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Regulation of Rad51 function by phosphorylation

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Rad51 is a key enzyme involved in DNA double-strand break repair by homologous recombination. Here, we show that in response to DNA damage, budding yeast Rad51 is phosphorylated on Ser 192 in a manner that is primarily mediated by the DNA-damage-responsive protein kinase Mec1. We show that mutating Rad51 Ser 192 to Ala or Glu confers hypersensitivity to DNA damage and homologous-recombination defects. Furthermore, biochemical analyses indicate that Ser 192 is required for Rad51 adenosine triphosphate hydrolysis and DNA-binding activity *in vitro*, whereas mutation of Ser 192 does not interfere with Rad51 multimer formation. These data suggest a model in which Mec1-mediated phosphorylation of Rad51 Ser 192 in response to DNA damage controls Rad51 activity and DNA repair by homologous recombination.

Keywords: DNA repair; homologous recombination; Mec1; phosphorylation; Rad51

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INTRODUCTION

Cellular responses to DNA damage involve the recognition and repair of DNA lesions together with signalling processes that, among other things, delay cell-cycle progression (Jackson & Bartek, 2009). The main signalling proteins of the DNA damage response are the protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) that belong to the phosphatidylinositol 3-kinase-related protein kinase family. The budding yeast counterparts of ATM and ATR—Tel1 and Mec1, respectively—are activated by DNA lesions and phosphorylate several proteins at DNA-damage sites to promote cell survival and genome stability by various mechanisms (Traven & Heierhorst, 2005).

The most dangerous DNA lesions, DNA double-strand breaks (DSBs), are repaired by two main pathways: non-homologous end-joining and homologous recombination. Homologous recombination uses an intact homologous DNA sequence—usually a sister chromatid or a homologous chromosome—to repair a DSB, which involves processing of DNA ends to yield single-stranded 3′ overhangs, by a mechanism termed resection (Lisby & Rothstein, 2009). These 3′-overhanging single-stranded DNA (ssDNA) regions invade the homologous duplex DNA and anneal to the homologous sequence. Subsequent DNA synthesis then extends the annealed 3′ end using information from the intact template, and DNA repair synthesis is completed by ligation of DNA nicks and resolution of recombination structures to yield intact repaired products (San Filippo *et al*, 2008). A key homologous-recombination enzyme in all eukaryotes is Rad51, which catalyses annealing of resected DNA ends to homologous DNA sequences (Krogh & Symington, 2004; Aylon & Kupiec, 2004). Rad51 contains conserved Walker-A and Walker-B ATPase motifs and forms filaments on ssDNA that are thought to invade homologous DNA duplexes by Rad51-catalysed adenosine triphosphate (ATP)-dependent strand exchange (Paques & Haber, 1999; San Filippo *et al*, 2008; Lisby & Rothstein, 2009).

Evidence from budding yeast implicates DNA damage response kinases in regulating DNA repair, as cells lacking *MEC1* are defective in homologous recombination (Fasullo & Sun, 2008; Suetomi *et al*, 2010). Although the underlying mechanisms for this are not clear, Mec1- and Tel1-dependent phosphorylation of the Sae2 protein has been shown to promote DSB resection (Baroni *et al*, 2004), and homologous recombination is further promoted by Mec1-dependent phosphorylation of Rad55 (Bashkirov *et al*, 2006; Herzberg *et al*, 2006). Here, we establish that Rad51 is phosphorylated on Ser 192 in response to DNA damage in a Mec1-dependent manner, and that mutation of this site confers hypersensitivity to DNA damage and defective homologous-recombination-mediated repair of a homothallic switching (HO) endonuclease-induced DSB. Furthermore, through biochemical analyses, we establish that Ser 192 is important for Rad51 ATP hydrolysis activity, but not for Rad51 oligomer formation.

