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Supplemental Data

Regulation of Meiotic Recombination via Mek1-Mediated Rad54 Phosphorylation

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Supplementary Experimental Procedures

Strains

All strains are derivatives of the fast sporulating SK1 background. To construct strains where the only allele of *RAD54* is contained on a plasmid integrated at *ura3*, a *rad54Δ::kanMX6/RAD54* heterozygous diploid was first created using the PCR based method of Longtine et al. (1998) to replace one copy of *RAD54* with *rad54Δ::kanMX6*. This diploid was then transformed with pRS306 digested with StuI or pHN103 (*RAD54*), pHN104 (*RAD54-T132A*) and pHN105 (*RAD54-T132D*) digested with NcoI. The diploids were sporulated and the dissected tetrads were analyzed for spore colonies that were **a** His^- Arg^+ $G418^R$ Ura^+ or α His^+ Arg^- $G418^R$ Ura^+. The haploids were then mated to produce NH938, NH939, NH940 and NH941. The haploids containing an integrated allele of *RAD54* were deleted for the second exon of *DMC1* (*dmc1Δ::hphMX4*) as described in Goldstein and McCusker (1999) and mated to make NH949, NH950 and NH951. *HED1* (*hed1Δ::NAT*) was deleted as described by Tong et al., (2001) from the *DMC1* haploids and the haploids mated to make NH944, NH945 and NH946, as well as from the *dmc1Δ::hphMX4* haploids to make the diploids NH952, NH953 and NH954. The *HED1/hed1Δ* heterozygous diploids (NH955, NH956 and NH957) were created by

mating *HED1 dmc1Δ::hphMX4* haploids containing the various *RAD54* alleles to the appropriate *hed1Δ::NAT dmc1Δ::hphMX4* haploids. *RAD54* was tagged with Flag in the S2683 and RKY1145 strains (de los Santos and Hollingsworth, 1999) using p3FLAG-kanMX6 (Gelbart et al. 2001) and the haploids mated to make NH1009. *MEK1* was deleted from the RAD54-Flag haploids using *mek1Δ::LEU2* and the haploids were mated to make NH1003 (de los Santos and Hollingsworth, 1999). To make the *dmc1Δ mek1-as RAD54* series, *MEK1* was first deleted with *NAT* in the *dmc1Δ::hphMX4* haploids containing various alleles of *RAD54*. The plasmid, pLW87, carrying *mek1-as* was digested with RsrII to target integration downstream of *mek1Δ::NAT* and transformed into the haploids. The appropriate haploids were then mated to generate the diploids NH968::pLW87, NH969::pLW87 and NH970::pLW87. All deletions were confirmed by PCR.

Plasmids

Mutations were introduced into *RAD54* or *RDH54* by site-directed mutagenesis (Quik-Change kit from Stratagene) using the plasmid, pNRB143 (Bishop et al., 1999) and pL23-1 (a gift from M. Dresser), respectively. All mutations were confirmed by DNA sequencing. For pHN103, pHN104 and pHN105, a 3.5 kb NotI/SmaI fragment from pNRB143 (*RAD54*), pNRB143-T132A (*RAD54-T132A*) or pNRB132-T132D (*RAD54-T132D*) was cloned into NotI/SmaI-digested pRS306. Plasmids were digested with NcoI to target integration to *ura3*. To generate a *mek1-as LEU2* plasmid, the Q241G mutation was introduced into pB131 (Rockmill and Roeder, 1991) to make pB131-Q241G. A 3.2 kb EcoRI/SalI fragment was then subcloned into pRS402 digested with EcoRI and SalI to make pLW21. Finally a 3.2 kb SalI/PstI fragment from pLW21 was

cloned into SalI/PstI digested pRS305 to create pLW87. This plasmid was digested with RsrII to target integration downstream of the *MEK1* ORF. The plasmids, pLW1 and pLW3 are described in (Wan et al., 2004). pHN26 and pBL12 are described in Niu et al. (2005) and (2007), respectively.

