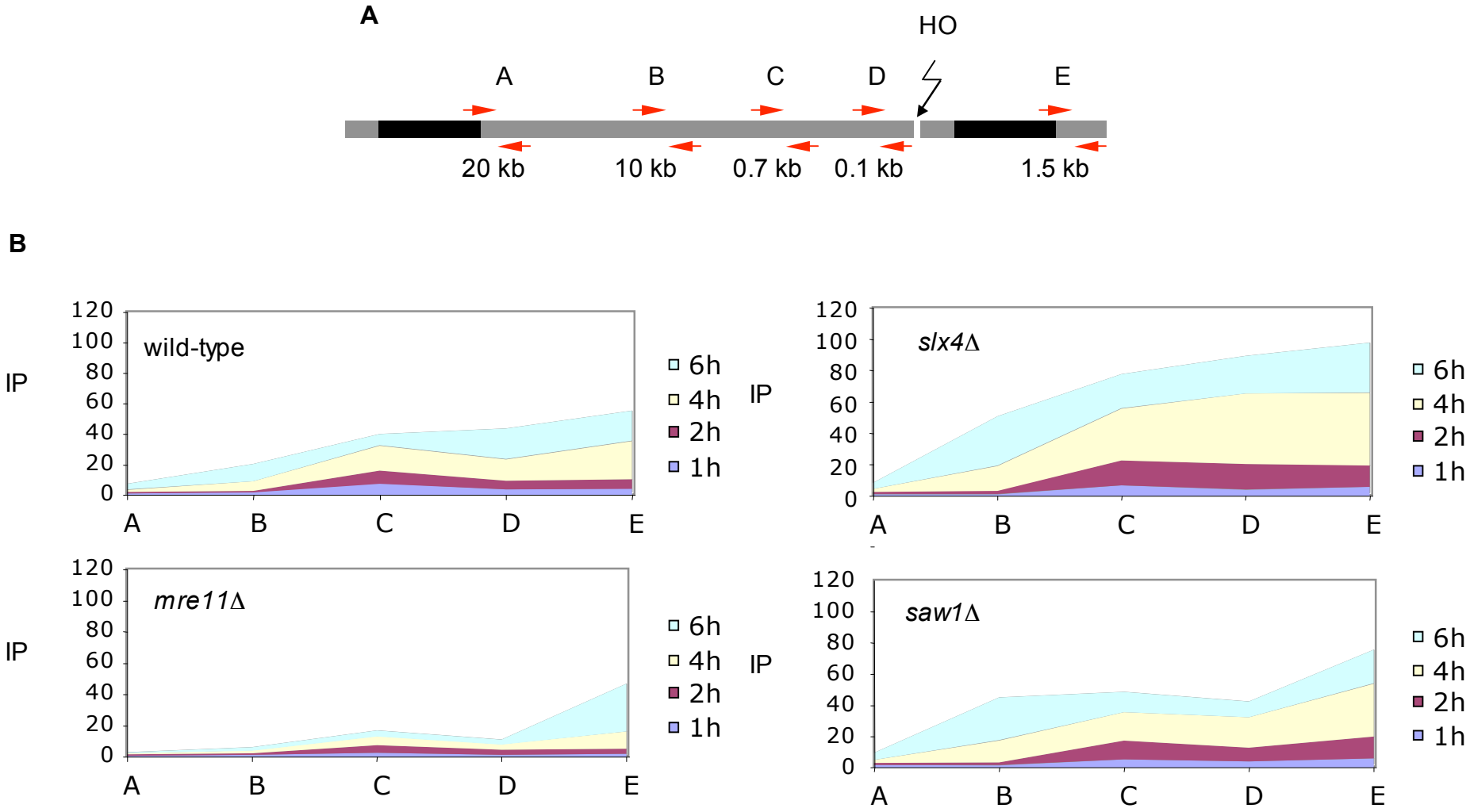
Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793-1808.

Yuan, D.S., Pan, X., Ooi, S.L., Peyser, B.D., Spencer, F.A., Irizarry, R.A., and Boeke, J.D. (2005). Improved microarray methods for profiling the Yeast Knockout strain collection. Nucleic Acids Res *33*, e103.

