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The putative nuclear localization signal of the human RAD52 protein is a potential sumoylation site

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RAD52, a key factor in homologous recombination (HR), plays important roles in both RAD51-dependent and -independent HR pathways. Several studies have suggested a link between the functional regulation of RAD52 and the protein modification by a small ubiquitin-like modifier (SUMO). However, the molecular mechanism underlying the regulation of RAD52 by SUMO is unknown. To begin investigating this mechanism, we identified possible target sites for sumoylation in the human RAD52 protein by preparing a RAD52–SUMO complex using an established *Escherichia coli* sumoylation system. Mass spectrometry and amino acid sequencing of the enzymatically digested fragments of the purified complex revealed that the putative nuclear localization signal located near the C terminus of RAD52 was sumoylated. Biochemical studies of the RAD52–SUMO complex suggested that sumoylation at the identified site has no apparent effect on the DNA binding, D-loop formation, ssDNA annealing and RAD51-binding activities of RAD52. On the other hand, visualization of the GFP-fused RAD52 protein in the human cell that contained mutations at the identified sumoylation sites showed clear differences in the cytosolic and nuclear distributions of the protein. These results suggest the possibility of sumoylation playing an important role in the nuclear transport of RAD52.

Keywords: homologous recombination/RAD52/self-association/sumoylation/enzymes/post translational modification.

DNA double-strand breaks (DSBs) pose a threat to genome integrity. Failure to repair such lesions may cause cell death or induce genomic rearrangements leading to various genetic disorders (*1*). Homologous recombination (HR) is a widely conserved mechanism for the accurate repair of DSBs. The RAD52 protein, which is conserved from yeast to human, is one of the key components of the eukaryotic HR machinery (*2*, *3*). RAD52 possesses a multitude of biochemical activities, which are likely to be important in the HR pathways that are both dependent and independent of RAD51, a recombinase that is conserved from bacteria to humans. Much of the biochemical studies were performed using the yeast and human RAD52 proteins. RAD52 has been shown to facilitate the assembly of the RAD51 recombinase on ssDNA coated with replication protein A (*4*, *5*), stimulate the DNA strand exchange activity of RAD51 (*6–9*) and catalyse the annealing reaction between homologous single-stranded DNAs (ssDNA) (*10–12*), as well as the heteroduplex formation between homologous ssDNA and double-stranded DNA (*13–15*). More recent studies suggest that RAD52 promotes the capturing of the second end of the DSB by annealing it to the displaced strand of the D-loop (*16–19*). Although the yeast and human RAD52 proteins display similar biochemical activities, these proteins may have distinct functions *in vivo*. In yeast, inactivation of RAD52 results in severe recombination and repair deficiencies, whereas in vertebrates, recombination is only mildly affected (*2*, *20*, *21*). This phenotypic difference may be attributed to the differential regulation of the functions of yeast and human RAD52 proteins.

The small ubiquitin-related modifier (SUMO) protein plays essential roles in maintaining genomic stability in eukaryotes (*22*). Sumoylation is one of the major post-translational modifications, in which the C terminus of SUMO covalently attaches to an internal lysine residue of the target protein. This reaction is mediated by a cascade of enzymes involved in the activation of SUMO (E1), as well as its transfer (E2) and substrate selection (E3) (*23*). Sumoylation has been shown to play important roles in the protein transport between the nucleus and the cytoplasm, the maintenance of the sub-nuclear architecture and the regulation of DNA replication, repair and recombination and of gene expression (*24*, *25*). In higher eukaryotes, defects in sumoylation can lead to cancer and developmental abnormalities (*26*).

Genomic and proteomic approaches have revealed several SUMO substrates, including RAD52 (*27*, *28*). In yeast, the sumoylation of RAD52 was demonstrated to be important for the exclusion of RAD52 from the nucleoli and for the regulation of recombinational

repair that occurs at the ribosomal gene locus (*29*). Others (*28*) have shown that RAD52 sumoylation shelters the protein from proteasomal degradation, which may be a useful regulatory mechanism to control the amount of RAD52 at the recombination site. The human RAD52 protein is also sumoylated *in vivo* (*28*). However, the molecular details concerning the regulatory roles of the human RAD52 sumoylation remain ill defined.