RESULTS AND DISCUSSION

Identification of Rad51 phosphorylation sites

To investigate whether Rad51 is phosphorylated in budding yeast, we generated a *Saccharomyces cerevisiae* strain in which a carboxy-terminally tandem affinity purification (TAP)-tagged

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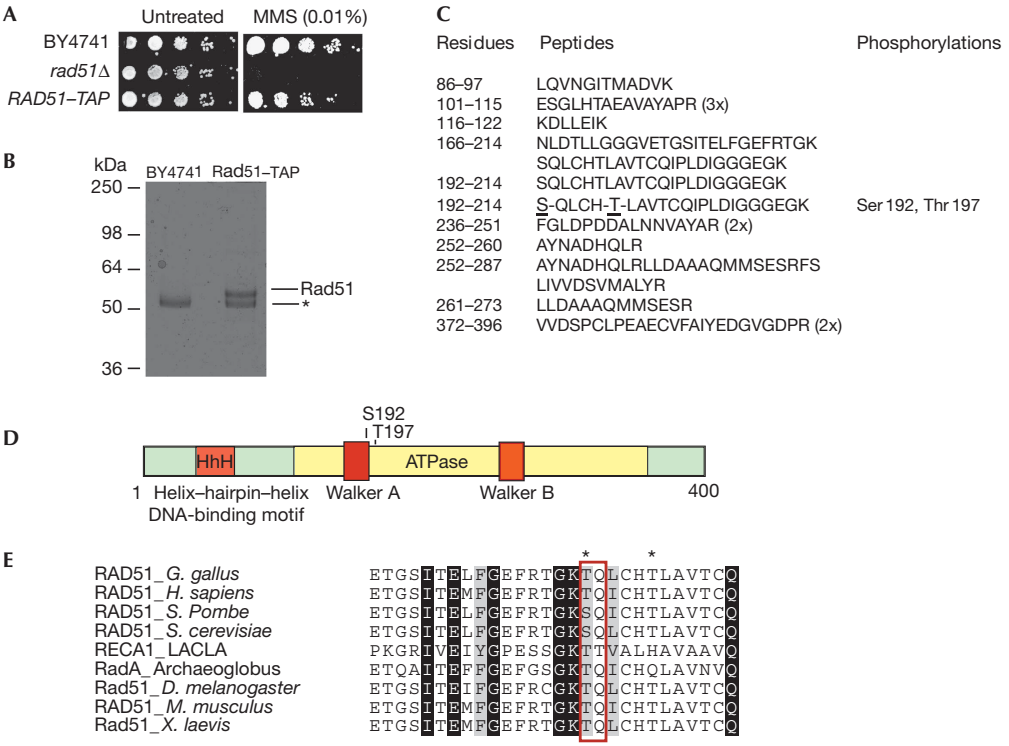

Fig 1 | Rad51 is phosphorylated on Ser 192 and Thr 197. (**A**) Wild-type (BY4741), *rad51*Δ (SFY103) and TAP-tagged Rad51 (SFY100) yeast cells were grown on plates in the presence or absence of MMS (0.01%). (**B**) SDS–PAGE and Coomassie staining of TAP purification from wild-type (BY4741) and TAP-tagged Rad51 (SFY100) yeast cells; asterisk indicates a contaminating protein. (**C**) Identified Rad51 peptides after phosphopeptide mapping mass spectrometry of purified Rad51. Phosphorylated residues are underlined. (**D**) Schematic showing the positions of Ser 192 and Thr 197 in Rad51, adjacent to the Walker-A box. (**E**) Alignment of the Rad51 Walker-A box sequences from different organisms, highlighting the identified Rad51 phospho residues, Ser 192 and Thr 197, with asterisks; Ser 192 and Gln 193 are in a red box. MMS, methyl-methane sulphonate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis, TAP, tandem affinity purification.

Rad51 derivative was expressed from the chromosomal *RAD51* locus under the control of its endogenous promoter. Importantly, TAP-tagged Rad51 was functional; in comparison to control cells, Rad51–TAP-expressing cells were only mildly hypersensitive to the DNA alkylating agent methyl-methane sulphonate (MMS) that causes replication-fork stalling and DSBs in S-phase, whereas *rad51*Δ cells were extremely hypersensitive to this drug (Fig 1A). We then used TAP to retrieve the tagged Rad51 from extracts of asynchronously growing yeast cultures (Fig 1B) and subjected the purified protein to phosphopeptide-mapping mass spectrometry. Of the Rad51 peptides detected, only one Rad51 phosphopeptide was identified, which was phosphorylated on two residues: Ser 192 and Thr 197 (Fig 1C). Notably, S192 and T197 are located in a highly conserved region of Rad51, adjacent to the Walker-A box that constitutes the ATP-binding site of the Rad51 ATPase domain (Fig 1D). Furthermore, as shown in Fig 1E, whereas T197 does not conform to a clear consensus for a known yeast kinase, S192 is followed by a highly conserved Gln (Q193), constituting a SQ sequence that is the preferred phosphorylation motif for Mec1 and Tel1 (Traven & Heierhorst, 2005).