Purification of Soluble GST-Mek1

A. For *In Vitro* Kinase Assays Using Radioactively Labeled ^{32}P

GST-Mek1 was purified from the *dmc1*Δ diploid NH520::pBL12 (GST-MEK1) or NH520/pLW1 (2μ GST-MEK1). For GST-mek1-K199R, NH520::pHN26 was used. Each strain was sporulated for five hours at 30°C and then 50 ml were collected, washed once with 10 ml 25% glycerol, resuspended in 800 μl 25% glycerol and frozen at -80°C. To make a lysate, cells were washed once with lysate buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 300 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 μg/ml Leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 10 mM NaF, 10 mM $Na_4P_2O_7$), resuspended in 1 ml lysis buffer and transferred to a 12 ml Falcon tube. ~800 μl glass beads were added and the cells were vortexed at maximum speed for 30 sec followed by a 1 min incubation on ice. This protocol was repeated six times, the cells were incubated on ice for five minutes and then a further six rounds of vortexing were performed. The lysate was transferred to a 2 ml microfuge tube and 10% Triton X-100 and 10% SDS were added to a final concentration of 0.5% (~80 μl) and 0.05% (~8 μl), respectively. The extract was incubated on a rotating platform at 4°C for 15 min and then clarified by centrifugation in a microfuge for 10 min at 13,200 rpm at 4°C. 50 μl of glutathione-S-Sepharose beads (1:1 slurry, GE Health Sciences) were equilibrated by washing once with lysis buffer and added to the lysate. After an additional 1.5 hr at 4°C

on the rotating platform, the beads were pelleted, washed twice with lysis buffer and three times with elution buffer lacking glutathione (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM DTT). Excess liquid was removed using a 30 ½ gauge needle. The beads were resuspended in 50 μl elution buffer plus 10 mM glutathione and incubated 10 min at room temperature. During this incubation, the beads were gently resuspended every two minutes by flicking. After pelleting the beads, the eluate containing soluble Gst-Mek1 was transferred to a new tube and used immediately for *in vitro* kinase assays.

B. For *In Vitro* Kinase Assays Using the Semisynthetic Epitope System

Two liters of NH520/pLW1 (2μ *GST-MEK1*) and three liters of NH520/pLW4 (2μ *GST-mek1-K199R*) were sporulated for 5 hours. The cells were pelleted and resuspended in 60 ml lysis buffer with protease inhibitors and 60 g glass beads. Using a Biospec bead beater, the cells were lysed using 10 X 30 sec pulses with two minute rests on ice between pulses. The lysate was removed and 20% Triton X-100 was added to a final concentration of 1%. After 10 min on ice, the lysate was centrifuged in a Sorvall SS34 rotor for 30 min at 15,000 rpm. The soluble extract was moved a clean tube and 500 μl glutathione-sepharose beads (prewashed with lysis buffer) were added and mixed on a nutator at 4^{o} for 2 h. The beads were loaded onto a Biorad Econo-column and washed 5 times with 2 ml lysis buffer. Five ml of elution buffer was loaded onto the column and the eluate collected in 1 ml fractions. The fraction containing the largest amount of kinase was assessed by Coomassie staining of an SDS-polyacrylamide gel and dialyzed with 4 X 1 L storage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM DTT, 20% glycerol). The proteins were concentrated by centrifugation in Centricon-10

filter units (Millipore) into 200 μl for a final concentration of 300 ng/μl (wild type) and 100 ng/μl (K199R). The kinase was divided into 10 μl aliquots and frozen at -80°C.

C. GST-Mek1-as Pull-Down

NH520/pLW3 (2μ *GST-mek1-as1*) cells were sporulated and 6 ml soluble extract was incubated with 100 μl glutathione-sepharose as described above. After incubation at 4°C for 1 h, the beads were washed four times with 5 ml elution buffer, and two times with kinase buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$ and 0.1 mM ATP or ATPγS). The beads were resuspended in 200 μl kinase buffer and divided into four tubes for immediate use in kinase assays.

Purification of His6-Hop1 from *E. Coli*

An overnight culture of BL21 codon plus RIL (Stratagene) containing pET15b-HOP1 (*His_6-HOP1*), was diluted 1:50 in 2 L LB with 100 mg/ml ampicillin and incubated on a shaker at 37°C until the OD_{660} reached 0.8. The culture was then cooled to room temperature and *His_6-HOP1* expression was induced by adding 1 M IPTG to a final concentration of 0.3 mM and the culture was incubated on a shaker for 14 hr at room temperature. Cells were harvested and resuspended in 20 ml lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 500 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 μg/ml each bestatin, aprotinin, pepstatin, 1 mM benzamidine). A lysate was made using a French Pressure Cell Press (SIM-AMINCO Spectronic Instrument) at pressure between 1000 and 15000 psi and clarified by centrifugation at 15,000 rpm in an SS-34 rotor at 4°C for 20 min. 500 ml Ni-NTA Sepharose beads (Qiagen), after equilibration with lysis buffer, were added to the supernatant, which was then incubated on a rotating platform for 1.5 hr at 4°C. The lysate with Ni-NTA beads was poured into a Biorad polyprep column

with reservoir. The beads were packed by gravity and washed five times with 10 ml lysis buffer. His_6-Hop1 was eluted with 5 ml elution buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 500 mM NaCl, 1 mM DTT, 200 mM imidazole). The eluate was dialyzed with 20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM NaCl, 1 mM DTT overnight and then loaded onto a heparin column using an Akta purifier FPLC (Fast Protein Liquid Chromatography) (GE Health Science). His6-Hop1 was eluted from this column using a NaCl gradient from 100 to 500 mM in 20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1 mM DTT. Fractions containing His_6-Hop1 were confirmed by immunoblot analysis using α-Hop1 antibodies (de los Santos and Hollingsworth, 1999). The fractions were pooled and concentrated to 4.5 mg/ml using a Centricon-30 column (Millipore).