In the present study, we have prepared an SUMO-modified human RAD52 protein, using an established *Escherichia coli* sumoylation system. The RAD52–SUMO complex has been purified to homogeneity in milligram quantities, which allowed us to biochemically characterize the complex. Mass spectrometry and amino acid sequencing of the enzyme-digested RAD52–SUMO fragments revealed sumoylation sites at the putative nuclear localization signal (NLS), near the C terminus of the protein. SUMO modification did not affect either the DNA binding, D-loop formation, ssDNA annealing, or RAD51-binding activities of RAD52. On the other hand, mutations in the sumoylation sites significantly altered the subcellular localization of RAD52. These results suggest that RAD52 sumoylation at the putative NLS may regulate the subcellular localization of the protein.

Experimental Procedures

RAD52 sumoylation in E. coli

RAD52 and its lysine-to-arginine (KR) mutants were sumoylated using the *E. coli* SUMO modification system (*30*). To facilitate the expression of RAD52 and its mutants, the pT-E1E2S1 vector was modified to express low-abundance tRNAs. The region of the pArg3Arg4 vector (a gift from K. Sakamoto, Systems and Structural Biology Center, RIKEN, Yokohama, Japan) containing the tRNA genes was polymerase chain reaction (PCR)-amplified with a pair of primers, tRNA-Arg34-forward (TTTTTTCCGCGGTTGCACCTG AAGTCAGCCCCAT) and tRNA-Arg34-reverse (TTTTTTCCGC GGTTCACGATGCGTCCGGCGTAGA). The fragment was digested with SacII and was inserted into the SacII site of the pT-E1E2S1 vector. The human *RAD52* gene was cloned into the pET21a vector (Novagen, Darmstadt, Germany) to express RAD52 as a C terminally His_6-tagged recombinant protein. The open reading frame (ORF) was PCR-amplified with a pair of primers, RAD52-NdeI-forward (TTTCATATGTCTGGGACTGAGG AAGCAATTCTTGGAGGACGT) and RAD52-tpXhoI-reverse (T TTCTCGAGGCTGCCGCGCGGCACCAGAGATGGATCATA TTTCCTTT). The PCR product was subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The clone with the correct DNA sequence was double digested by NdeI and XhoI, and the fragment was inserted between the NdeI and XhoI sites of the pET21a vector. The translated protein has a thrombin-cleavage site before the His_6-tag, and the cleavage of the recombinant protein with thrombin protease leaves the amino acid residues, leucine-valine-proline-arginine (LVPR), at the C terminus of RAD52. Expression vectors for the RAD52 KR mutants were constructed using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA).

The modified vector (pT-E1E2S1 Arg3,4) and the vector bearing the wild-type or mutant *RAD52* gene were cotransformed into the *E. coli* JM109 (DE3) strain. For the purification, 4 l of an lysogeny broth (LB) culture was incubated at 37°C until the optical density (A_{600}) reached 0.6–0.8. The temperature was lowered to 25°C, and the culture was further incubated for 2 h. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce protein expression. Cells were harvested after overnight induction. Sumoylated proteins were purified as described previously (*31*), with the following exceptions. For the RAD52–SUMO complex, SP-sepharose (GE Healthcare, Uppsala, Sweden) was used instead of heparin–sepharose (GE Healthcare, Uppsala, Sweden) as the final purification column. For the sumoylated KR mutants, the purification step was omitted using the heparin–sepharose column. Unmodified RAD52 was purified using the same protocol as described above for the RAD52–SUMO complex.

The concentrations of all proteins were determined using a Bio-Rad protein assay kit, with bovine serum albumin (Nakalai Tesque, Kyoto, Japan) as the standard.

In vitro *RAD52 sumoylation*

RAD52 was sumoylated in a 10-μl reaction mixture, containing 50 mM Tris–HCl (pH 7.5), 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM ATP, Aos1-Uba2 heterodimer (E1, 0.5 μg), Ubc9 (E2, 1.25 μg), SUMO-1 (4 μg) and RAD52 (2 μg). The reaction mixture was incubated at 30°C for 2 h. The product was fractionated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Bands were visualized by Coomassie brilliant blue staining. E1 was purchased from BIOMOL International, L. P., Plymouth Meeting, PA, USA. Expression vectors for E2 and SUMO-1 were a kind gift from H. Saitoh (Kumamoto University, Kumamoto, Japan). The E2 enzyme was expressed as an N terminally His_6-tagged recombinant protein and was purified as described (*32*). The SUMO-1 protein was expressed as an N terminally glutathione S-transferase (GST)-fused recombinant protein and was purified as described (*33*), with the exception of chromatography on Q Sepharose (GE Healthcare, Uppsala, Sweden) and Glutathione Sepharose 4B (GE Healthcare, Uppsala, Sweden) columns, instead of the G75 Superdex column. The GST-SUMO-1 fusion protein was eluted from the Q Sepharose column in a linear gradient of 0–1.0 M NaCl in the described buffer. The fusion protein was digested with thrombin protease to remove the GST and was passed through a Glutathione Sepharose 4B column. The flow-through fraction, containing only SUMO-1, was collected. Both E2 and SUMO-1 were stored at −80°C.