Rad51 Ser 192 is phosphorylated after DNA damage

In light of the above, we decided to focus on S192 phosphorylation by raising antibodies against a phosphopeptide corresponding to this site. The resulting antibodies preferentially recognized the S192 phosphopeptide over its non-phosphorylated equivalent (Fig 2A), and detected Rad51 that had been *in vitro* phosphorylated by purified DNA-dependent protein kinase (DNA-PK)—a phosphatidylinositol 3-kinase-related protein kinase enzyme with a similar SQ target consensus sequence to Mec1 and Tel1 (Smith & Jackson, 1999)—more strongly than untreated or mock-treated Rad51 (Fig 2B; data not shown). Moreover, the Rad51-phospho-S192 antibody only recognized immunoprecipitated Rad51 when it was derived from cells that had been treated with DNA-damaging agents (Fig 2C,D). Furthermore, in accord with S192 residing in a Mec1 consensus motif, we observed DNA-damage-induced phosphorylation of Rad51 in wild-type cells; however, this was reduced in cells lacking Mec1 and was almost absent in cells lacking both Mec1 and Tel1 (Fig 2E).

Rad51 Ser 192 mutations impair Rad51 function *in vivo*

To investigate the functional importance of Rad51–S192, we tested the impact of mutating it to an unphosphorylatable Ala (A) or to a negatively charged Glu (E) residue, with the rationale that the latter mutant might mimic constitutive phosphorylation. Thus, we analysed the DNA-damage sensitivities of *rad51*Δ cells expressing, from the chromosomal *RAD51* promoter, wild-type Rad51, Rad51–S192A or Rad51–S192E fused