***In Vitro* Kinase Assays Using ATP**

All kinase assays were conducted using a total volume of 24 μl of which 20 μl consisted of the Gst-Mek1 eluate and 1 μl 250 mM $MgCl_2$ (10 mM final concentration). For assays using radioactively labeled ^{32}P, 1 μl 2 mM ATP and 1 μl $^{32}P\gamma ATP$ (6000 Ci/mmol) were included. Recombination proteins purified out of yeast were diluted to a concentration of 1 mg/ml in 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM DTT and 1 μl was added to the kinase reaction. For the non-radioactive kinase assays, 2 μl of 2 mM ATP and 1 μl of 2.5 mg/ml recombinant Rad54. After addition of ATP, the reactions were incubated at 30^{o}C for 30 min. Reactions involving radioactivity were stopped by adding 6 μl 5 X Protein Sample buffer and heating the samples at 95^{o}C for five min. 25 μl of reaction were loaded onto an 8% SDS-polyacrylamide gel, run at 6 mA for 14 hr, transferred to a nitrocellulose membrane and exposed to film or a phospho-imager screen. For non-

radioactive labeling of Rad54 for analysis by MS, kinase reactions were stopped by adding 10 μl of 3 X Protein Sample buffer [1:100 TCEP-HCl, Tris(2-Carboxyethyl) phosphine hydrochloride, Pierce] to 10 μl kinase reaction and heating the samples at 95°C for five min. 10 μl 50 mM Tris-HCl pH8.0, 100 mM iodoacetamide were added and the sample was incubated at room temperature for 30 min for reduction and alkylation of cystidine residues. 15 μl sample containing ~500 ng phosphorylated Rad54 was fractionated using a 4-12% Bis-Tris NuPAGE gel (Invitrogen). The remainder was frozen at 80°C. After Gel-Code Blue staining (Pierce), the Rad54 band was cut out of the gel and analyzed by mass spectrometry as described below.

***In Vitro* Kinase Assays Using the Semisynthetic Epitope System**

For the experiments using Gst-Mek1 and Gst-mek1-K199R, 100 ng of soluble kinase were used in each reaction along with 1 μg recombinant Rad54. For Gst-mek1-as, 50 μl bead/kinase buffer slurry were pelleted and resuspended in 22 μl kinase buffer. To inhibit GST-mek1-as, 10 μM 1-NA-PP1 was added to the reaction prior to the addition of the nucleotide. 1 μl of 10 mM ATPγS (Sigma) or analog was added along with 1 μl 1μg/μl recombinant Rad54 and the reaction was put at room temperature for 30 min. 1.3 μl of 50 mM PNBM dissolved in dimethyl sulfoxide (final concentration 2.5 mM) was added and the aklyation reaction proceeded at room temperature for one hr. After addition of 5 μl of 5 X Protein Sample Buffer, the samples were heated at 95°C for five minutes and 10 μl of each reaction were loaded onto an 8% SDS-polyacrylamide gel. The gel was run at 20 mAmp for one hour. The proteins were transferred to nitrocellulose, and the filter was blocked by incubation in 4% milk for one hour at room temperature. The α-hapten antibody was diluted 1:15,000 by adding 1 μl antibody to 15

ml milk. The filter was placed in 3 ml milk containing the antibody and placed on a rotating platform at room temperature for 1 hr, followed by 4°C overnight. The blot was washed four times for ten min with TBST and then a 1:2500 dilution of goat anti-rabbit secondary antibody coupled to horseradish peroxidase (BioRad) was added. The blot was rotated for one hr at room temperature and the processed per the manufacturer's instructions.

Synthesis of furfuryl-ATPγS

For N^6-furfuryl-ATPγS, N^6-furfuryl-adenosine was first prepared by refluxing 6-chloro-adenosine with furfurylamine and Et_3N in ethanol overnight, as described for tetrahydrofurfuryladenosine (Fleysher, 1972). N^6-furfuryl-ADP was prepared as described for ADP, through reaction with $POCl_3$ followed by H_3PO_4 in the presence of 1,8-diazabicyclo[5.4.0]undec-7-en (DBU)(Hoffman et al., 1996). N^6-furfuryl-ATPγS was synthesized as described for ATPγS (Kowalska et al., 2007).