Densitometric analysis

The sumoylated RAD52 was fractionated by 10% SDS–PAGE, and the Commassie brilliant blue-stained bands were quantitated with the ImageGauge software (Fuji Film Co., Tokyo, Japan).

Gel filtration analysis

The RAD52 and RAD52–SUMO proteins were concentrated to 1 mg/ml, and 200 μl of each concentrated protein was fractionated through a 25-ml Superdex 200 10/30 GL column (GE Healthcare, Uppsala, Sweden) using a buffer (pH 7.5) containing 20 mM HEPES–KOH, 0.3 M KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol and 5% glycerol.

Analytical ultracentrifugation

Sedimentation velocity distributions of sumoylated and unmodified RAD52 were obtained using an Optima XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). The proteins were centrifuged at 20°C in two separate cells using a Beckman An-60Ti rotor. Both proteins were dialysed against a buffer (pH 7.5) containing 20 mM HEPES–KOH, 0.3 M KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol and 5% glycerol. Protein concentrations were adjusted so that the absorbance at 280 nm was near 0.9. Velocity distributions were analysed at 5-min intervals for 5 h at 40,000 r.p.m. For the analysis, the protein partial-specific volumes were calculated based on the amino acid composition using the SEDNTERP program (0.722 cm^3/g for sumoylated and unmodified RAD52). The solvent density (1.033 g/cm^3) was determined by weighing the solvent equilibrated at 20°C. The velocity scans were analysed with the SEDFIT program (*34*).

Identification of sumoylation sites

RAD52 and RAD52–SUMO were each fractionated by 10% SDS–PAGE. The bands were excised from the gel and were subjected to an overnight, in-gel digestion with 40 ng of endoproteinase AspN (selectively cleaves peptide bonds at N-terminal to aspartic acid and cysteine residues) in 40 μl of 50 mM Tris–HCl, pH 8.0, at 37°C. The peptides generated were extracted from the gel and separated on a column of Inertsil ODS-3 (1 × 100 mm; GL Sciences Inc., Tokyo, Japan) connected in series with a model 1100 series liquid

chromatography system (Agilent Technologies, Waldbronn, Germany). Peptides were eluted at a flow rate of 20 μl/min using a linear gradient of 0–60% solvent B, where solvents A and B were 0.09% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile, respectively. Peptides specific for RAD52–SUMO were subjected to Edman degradation using a Procise cLC protein sequencing system (Applied Biosystems, Carlsbad, CA, USA) and to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Ultraflex MALDI-TOF (Bruker-Franzen Analytik, Bremen, Germany) in a reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix.

Assays for DNA binding, D-loop formation and ssDNA annealing

The ssDNA binding, dsDNA binding, D-loop formation and ssDNA annealing assays were performed as described (*31*).

Assay for RAD51 binding

RAD51 was purified as described previously (*35*). RAD51 (1 mg) was covalently conjugated to Affi-Gel 15 beads (150 μl, Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. The unbound proteins were removed by washing the beads five times with binding buffer (pH 7.5), which contained 20 mM HEPES–KOH, 0.15 M KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, 10% glycerol and 0.05% Triton X-100. To block the residual active ester sites, ethanolamine (pH 8.0) was added to a final concentration of 0.1 M, and the resin was incubated at 4°C overnight. After washing the resin five times with 150 μl aliquots of the binding buffer, the Affi-Gel 15-protein matrices were adjusted to 50% slurries with the binding buffer and were stored at 4°C. For the binding assay, 8 μl of the Affi-Gel 15-protein slurry (~20 μg of RAD51) were mixed with 30 μg of RAD52 or RAD52–SUMO at room temperature for 1.5 h. The Affi-Gel 15-protein beads were then washed three times with 500 μl of the binding buffer. A denaturing buffer, which contained 0.1 M Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol and 0.7 M 2-mercaptoethanol, was mixed directly with the washed beads. After heating the mixture at 95°C for 5 min, the proteins were fractionated by 10% SDS–PAGE. Bands were visualized by Coomassie brilliant blue staining.