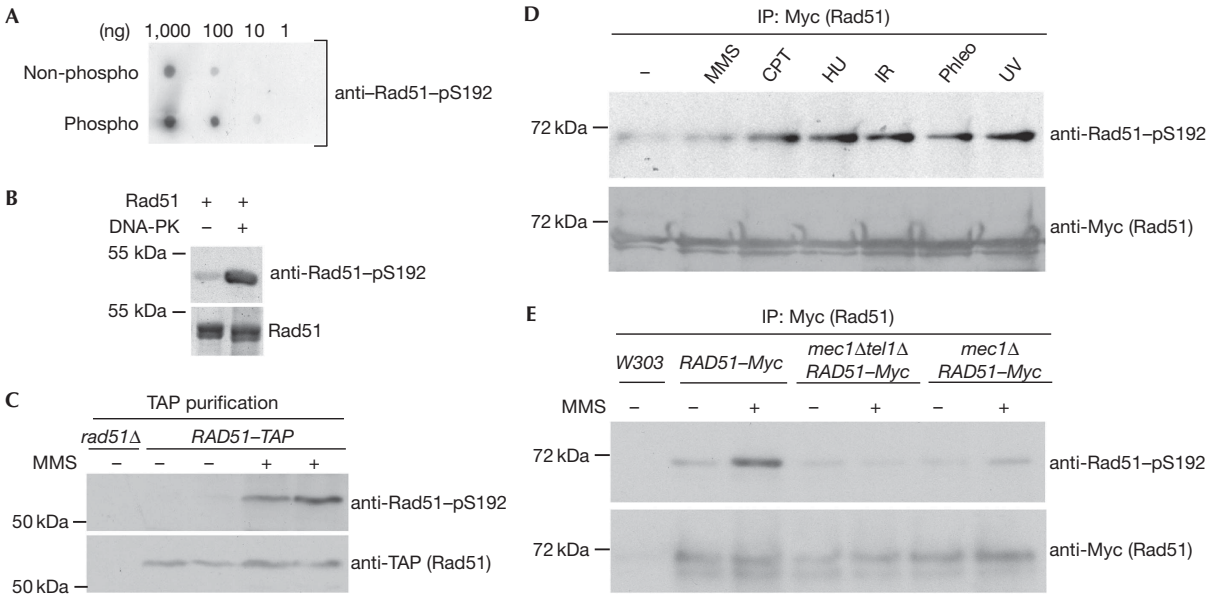

Fig 2 | Rad51 phosphorylation in response to DNA damage. (**A**) Rad51–S192 phospho-specific antibody (Rad51–pS192) preferentially recognizes Rad51–phospho-S192 peptide over Rad51–non-phospho-S192 peptide; amounts of peptides on membrane are indicated. (**B**) Kinase assay with purified DNA-PK and bacterially purified Rad51. Rad51 phosphorylation by DNA-PK was detectable with the Rad51–pS192 antibody (top), whereas protein levels are visualized by Coomassie staining (bottom) after SDS–PAGE. (**C**) TAP-purification from *rad51*Δ (SFY103) and TAP-tagged Rad51 (SFY100) yeast cells that were treated with MMS (0.02%) for 2 h, followed by western blot analysis with Rad51–pS192 and TAP antibodies; lanes 2 and 3 are derived from duplicate assays, as are lanes 4 and 5. (**D**) Rad51 was immunoprecipitated from lysates of Myc-tagged Rad51 yeast cells (SFY101) that were treated with MMS (0.02%), camptothecin (CPT; 5 μg/ml), hydroxyurea (HU; 0.2 M) or phleomycin (Phleo; 10 μg/ml) for 2 h, or with ultraviolet radiation (UV; 40 J/m^2) or ionizing radiation (IR; 180 Gy) and left to recover for 2 h. Immunoprecipitated Rad51 was detected by western blot analysis with Rad51–pS192 followed by Myc antibody. (**E**) Immunoprecipitations with a Myc antibody were performed from lysates of wild-type (W303-1A), Myc-tagged Rad51 wild-type (SFY101), Myc-tagged Rad51 *mec1*Δ*tel1*Δ (SFY102) and Myc-tagged Rad51 *mec1*Δ (SFY106) yeast cells that were treated with MMS (0.02%) for 2 h. Immunoprecipitated Rad51 was detected by western blot analysis with Rad51–pS192 followed by Myc antibody. DNA-PK, DNA-dependent protein kinase; IP, immunoprecipitation; MMS, methyl-methane sulphonate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TAP, tandem affinity purification.

to a C-terminal haemagglutinin tag (Fig 3A). This revealed that cells expressing Rad51–S192A or Rad51–S192E were almost as hypersensitive to the DNA-damaging agent MMS as were *rad51*Δ cells (Fig 3A). This effect was not due to lower protein expression, as both Rad51 mutants were expressed at levels similar to that of the wild-type protein (Fig 3B).