Purification of Rad54-3Flag from *dmc1*Δ and *dmc1*Δ *mek1*Δ Meiotic Cells

Two liters of log phase NH1003 or NH1009 cells grown in YPA were transferred to sporulation medium and harvested after five hours. The cell pellet (20g) was ground with dry ice in a coffee grinder for 3 min and the resulting powder was resuspended in 20 mL lysis buffer (50mM Tris pH8.0, 10mM EDTA pH 8.0, 600mM KCl, 0.5mM DTT, 5% Glycerol, 1% Triton x-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 2 mM benzamidine, 1 mM NaF, 1 mM $Na_4P_2O_7$). The crude lysate was cleared by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor. The cell extract (~30 mL) was incubated with 0.5 mL anti-M2-Flag beads (Sigma) for 3 hrs at 4° C followed by one wash with 20mM Tris, 1mM EDTA, 600mM KCl 1% Triton x

-100 and three washes with the same buffer without Triton X-100. Rad54-3Flag was then eluted from the beads with 1.5 mL 0.1M glycine pH 2.5. One half of the eluate was TCA precipitated as described in (Niu et al., 2007). The proteins were fractionated on a 10% SDS-polyacrylamide gel. The identity of Rad54-Flag was confirmed by Western blot analysis using anti-flag antibodies. The remainder of the proteins were stained with Coomassie and the two major Rad54-Flag bands were cut for MS analysis.

Mass Spectrometry Analysis to Map Phosphorylation Sites on Rad54

Gel bands containing Rad54 were cut into approximately 1 mm cubes. The gel pieces were destained with 50% acetonitrile + 50 mM ammonium bicarbonate for 30 min at 37 ^{o}C and then dehydrated by addition of 100% acetonitrile and dried under vacuum for 30 min. For in-gel digestion, the gel pieces were rehydrated with 5 ng/µL trypsin (50 mM ammonium bicarbonate, pH 8.0) on ice for 30 min. After rehydration the in-gel digestion was allowed to incubate at 37 ^{o}C overnight. The peptides were extracted from the gel pieces using a solution of 50% ACN + 5% formic acid followed by a second extraction using 50% MeOH + 5% formic acid and dried under vacuum. The dried peptide mixture was resuspended in 1% formic acid. The resuspended peptide mixture was desalted using homemade StageTips packed with C_{18} resin from an Empore extraction disk (3M) and equilibrated with 1% formic acid (Rappsilber et al., 2007). The peptides were eluted from the StageTips using 50% ACN + 1% acetic acid followed by a second elution step using 50% MeOH + 1% acetic acid. The desalted peptides were dried under vacuum followed by resuspension in 4 µL 5% ACN + 5% formic acid.

Liquid chromatography-tandem MS experiments were performed on an LTQ Orbitrap mass spectrometer (Thermo Fisher). Approximately 0.5 µL of resuspended

peptide was loaded onto a 125 µm inner diameter fused-silica microcapillary column packed in-house to a length of 17 cm with a C_{18} reverse phase resin (200 Å pore size, 5 µm particle size, Magic C_{18}AQ beads, Michrom Bioresources Inc.). The peptides were separated using a 35 min linear gradient from 7% to 32% buffer B (100% ACN + 0.15% formic acid) equilibrated with buffer A (3% ACN + 0.125% formic acid) at a flow rate of 100 µL/min. The LTQ Orbitrap mass spectrometer was operated in the data-dependent mode using the TOP10 strategy. In brief, one full MS scan per cycle was acquired, followed by MS/MS scans on the 10 most abundant precursor ions with dynamic exclusion of previously selected ions. MS/MS spectra were searched via the SEQUEST algorithm against a composite database containing the yeast NCI protein database and its reversed complement. Parameters allowed for static alkylation of cysteine (57.02146 Da) and dynamic oxidation of methionine (15.99491 Da), and dynamic phosphorylation on tyrosine, threonine, and serine (79.96533 Da).

Protein Purification

The pET32a plasmid carrying the *RAD54* gene (Raschle et al., 2004) was mutagenized to create *RAD5-T132A* and *RAD54-T132D* using the site directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The mutant proteins were expressed and purified following the procedure described for wild-type Rad54 (Raschle et al., 2004). Rad51 protein was purified as described (Sung and Stratton, 1996).

ATPase Assays

Rad54, Rad54-T132A, or Rad54-T132D (40 nM) was incubated with or without Rad51 (890 nM) in 9.5 µl of Buffer D (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT) containing 1 mM ATP, 0.5 µCi [γ-^{32}P] ATP, and the indicated concentration of KCl on ice

for 10 min. Linearized ϕX174 RF I DNA (22 μM base pairs, in 0.5 μl) was added to initiate the ATP hydrolysis reaction. The reaction was incubated at 30°C for 15 min. EDTA was added to 250 mM to halt the reaction, followed by thin layer chromatography and phosphorimaging analysis to assess the level of ATP hydrolysis (Petukhova et al., 1998).