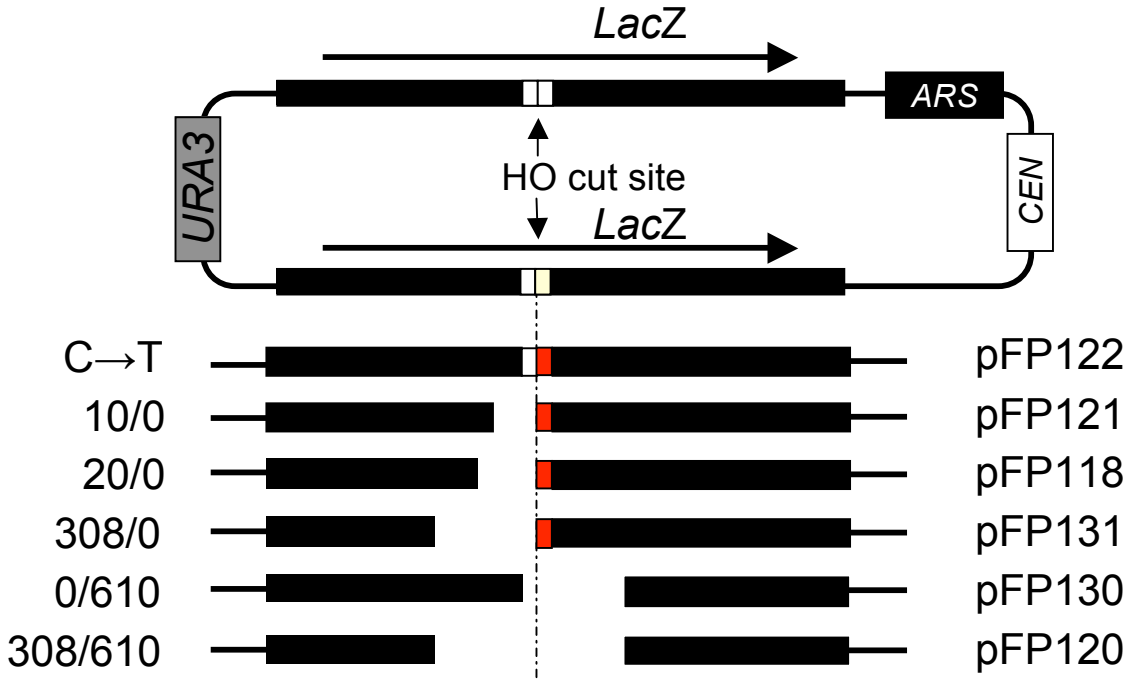
Li et al., 2008. Supplemental Figure 2.