To directly measure the effects of S192 mutations on DNA repair, we used an HO endonuclease-induced yeast mating-type (*MAT*) switching assay, using the *HML* locus as the donor template (Haber, 2006; Ohuchi *et al*, 2008). The cells used for these assays contained a galactose-inducible HO endonuclease and expressed wild-type Rad51, Rad51–S192A or Rad51–S192E. After generating a DSB at the *MAT* locus by HO endonuclease induction, we prepared genomic DNA at various times and assessed repair by polymerase chain reaction (PCR) analysis. As shown in Fig 3C, although a strong PCR signal for DNA strand invasion was obtained with cells expressing wild-type Rad51, this signal was reduced in cells lacking Rad51 or in cells expressing Rad51–S192A or Rad51–S192E. Accordingly, although the homologous-recombination-ligation step was readily detectable in cells expressing wild-type Rad51, the PCR product indicative of DSB ligation was diminished in cells expressing Rad51–S192A or Rad51–S192E to a degree comparable with that of *rad51*Δ cells (Fig 3D). These data therefore indicated that Rad51 requires S192 to promote DSB repair by homologous recombination, and indicated that S192 phosphorylation might regulate homologous recombination.

Rad51 Ser 192 affects Rad51 ATPase activity

In the published structure of a Rad51 filament that is thought to represent the active form of Rad51 filaments (Conway *et al*, 2004), S192 lies at the interface of two Rad51 monomers and near the phosphate-binding loop (P-loop; Fig 4A). This suggests that phosphorylation on S192 might affect Rad51 filament formation. Furthermore, in the Rad51 filament structure, a sulphate ion resides in the P-loop, where the middle phosphate group of ATP would bind (Grigorescu *et al*, 2009), and a phosphate group covalently attached to S192 would also be able to occupy this position (Fig 4A). To address the potential impact of S192 on Rad51 multimer formation, we expressed wild-type Rad51, Rad51–S192A and Rad51–S192E, purified them from bacteria and monitored their native oligomer states by gel filtration. Rad51 multimers were fractionated through a Superdex-200 column and visualized by SDS–polyacrylamide gel electrophoresis analysis (Fig 4B). High-molecular-weight Rad51 oligomers were detectable for wild-type Rad51, as well as for Rad51–S192A and Rad51–