Visualization of EGFP-fused RAD52 KR mutants in human cells

GM0637 cells, a simian virus 40-transformed fibroblast cell line, were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were grown on glass cover slips and transfected with each expression vector using a GeneJuice transfection reagent (Novagen, Darmstadt, Germany). Cells were fixed 24 h after transfection with 4% paraformaldehyde in 1× phosphate buffered saline (PBS) for 10 min and then permeabilized with 0.5% Triton X-100 in 1× PBS for 10 min. Nuclei were stained with 10 μM Hoechst 33342 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 1 min. Cover slips were mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Images were obtained with an Axioplan2 epifluorescence microscope equipped with AxioCam MRm controlled by Axiovision (Carl Zeiss, Göttingen, Germany). Adobe Photoshop was used for the presentation of images.

Results

Preparation of the RAD52–SUMO complex

The human RAD52–SUMO complex was prepared using the *E. coli* sumoylation system developed by Saitoh and co-workers (*30*). This system has been successfully used to reconstitute functionally relevant sumoylated states of several proteins (*36–39*). In this system, a target protein is co-expressed with SUMO and its conjugating enzymes (E1 and E2) in *E. coli*. As RAD52 requires co-expression of low-abundance tRNAs for optimal expression, we modified the system by inserting the *Arg3* and *Arg4* tRNA genes from the pArg3Arg4 vector into the pT-E1E2S1 vector, which contains the SUMO-1, Aos1-Uba2 fusion (E1) and Ubc9 (E2) genes (Fig. 1A). The C terminally hexahistidine-tagged RAD52 was sumoylated, and the resulting complex was purified from the cell lysate by Ni column chromatography, followed by the removal of the His-tag with thrombin protease and cation exchange column chromatography. The final purification fraction contained products that migrated slower than the unmodified RAD52 in SDS–PAGE and was clearly visible with Commassie brilliant blue staining (Fig. 1B, lane 4). To confirm that the product was RAD52–SUMO, we treated the purified fraction with SUMO protease, an enzyme that specifically deconjugates SUMO from its target protein. The treatment with SUMO protease completely eliminated the product, and a band corresponding to the size of the SUMO molecule appeared at the bottom of the gel (Fig. 1B, lane 5). These observations indicate that RAD52 was sumoylated in this system. *In vitro* sumoylation of RAD52 using purified SUMO and its conjugating enzymes also yielded a product with the same migration distance (Fig. 1C).

Identification of the sumoylation site in RAD52

Previously, several sumoylation sites of *Saccharomyces cerevisiae* RAD52 were identified, and they were located near the N terminus and near the NLS of the protein (*28*). The human RAD52 protein is also known to be sumoylated *in vivo* (*28*). However, the target sumoylation sites have not been identified. RAD52 has two structurally and functionally distinct regions, the N terminal half and the C terminal half (Fig. 2A). The isolated, N terminal half self-associates into a ring structure (*40–42*) and is responsible for the DNA binding, DNA annealing and D-loop formation activities of RAD52 (*13*, *40*, *31*). On the other hand, the isolated, C terminal half does not self-associate into a ring structure (*40*), and it contains regions for replication protein A (RPA) and RAD51 binding, along with the putative NLS near the C terminus of the protein (*43*, *44*). Elucidating the sumoylation sites relative to these known functional regions may provide clues to the possible roles of sumoylation in RAD52 function. It is also of interest to see whether sumoylation sites are conserved between human and yeast. To identify potential target sites for the human RAD52 protein, the SUMO attachment sites in the purified RAD52–SUMO complex were determined by mass spectrometry and amino acid sequencing (Fig. 2B). The RAD52–SUMO complex was first isolated by SDS–PAGE fractionation. The band containing RAD52–SUMO was excised from the gel and was treated with endoproteinase AspN using the in-gel digestion method. As a negative control, unmodified RAD52 was similarly isolated and digested. The fragments of RAD52–SUMO and unmodified RAD52 were then separated by reverse phase liquid chromatography, and those unique to RAD52-SUMO were isolated, and identified by amino acid sequencing and mass spectrometry (MALDI TOF-MS). One of the identified peptide fragments corresponded to a RAD52 fragment ($RAD52_{409\text{-}415}$, DMKKRKY) that was covalently attached to the C terminal fragment of SUMO