Affinity Pull-Down Assays

For pull-down assays, Rad51 (5 μg) and S-tagged Rad54 (5 μg), Rad54-T132A (5 μg), or Rad54-T132D (5 μg) were used. The indicated protein pairs were incubated in 30 μl of Buffer B (25 mM Tris-HCl, pH 7.5, 0.005% Triton, 200 mM KCl, and 1 mM DTT) for 30 min at 4°C. The reactions were mixed with 11 μl of S-Protein agarose beads (which recognize the S-tag; Novagen) at 4°C for 30 min. After washing the beads twice with 200 μl of the same buffer, bound proteins were eluted with 25 μl of 2% SDS. The supernatant (S), wash (W), and SDS eluate (E) from these reactions, 5 μl each, were analyzed by 10% SDS-PAGE and Coomassie Blue staining. The gels were scanned and the images were analyzed by the Quantity One software (BioRad) to determine the proportion of bound Rad51.

D-Loop Assay

The D-loop reaction was performed as described previously (Raschle et al., 2004). Briefly, Rad51 (1 μM) was incubated with the ^{32}P-labeled 90-mer oligonucleotide substrate (3 μM nucleotides) in 11 μl Buffer D containing 4 mM ATP and an ATP-regenerating system (20 mM creatine phosphatase and 30 μg/ml creatine kinase) and 150 mM KCl for 5 min at 37°C. Following the addition of the indicated amount of Rad54, Rad54-T132A, or Rad4-T132D (in 0.5 μl) and a 3-min incubation at 25°C, the D-loop reaction was initiated by the addition of pBluescript SK replicative form I DNA (45 μM

base pairs, in 1 µl). The reactions were incubated at 30°C for 1 min and stopped by the addition of an equal volume of 1% SDS containing 1 mg/ml of proteinase K. After a 3-min incubation at 37°C, the de-proteinized samples were subject to electrophoresis in a 0.9% agarose gel. The gel was dried and analyzed in the phosphorimager.

Table S1. Summary of Phosphorylated Peptides Found in Rad54 and Rdh54

Relevant amino acid	Peptide[a]	m/z	Charge State	X_{corr}[b]
[c]Rad54 Thr_{58}	IAT#GSDNIVGGR	620.4	2^+	2.96
[c]Rad54 Thr_{132}	SFT#VPIK	437.1	2^+	2.46
[c]Rad54 Thr_{231}	HPALM^{ox}T#NGVR	596.6	2^+	2.40
[d]Rad54 Thr_{132}	SFT#VPIK	436.2	2^+	2.18
[e]Rad54 Thr_{132}	RSFTVPIK	474.3	2^+	2.12
[f]Rdh54 Ser_{85}, Thr_{89}	SIS#AGPT#LNLAK	666.3	2^+	2.57

[a]# sign indicates a phosphorylated amino acid; M^{ox}, methionine sulfoxide

[b]X_{corr} values were generated from the SEQUEST algorithm.

[c]Phosphopeptide from Mek1-phosphorylated recombinant Rad54

[d]Phosphopeptide from Rad54-3FLAG purified from *dmc1*Δ cells after five hours in Spo medium.

[e]Phosphopeptide from Rad54-3FLAG purified from *mek1*Δ *dmc1* cells after five hours in Spo medium.

[f]Phosphopeptide from Mek1-phosphorylated Rdh54

Table S2. *S. Cerevisiae* Strains

Name	Genotype	Source
DKB201	*MAT**a** leu2::hisG his4-X dmc1Δ::LEU2 hoΔ::LYS2 lys2 ura3* *MATα leu2::hisG his4-B dmc1Δ::LEU2 hoΔ::LYS2 lys2 ura3*	Doug Bishop
NH520	*Same as DKB201 only mek1Δ::kanMX6* *mek1Δ::kanMX6*	Niu et al. 2007
NH938	*MAT**a** leu2Δ::hisG his4-x ARG4 rad54Δ::kanMX6 ura3::URA3 hoΔ::LYS2 lys2* *MATα leu2-K HIS4 arg4 rad54Δ::kanMX6 ura3::URA3 hoΔ::LYS2 lys2*	This work
NH939	Like NH938 only *ura3::URA3-RAD54* *ura3::URA3-RAD54*	This work
NH940	Like NH938 only *ura3::URA3-RAD54-T132A* *ura3::URA3-RAD54-T132A*	This work
NH941	Like NH938 only *ura3::URA3-RAD54-T132D* *ura3::URA3-RAD54-T132D*	This work
NH944	Like NH938 only *ura3::URA3-RAD54 hed1Δ::NAT* *ura3::URA3-RAD54 hed1Δ::NAT*	This work
NH945	Like NH938 only *ura3::URA3-RAD54-T132A hed1Δ::NAT* *ura3::URA3-RAD54-T132A hed1Δ::NAT*	This work
NH946	Like NH938 only *ura3::URA3-RAD54-T132D hed1Δ::NAT* *ura3::URA3-RAD54-T132D hed1Δ::NAT*	This work
NH949	Like NH938 only *ura3::URA3-RAD54 dmc1Δ::hphMX4* *ura3::URA3-RAD54 dmc1Δ::hphMX4*	This work