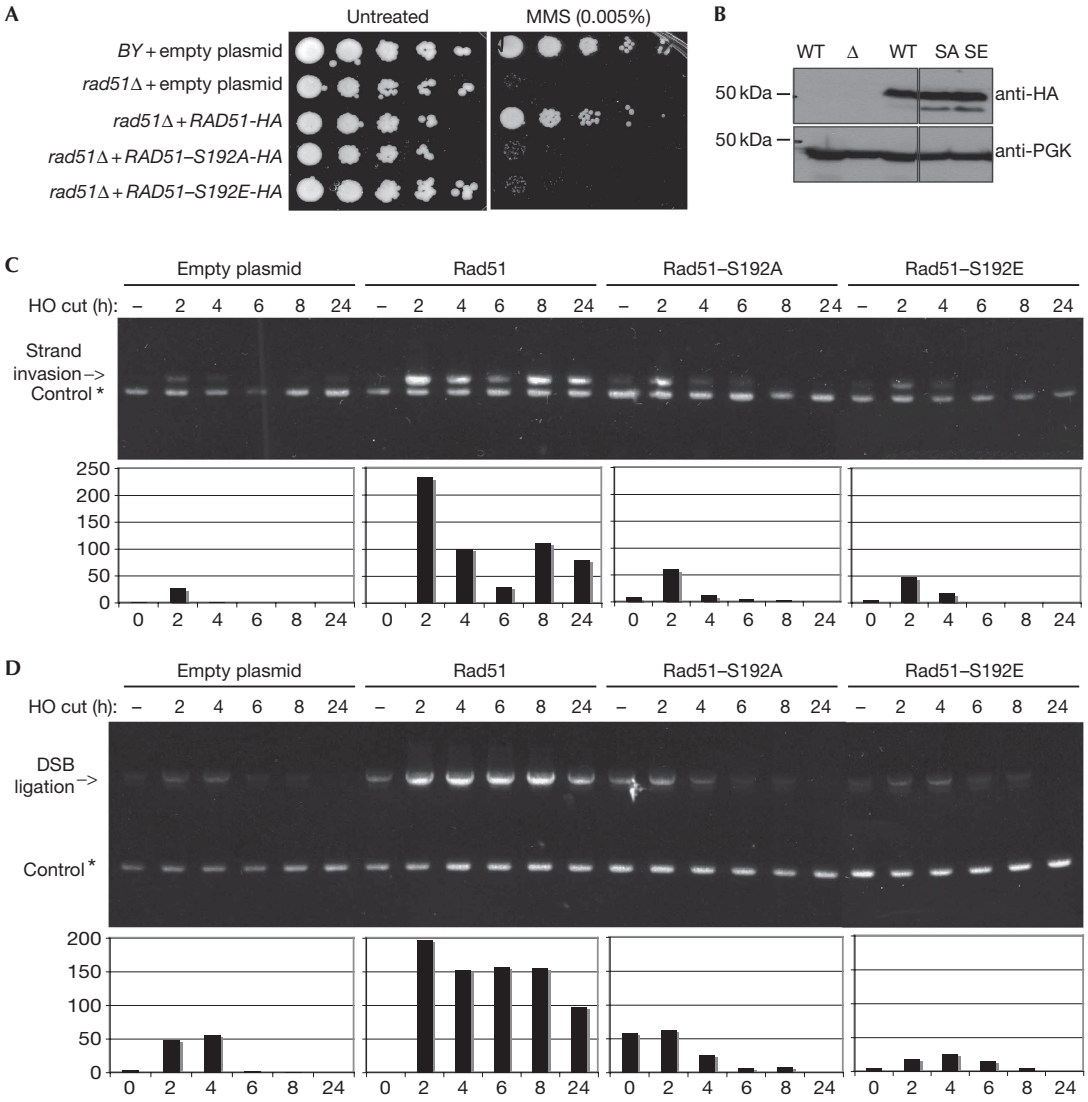

Fig 3 | Functional analyses of Rad51–S192 phosphorylation in cells. (**A**,**B**) MMS hypersensitivity assay of wild-type (WT; BY4741) and *rad51*Δ (Δ) cells (SFY103) that contained an empty plasmid or a plasmid expressing WT Rad51, Rad51–S192A (SA) or Rad51–S192E (SE), respectively, under the *RAD51* promoter with a C-terminal haemagglutinin (HA) tag. In **A**, cells were tested for survival in the presence or absence of MMS (0.005%). In **B**, TCA protein lysates were prepared from the indicated yeast strains, and Rad51–HA protein expression levels were monitored by western blot analysis with a HA antibody; a 3-phosphoglycerate kinase (PGK) antibody was used as a loading control; the image is formed from different parts of the same gel at the same exposure. (**C**,**D**) SFY105 (*rad51Δ*) cells expressing C-terminal HA-tagged Rad51 WT, Rad51–S192A or Rad51–S192E were transformed with a galactose-inducible HO endonuclease plasmid. A DNA DSB was introduced by induction of HO endonuclease, and genomic DNA samples were prepared from untreated cells at 2, 4, 6, 8 and 24 h after galactose induction. Repair of the break was monitored by PCR with primers to detect strand invasion (**C**) or ligation of the break (**D**). PCR products indicative of strand invasion or ligation of the break were quantified with Image J and normalized to band intensity of control PCRs (shown as histograms under the corresponding gel; similar results were obtained in three independent replicates of the experiments carried out in **C** and **D**, the histogram quantifications shown are derived from the primary data displayed in the upper panels). In **C**, we do not think that the reduction in strand-invasion product at the 6-h time point for WT Rad51 is of biological significance, as it was not observed in repeats of this experiment. DSB, double-strand break; HA, haemagglutinin; MMS, methyl-methane sulphonate; PCR, polymerase chain reaction; WT, wild type.

S192E (Fig 4B), indicating that S192 mutation does not alter the ability of Rad51 to form multimers in the absence of DNA, at least as detected by such assays.

To determine whether S192 influences Rad51 ATP hydrolysis and/or DNA binding, purified Rad51 wild type, Rad51–S192A and Rad51–S192E were incubated with ATP in the presence or absence of ϕX174 viral (+)-strand DNA to analyse ATP hydrolysis (Fig 4C), or with an 80-mer ssDNA oligonucleotide to assess DNA binding in the presence of ATP (Fig 4D). Although wild-type Rad51 hydrolysed ATP in the presence of DNA and readily formed