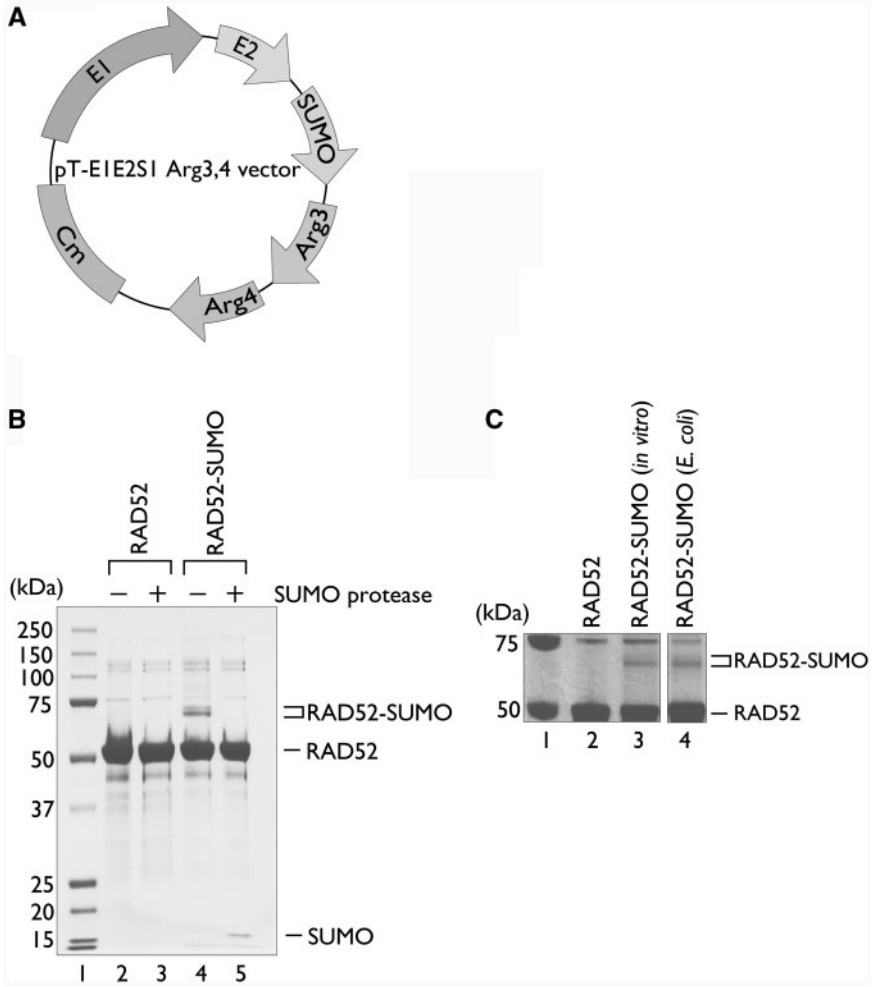

Fig. 1 Preparation of the RAD52-SUMO complex. (A) Schematic diagram of the *E. coli* sumoylation vector. The *Arg3* and *Arg4* tRNA genes were inserted at the SacII site of the vector to express low-abundance tRNAs for enhanced expression of the target protein. (B) About 4 μg of purified RAD52 (lanes 2 and 3) and purified RAD52 that was co-expressed with the sumoylation components (lanes 4 and 5) were fractionated by 10% SDS–PAGE. The sumoylation was confirmed by treating the purified complex (30 μg) with the *S. cerevisiae* SUMO protease (15 U) in the SUMO protease buffer provided by the manufacturer (Invitrogen, Carlsbad, CA, USA) (lane 5). (C) *In vitro* sumoylation of RAD52, using the purified Aos1-Uba2 heterodimer (E1), Ubc9 (E2) and SUMO-1.

($SUMO_{86-97}$, DVIEVYQEQTGG) (Fig. 2C). The identified RAD52 fragment overlaps with the putative NLS of RAD52 and contains three candidate sumoylation sites (Lys411, Lys412 and Lys414).

When RAD52 mutants containing arginine substitutions at Lys411, Lys412 and Lys414 were co-expressed with SUMO and its conjugating enzymes (E1 and E2) in *E. coli*, we observed lower levels of sumoylation compared with that of the wild-type RAD52. The double- and triple-point mutations nearly abolished sumoylation (Fig. 2D, lanes 7–10), indicating that sumoylation of RAD52 in the *E. coli* system mainly occurred at the lysine residues in the putative NLS. Notably, the single-point mutations did not reduce the efficiency of sumoylation (Fig. 2D, lanes 4–6). Sumoylation may have taken place at other non-mutated, lysine residues.