NH950	Like NH938 only *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4* *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4*	This work
NH951	Like NH938 only *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4* *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4*	This work
NH952	Like NH938 only *ura3::URA3-RAD54 dmc1Δ::hphMX4 hed1Δ::NAT* *ura3::URA3-RAD54 dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH953	Like NH938 only *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4 hed1Δ::NAT* *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH954	Like NH938 only *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4 hed1Δ::NAT* *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH955	Like NH938 only *ura3::URA3-RAD54 dmc1Δ::hphMX4 HED1* *ura3::URA3-RAD54 dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH956	Like NH938 only *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4 HED1* *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH957	Like NH938 only *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4 HED1* *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4 hed1Δ::NAT*	This work
NH968:: pLW87	Like NH938 only *ura3::URA3-RAD54 dmc1Δ::hphMX4 mek1Δ::NAT::LEU2-mek1-as1* *ura3::URA3-RAD54 dmc1Δ::hphMX4 mek1Δ::NAT::LEU2-mek1-as1*	This work
NH969:: pLW87	Like NH938 only *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4* *ura3::URA3-RAD54-T132A dmc1Δ::hphMX4* *mek1Δ::NAT::LEU2-mek1-as1* *mek1Δ::NAT::LEU2-mek1-as1*	This work
NH970:: pLW87	Like NH938 only *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4* *ura3::URA3-RAD54-T132D dmc1Δ::hphMX4* *mek1Δ::NAT::LEU2-mek1-as1*	This work

	mek1Δ::NAT::LEU2-mek1-as1	
NH1009	Like NH938 only *ura3 RAD54-Flag::kanMX6 dmc1Δ::NAT* *ura3 RAD54-Flag::kanMX6 dmc1Δ::NAT*	This work
NH1003	Like NH938 only *ura3 RAD54-Flag::kanMX6 mek1Δ::LEU2 dmc1Δ::NAT* *ura3 RAD54-Flag::kanMX6 mek1Δ::LEU2 dmc1Δ::NAT*	This work

Supplemental Figures

Figure S1. MS Analysis of *In Vitro* Phosphorylation Events in Rad54

Assignment of b (red) and y (blue) ion series in MS/MS scan from the 620.4 and 596.6 precursor peptide ion(s) confirms a phosphorylation event at T58 (A) and T231 (B) in Rad54, respectively. X_{corr} values are given in Table S1.

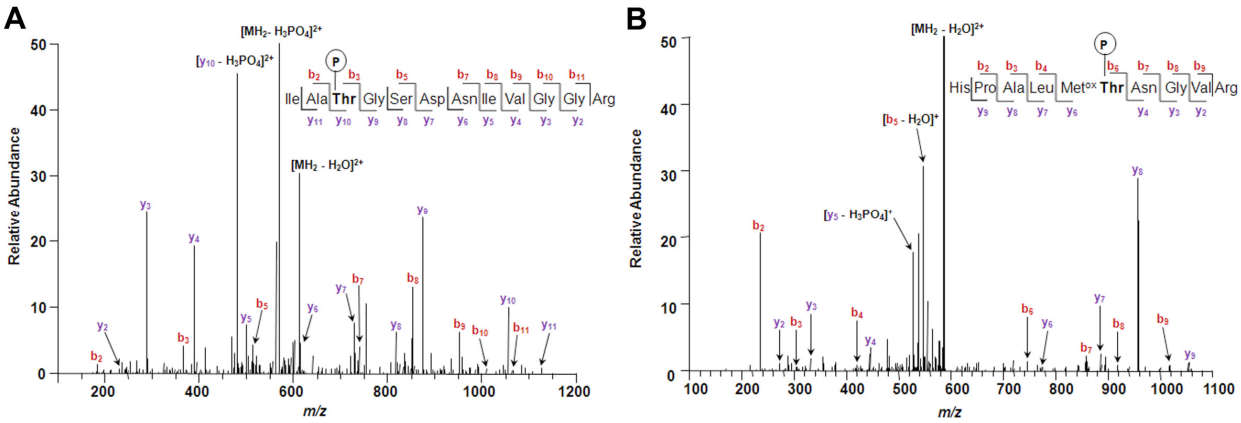


Figure S2. MS Analysis of an *In Vitro* Phosphorylation Events in Rdh54

Assignment of b (red) and y (blue) ion series in MS/MS scan from the 666.3 precursor peptide ion confirms a phosphorylation event at S85 and T89 in Rdh54. X_{corr} values are given in Table S1.