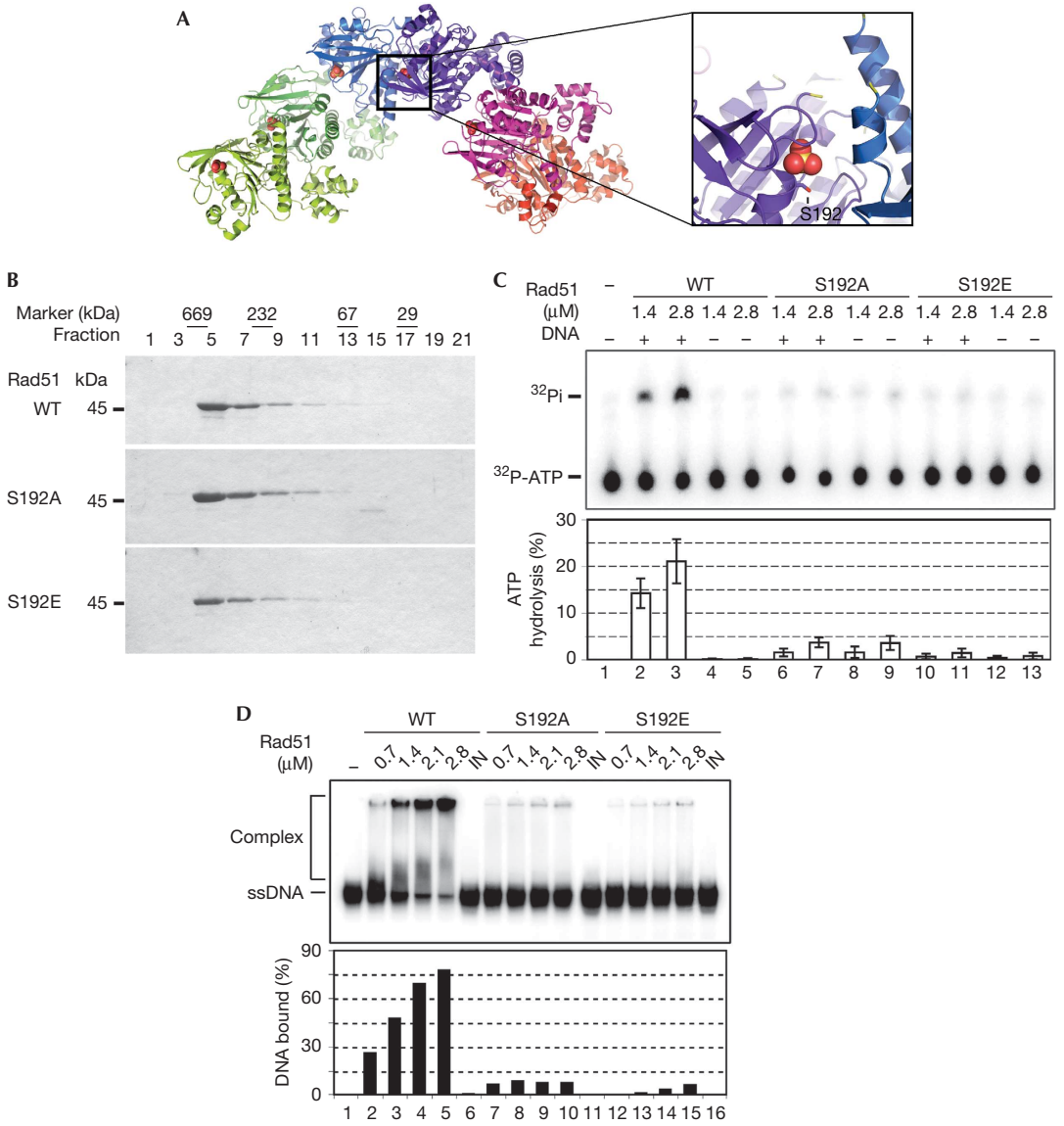

Fig 4 | Biochemical analysis of wild-type Rad51, Rad51–S192A and Rad51–S192E. (**A**) Crystal structure of a Rad51 filament, showing a sulphate in red at the monomer interface (Conway *et al*, 2004). Black box shows a close-up of the interface between two Rad51 monomers; S192 is depicted in stick representation. (**B**) Gel-filtration experiment to visualize Rad51 multimers. Bacterially purified wild-type Rad51, Rad51–S192A and Rad51–S192E were fractionated through a Superdex-200 column and analysed by SDS–PAGE and Coomassie staining. The elution positions of size markers are marked on the top; 669 kDa (thyroglobulin), 232 kDa (catalase), 67 kDa (BSA) and 29 kDa (carbonic anhydrase). (**C**) ATP hydrolysis assays on Rad51, Rad51–S192A and Rad51–S192E. Rad51 proteins were incubated with ATP and with or without DNA, as indicated. ATP hydrolysis was determined by thin-layer chromatography followed by phosphorimaging (top). Quantifications of ATP hydrolysis are presented in the histogram (bottom); error bars indicate the s.d. of three experiments. (**D**) DNA binding of Rad51, Rad51–S192A and Rad51–S192E. ^{32}P-labelled 80-mer ssDNA was incubated with indicated amounts of Rad51 protein and reaction mixtures were run on a native polyacrylamide gel and analysed by phosphorimaging (top). IN indicates proteinase/SDS-treated samples, and quantifications of DNA binding are presented in the histogram (bottom). The Rad51–DNA-binding gel shows various Rad51/DNA complexes, some of which are stuck in the wells (upper part of top panel) and some enter the gel (lower part of top panel). The complexes that are fully saturated with Rad51 might not enter the 8% polyacrylamide gel because of the size of the complex. ATP, adenosine triphosphate; MMS, methyl-methane sulphonate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; WT, wild type.

complexes with DNA in the DNA-binding assay, both Rad51–S192A and Rad51–S192E were defective in ATP hydrolysis and DNA binding (Fig 4C,D).