Biochemical characterization of the sumoylated RAD52

Throughout the course of the RAD52–SUMO complex preparation, the unmodified RAD52 was co-purified with the RAD52–SUMO complex. Densitometric analysis of the gel bands indicated an approximate 1 : 6 to 1 : 7 ratio between the RAD52–SUMO complex and the unmodified RAD52 (Fig. 3). Because RAD52 forms heptameric rings (*45*), a ring structure composed of both the RAD52–SUMO complex and the unmodified RAD52 may form. Consistent with these observations, a gel filtration analysis revealed that the RAD52–SUMO complex co-eluted with the unmodified RAD52 (Fig. 4). To further characterize the complex formed between RAD52–SUMO and RAD52, the purified complex was analysed by analytical ultracentrifugation. Sedimentation velocity analysis revealed that the complex existed primarily as a single species, as judged from the sedimentation coefficient distributions (Fig. 5). The average sedimentation coefficient of the complex was close to that of the RAD52 heptamer, suggesting that the size of the complex formed by sumoylated and unmodified RAD52 was similar to that of the heptameric RAD52 ring. These observations suggest that the

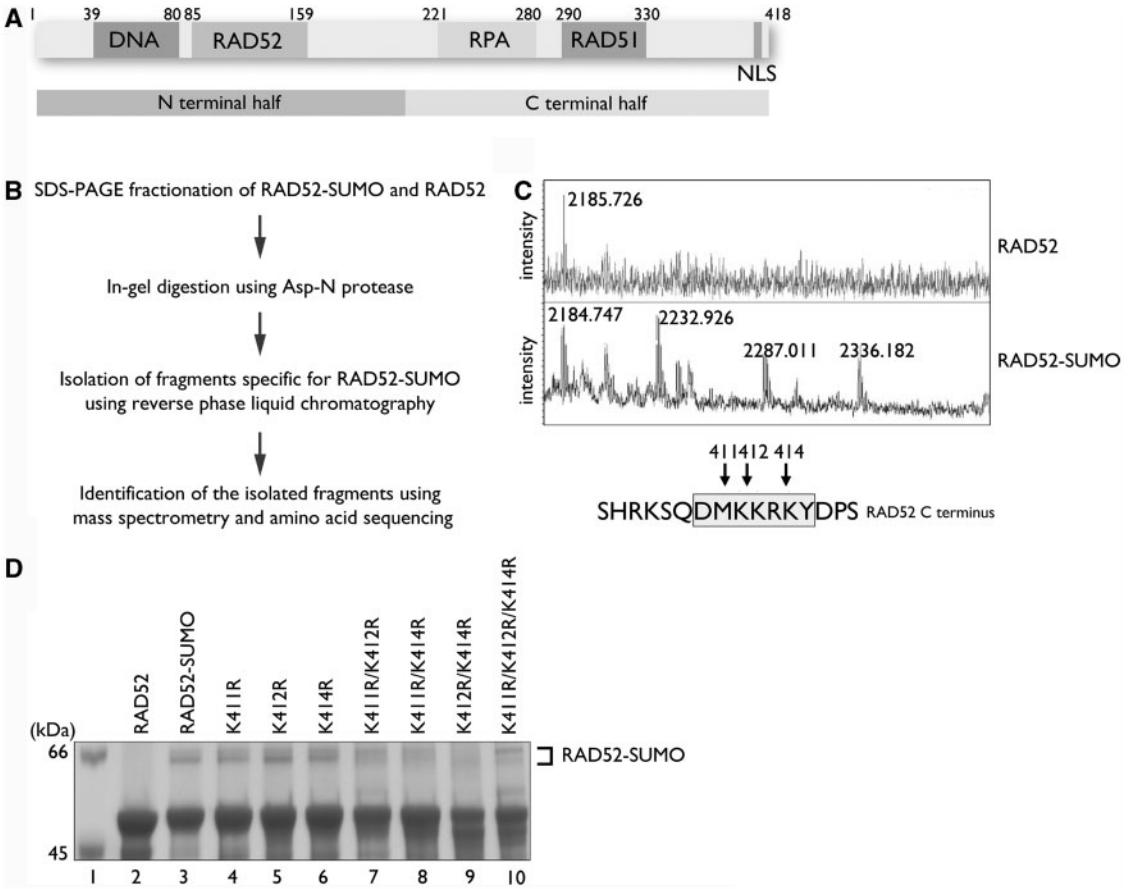

Fig. 2 Identification of the sumoylation site in RAD52. (A) The known functional regions of RAD52 mapped on its primary structure. The regions essential for DNA binding and self-association are located within the N terminal half. The C terminal half contains the putative NLS, as well as the interacting regions for RPA and RAD51. (B) Procedures for identifying the sumoylation site of RAD52. (C) Mass spectrometry of the digested fragments of RAD52 and RAD52–SUMO. The fragment with a molecular mass of 2,287 Da corresponds to the RAD52 sequence boxed in yellow (below) that is covalently attached to the C terminal SUMO-1 sequence (DVIEVYQEQTGG). Other major peaks corresponded to fragments of SUMO (2232.93 Da, $SUMO_{12\text{-}32}$, DLGDKKEGEYIKLKVIGQDSS) and RAD52 (~2185 Da, $RAD52_{13\text{-}33}$, DSHPAAGGGSVLCFGQCQYTA and 2336.18 Da, $RAD52_{123\text{-}144}$, DVGYGVSEGLKSKALSLEKARK). (D) About 8 μg of sumoylated RAD52 KR mutants were fractionated by 10% SDS–PAGE.