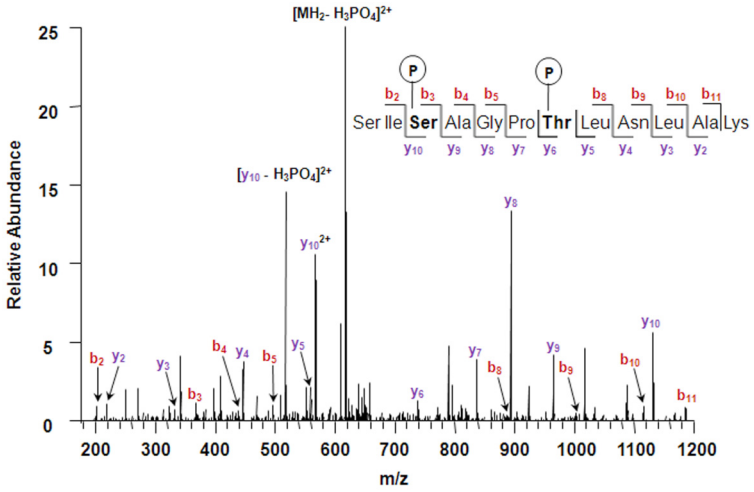


Figure S3. Purified Recombinant Rad54 Protein

Purified Rad54, Rad54T-132A, and Rad54-T132D, 1 ug each, were analyzed by SDS-PAGE and Coomassie staining.

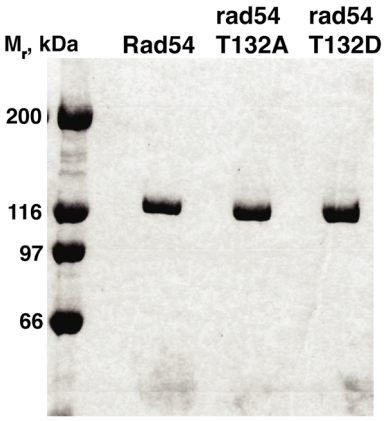
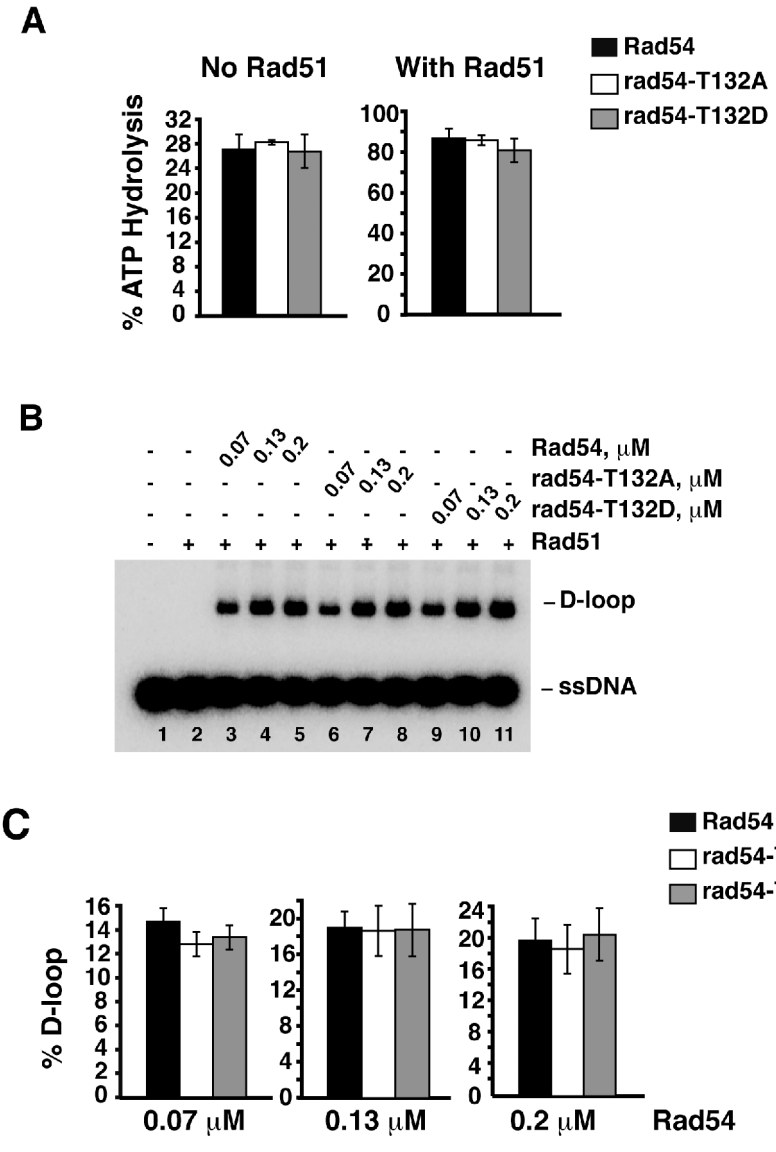