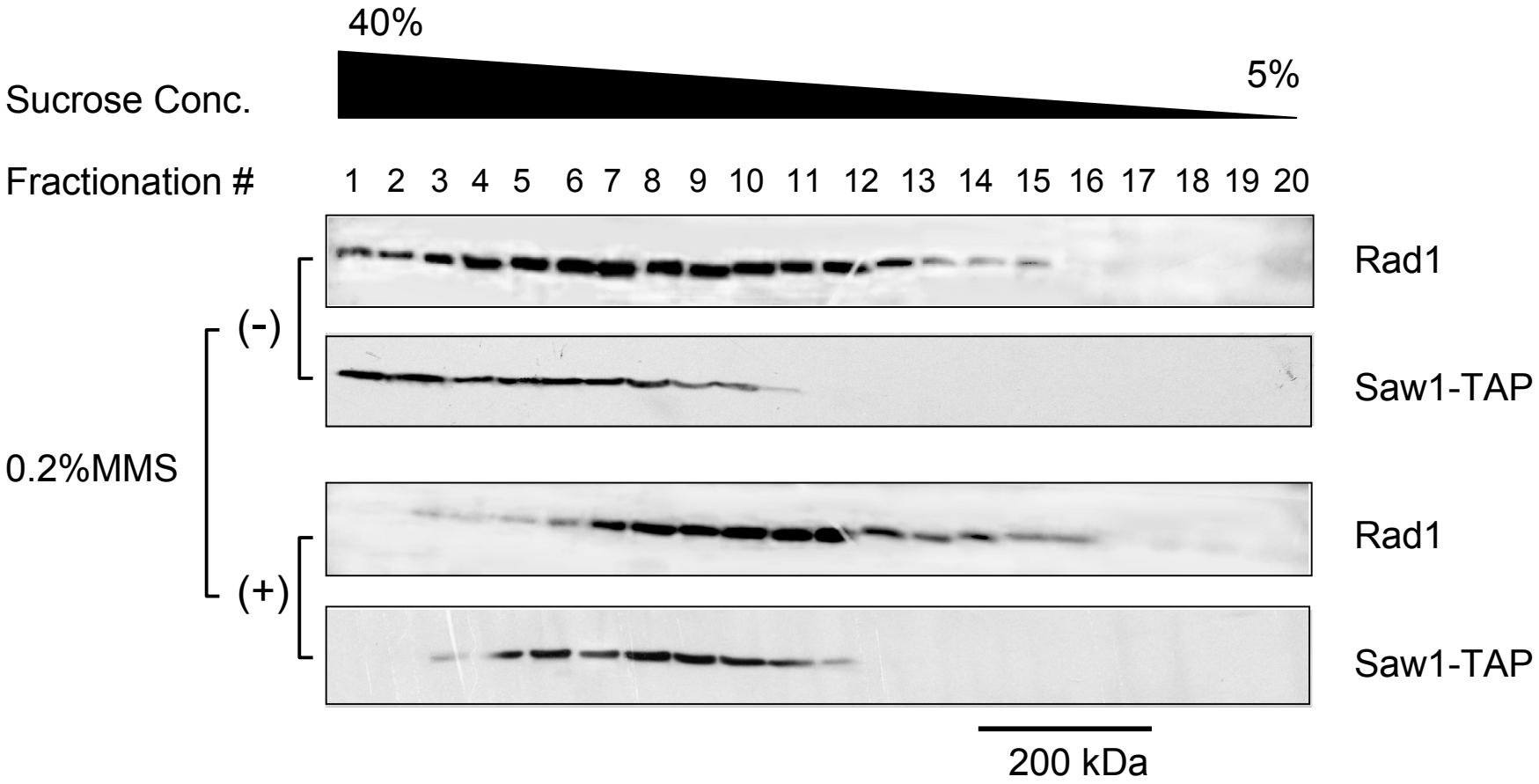
Li et al. 2008. Supplemental Figure 3



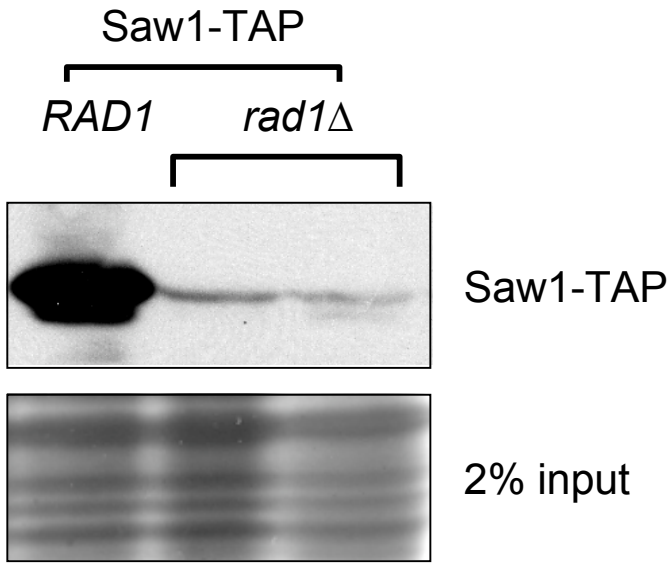
Li et al. 2008. Supplemental Figure 4



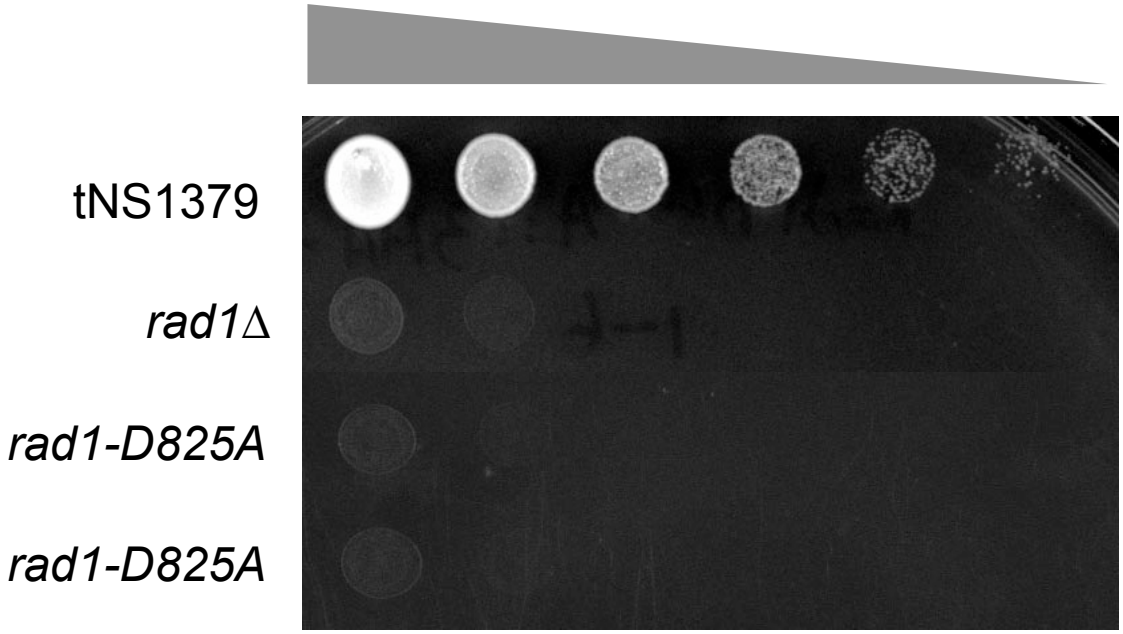
Li et al. 2008. Supplemental Figure 5



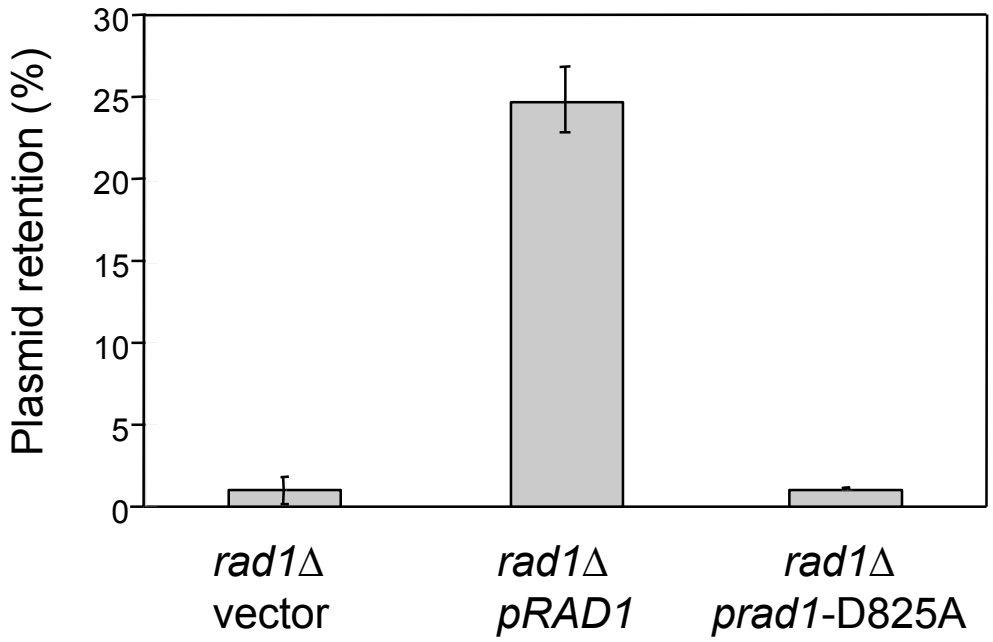
Li et al. 2008. Supplemental Figure 6



Li et al. 2008. Supplemental Figure 7



Li et al., 2008 Supplemental Figure 8



Supplemental References

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem *277*, 45099-45107.

Colaiacovo, M.P., Paques, F., and Haber, J.E. (1999). Removal of one nonhomologous DNA end during gene conversion by a RAD1- and MSH2-independent pathway. Genetics *151*, 1409-1423.

Gietz, R.D., and Schiestl, R.H. (1991). Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast *7*, 253-263.

Ooi, S.L., Shoemaker, D.D., and Boeke, J.D. (2001). A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae. Science *294*, 2552-2556.

Sugawara, N., Ira, G., and Haber, J.E. (2000). DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair. Mol Cell Biol *20*, 5300-5309.

Vaze, M.B., Pellicioli, A., Lee, S.E., Ira, G., Liberi, G., Arbel-Eden, A., Foiani, M., and Haber, J.E. (2002). Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell *10*, 373-385.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793-1808.

Yuan, D.S., Pan, X., Ooi, S.L., Peyser, B.D., Spencer, F.A., Irizarry, R.A., and Boeke, J.D. (2005). Improved microarray methods for profiling the Yeast Knockout strain collection. Nucleic Acids Res *33*, e103.