Our findings indicate that Rad51 S192 is crucial for Rad51 function, and provide a potential mechanism for the way in which Mec1-mediated phosphorylation of this site could influence homologous recombination. As mutation of Rad51–S192 causes severe defects in homologous recombination and DNA-damage resistance in cells, as well as in ATP hydrolysis and DNA binding *in vitro*, phosphorylation of this site provides a potential

mechanism to inactivate Rad51 after strand invasion has occurred. Rad51–S192A and Rad51–S192E formed multimers to a similar degree as wild-type Rad51, however, suggesting that their defects in ATP hydrolysis and DNA binding are probably not caused by improper folding. On the basis of these findings and structural considerations, we suggest that S192 phosphorylation could influence homologous recombination by mimicking the ATP- or ADP-bound form of Rad51, which is able to form a multimer but is catalytically inactive. It is tempting to speculate that Mec1-mediated S192 phosphorylation might have evolved as a mechanism to allow Rad51 molecules to be modified, presumably dynamically, in the context of recombination structures. This could allow for the formation of both ATP-bound and non-ATP-bound, phosphorylated versions of the protein, allowing homologous-recombination intermediates to become established and then regulating their maturation into alternative structures as recombination proceeds, and also possibly facilitating their removal once recombination is complete.

METHODS

Yeast strains, plasmids and antibodies. Plasmids and antibodies are described in the supplementary information online, and genotypes of strains are listed in supplementary Table S1 online.

DNA-damage sensitivity assays. Ten-fold serial dilutions of indicated strains were spotted onto agar plates with or without MMS. Plates were incubated at 30 °C for 3 days.

Rad51 phospho-site analysis. Rad51–TAP was purified by TAP (Huertas *et al*, 2008) and phosphopeptide analysis was performed by using immobilized metal affinity chromatography for enrichment of phosphopeptides before tandem mass spectrometry by the Albany Center for Functional Genomics (Proteomics and Mass Spectrometry Services, University of Albany, USA).

Kinase assays. Purified Rad51 was phosphorylated by DNA-PK *in vitro* (provided by KuDOS Pharmaceuticals). Each reaction included Rad51 (2 μg), DNA-PK (0.4 μg), kinase buffer (50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, pH 8.0, 0.1% β-mercaptoethanol, 10 mM magnesium acetate), double-stranded DNA (30 ng) and ATP (0.1 mM), and was incubated at 30 °C for 60 min.

Cell extracts, immunoprecipitations and western blots. Trichloroacetic acid lysates, native cell extracts, immunoprecipitations and western blots were performed as described previously (Flott & Rouse, 2005).

Detection of repair of HO endonuclease-induced DSBs. Galactose induction, genomic DNA isolation and PCR analysis for strand invasion and ligation were performed as described previously (Ohuchi *et al*, 2008).

Rad51 purification. Untagged yeast Rad51 and Rad51–S192 mutant proteins were overexpressed in *E. coli* Rosetta cells (Novagen), induced with isopropylthiogalactoside (0.1 mM) for 16 h at 16 °C and purified with ammonium sulphate precipitation, Q Sepharose fast flow, Macro Hydroxyapatite and Mono Q steps, as described previously (Van Komen *et al*, 2006).

Rad51 gel-filtration analysis. For gel-filtration analyses, purified Rad51 proteins (300–600 μg) were loaded onto a Superdex-200 10/300 column and were eluted with buffer T300 (25 mM Tris–HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.01% Igepal and 300 mM KCl) at a flow rate of 0.3 ml/min. After the first 6 ml elution, fractions (0.6 ml each) were collected, and eluted proteins were analysed by 10% SDS–polyacrylamide gel electrophoresis and Coomassie staining.

ATP hydrolysis assay. The indicated amounts of Rad51 or S192 mutants were incubated with 100 μM of ATP and 0.05 μCi/μl [γ-^{32}P] ATP in 10 μl reaction buffer (35 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 10 ng/μl φX174 viral DNA and 50 mM KCl) at 37 °C for 20 min. The reaction was stopped with equal volume of 0.5 M EDTA, and hydrolysed ATP was determined by thin-layer chromatography, followed by phosphorimaging analysis, as described previously (Sung & Stratton, 1996).

DNA-binding assay. DNA binding by Rad51 and S192 mutants was examined as described previously (Kwon *et al*, 2007), with minor modifications. The indicated amounts of proteins were incubated with ^{32}P-labelled 80-mer ssDNA (65 nM) for 5 min at 37 °C in 10 μl of binding buffer (35 mM Tris–HCl, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 100 μg/ml BSA, 1 mM DTT and 50 mM KCl). The reaction mixtures were resolved in an 8% polyacrylamide gel in 1 × Tris Acetate EDTA (TAE) buffer and the gel was visualized and quantified by phosphorimaging analysis.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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