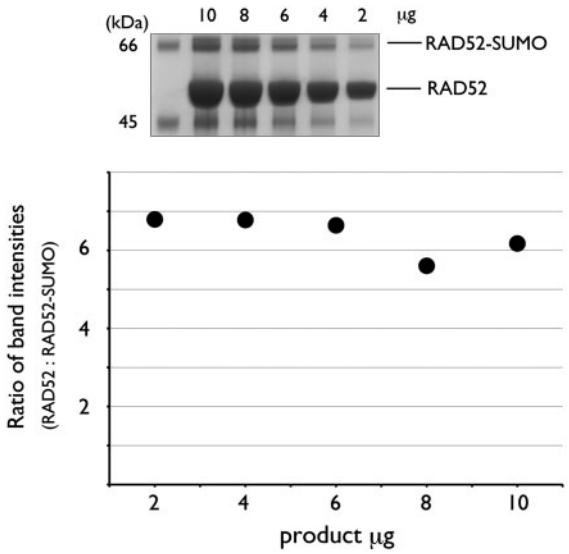

Fig. 3 Densitometric analysis of the sumoylated RAD52. Varying amounts (2–10 μg) of sumoylated RAD52 were fractionated by 10% SDS–PAGE. Bands corresponding to RAD52–SUMO and RAD52 were quantitated using the ImageGauge software (Fuji Film Co., Tokyo, Japan), and their ratios were plotted against the total amount of protein.

sumoylated RAD52 prepared in the present study existed as a mono-sumoylated, heptameric RAD52 ring.

We next investigated the effects of the sumoylation on various recombination activities reported for RAD52. The electrophoretic mobility shift assays (EMSAs) indicated that the ssDNA- and dsDNA-binding activities of the sumoylated RAD52 were nearly the same as those of the unmodified RAD52 (Fig. 6A). The D-loop formation and ssDNA annealing activities of RAD52 were also unaffected by sumoylation (Fig. 6B and C). We next examined whether sumoylation affects the RAD52 function that is mediated through its C terminal half. A key function of the C terminal half of RAD52 is RAD51 binding (*44*). A pull-down assay was performed, in which RAD51 was covalently conjugated to Affigel 15 beads, and either unmodified or sumoylated RAD52 was co-precipitated. The experiment revealed that the sumoylated RAD52 displayed similar affinities towards RAD51 as compared with the unmodified RAD52 (Fig. 6D).