Figure S4. Enyzmatic Activies of Rad54, Rad54-T132A, and Rad54-T132D at Low Salt

A. ATP hydrolysis by Rad54, Rad54-T132A, or Rad54-T132D (40 nM) with or without Rad51 (890 nM) was examined in the presence of 50 mM KCl. Error bars represent SEM. **B**. The D-loop reaction was carried out in the presence of 100 mM KCl. Rad51 (0.1 μM) was incubated with radiolabeled ss 90-mer oligo (3 μM nucleotides) followed by the addition of the indicated amount of Rad54, rad54-T132A, or rad54-T132D. The

reaction was initiated by the addition of the pBluescript SK replicative form I DNA (45 μM base pairs). **C**. Quantification of the D-loop product based on three independent experiments. Error bars represent SEM.



REFERENCES

Allen, J. A., Li, M., Brinkworth, C. S., Paulson, J. L., Wang, D., Hubner, A., Chou, W.-H., Davis, R. J., Burlingame, A. L., Messing, R. O*., et al.* (2007). A semisynthetic epitope for kinase substrates. Nature Methods *4*, 511-516.

Bishop, D. K., Nikolski, Y., Oshiro, J., Chon, J., Shinohara, M., and Chen, X. (1999). High copy number suppression of the meiotic arrest caused by a *dmc1* mutation: *REC114* imposes an early recombination block and *RAD54* promotes a *DMC1*-independent DSB repair pathway. Genes Cells *4*, 425-443.

de los Santos, T., and Hollingsworth, N. M. (1999). Red1p: A *MEK1*-dependent phosphoprotein that physically interacts with Hop1p during meiosis in yeast. J Biol Chem *274*, 1783-1790.

Fleysher, M. H. (1972). N6-substituted adenosine. Synthesis, biological activity and some structure-activity relations. J Med Chem *15*, 187-191.

Gelbart, M. E., Rechsteiner, T., Richmond, T. J. and Tsukiyama, T. (2001). Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. Mol. Cell. Biol. 21: 2098-2106.

Goldstein, A. L., and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast *15*, 1541-1553.

Hoffman, C., Genieser, H. G., Veron, M., and Jastorff, B. (1996). Novel synthesis of nucleoside 5'-polyphosphates. Bioorganic Med Chem Letters *6*, 2571-2574.

Kowalska, J., Lewdorowicz, M., Darzynkiewicz, E., and Jemielity, J. (2007). A simple and rapid synthesis of nucleotide analogues containing a phosphorothioate moeity at the terminal position of the phosphate chain. Tetrahedron Letters *48*, 5475-5479.

Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953-961.

Niu, H., Li, X., Job, E., Park, C., Moazed, D., Gygi, S. P., and Hollingsworth, N. M. (2007). Mek1 kinase is regulated to suppress double-strand break repair between sister chromatids during budding yeast meiosis. Mol Cell Biol *27*, 5456-5467.

Petukhova, G., Stratton, S., and Sung, P. (1998). Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. Nature *393*, 91-94.

Raschle, M., Van Komen, S., Chi, P., Ellenberger, T., and Sung, P. (2004). Multiple interactions with the Rad51 recombinase govern the homologous recombination function of Rad54. J Biol Chem *279*, 51973-51980.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc *2*, 1896-1906.

Rockmill, B., and Roeder, G. S. (1991). A meiosis-specific protein kinase homologue required for chromosome synapsis and recombination. Genes Dev *5*, 2392-2404.

Tong, A. H., Evangelista, M., Parsons, A. B., Xu, H., Bader, G. D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C. W., Bussey, H.*, et al.* (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science *294*, 2364-2368.

Wan, L., de los Santos, T., Zhang, C., Shokat, K., and Hollingsworth, N. M. (2004). Mek1 kinase activity functions downstream of *RED1* in the regulation of meiotic DSB repair in budding yeast. Mol Biol Cell *15*, 11-23.