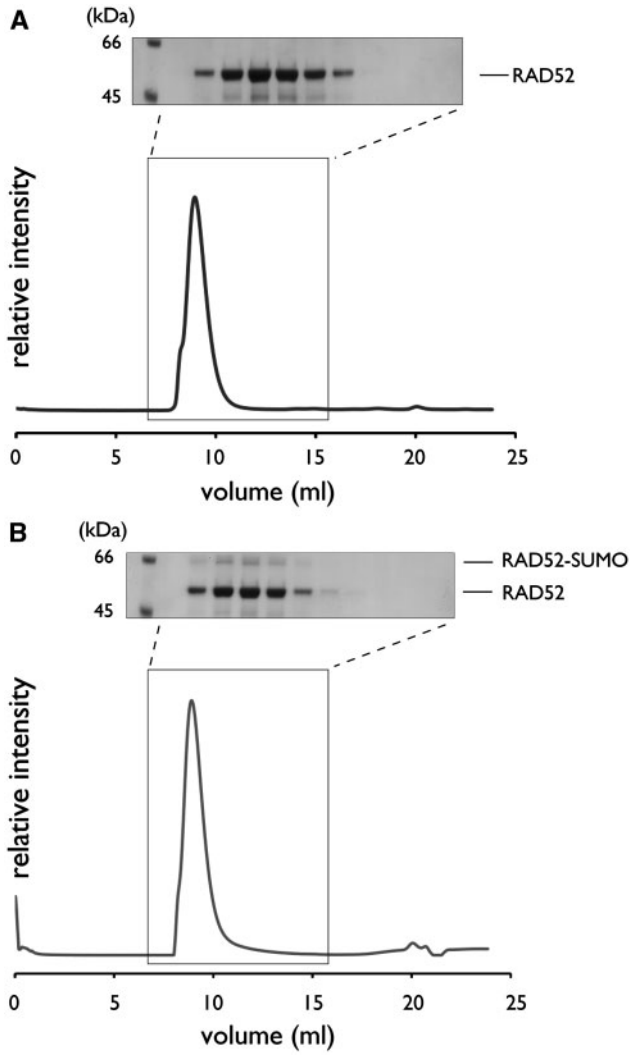

Fig. 4 Gel filtration analysis of the sumoylated RAD52 protein. Elution profiles of RAD52 and RAD52–SUMO from a Superdex 200 gel filtration column are shown. The boxed portion containing the peak was fractionated and analysed by SDS–PAGE.

Subcellular localization of RAD52 and its lysine mutants

The overlapping of the identified sumoylation site and the putative NLS region suggests a relationship between sumoylation and the subcellular distribution of RAD52. To examine whether the identified sumoylation sites (K411, K412 and K414) play a role in the subcellular distribution of RAD52, we constructed a vector containing an N terminally, EGFP-tagged RAD52, and transfected the human GM0637 cells with the vector. More than 80% of the wild-type RAD52 protein was located in the nucleus (Fig. 7B), which is consistent with previous studies (*46*). The K412R/K414R double mutant also exhibited similar nuclear localization as the wild type (Fig. 7A and B). In contrast, the K411R/K412R double mutant was mostly localized in the cytoplasm, suggesting that K411 is the key residue affecting the nuclear localization of RAD52 (Fig. 7A and B). Furthermore, K412 appears to also play a role in the nuclear

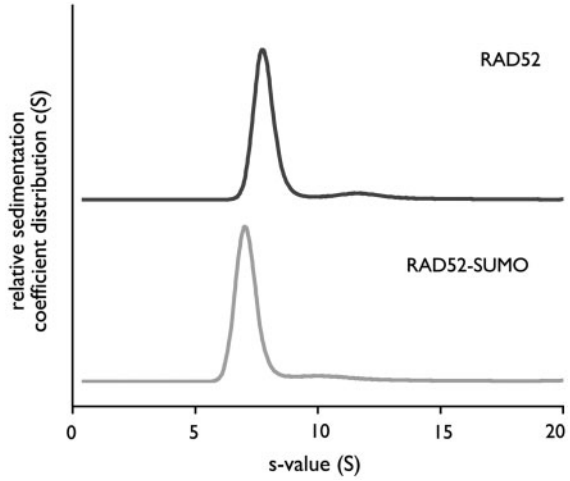

Fig. 5 Sedimentation coefficient distributions derived from sedimentation velocity profiles of RAD52 and RAD52–SUMO. Velocity distributions were analysed at 5-min intervals for 5 h at 40,000 rpm.

localization, because the K411R/K414R mutant was not completely defective in localizing at the nucleus (Fig. 7A and B). These results demonstrate that K411 and K412 are important for the nuclear localization of RAD52.

Discussion

The human RAD52 protein was shown to be sumoylated *in vivo* (*28*). However, the *in vivo* roles, as well as the biochemical characterization of the sumoylated RAD52, have not been reported. In the present study, we purified a RAD52–SUMO complex in milligram quantities, and studied the effects of the SUMO molecule on RAD52 by structural and biochemical means. Mass spectrometry and amino acid sequencing analyses identified sumoylation sites at the putative NLS, which is located near the C terminus of RAD52. The sumoylated RAD52, prepared in this study, was mostly homogeneous, forming a multimeric complex with the unmodified RAD52 in an approximate 1 : 6 ratio. Biochemical studies of the purified, sumoylated RAD52, however, did not reveal any clear differences in DNA binding, D-loop formation, ssDNA annealing or RAD51-binding activities with those of the unmodified RAD52. In contrast, mutations in the identified sumoylation sites affected the nuclear and cytosolic distributions of RAD52. Thus, these results suggest that the identified sumoylation sites play a role in nuclear–cytosolic transport. However, it remains a possibility that the apparent disinvolvement of SUMO in the biochemical activities of RAD52 was due to the insufficient number of SUMO molecules attached to the RAD52 ring. The *E. coli* sumoylation system utilized in the present study lacks the E3 ligase, which is believed to be essential for the efficient sumoylation of the target protein. The E3 ligase that specifically sumoylates RAD52 is unknown, but its identification and utilization in an *in vitro* sumoylation system may be essential to introduce multiple sumoylations in RAD52.

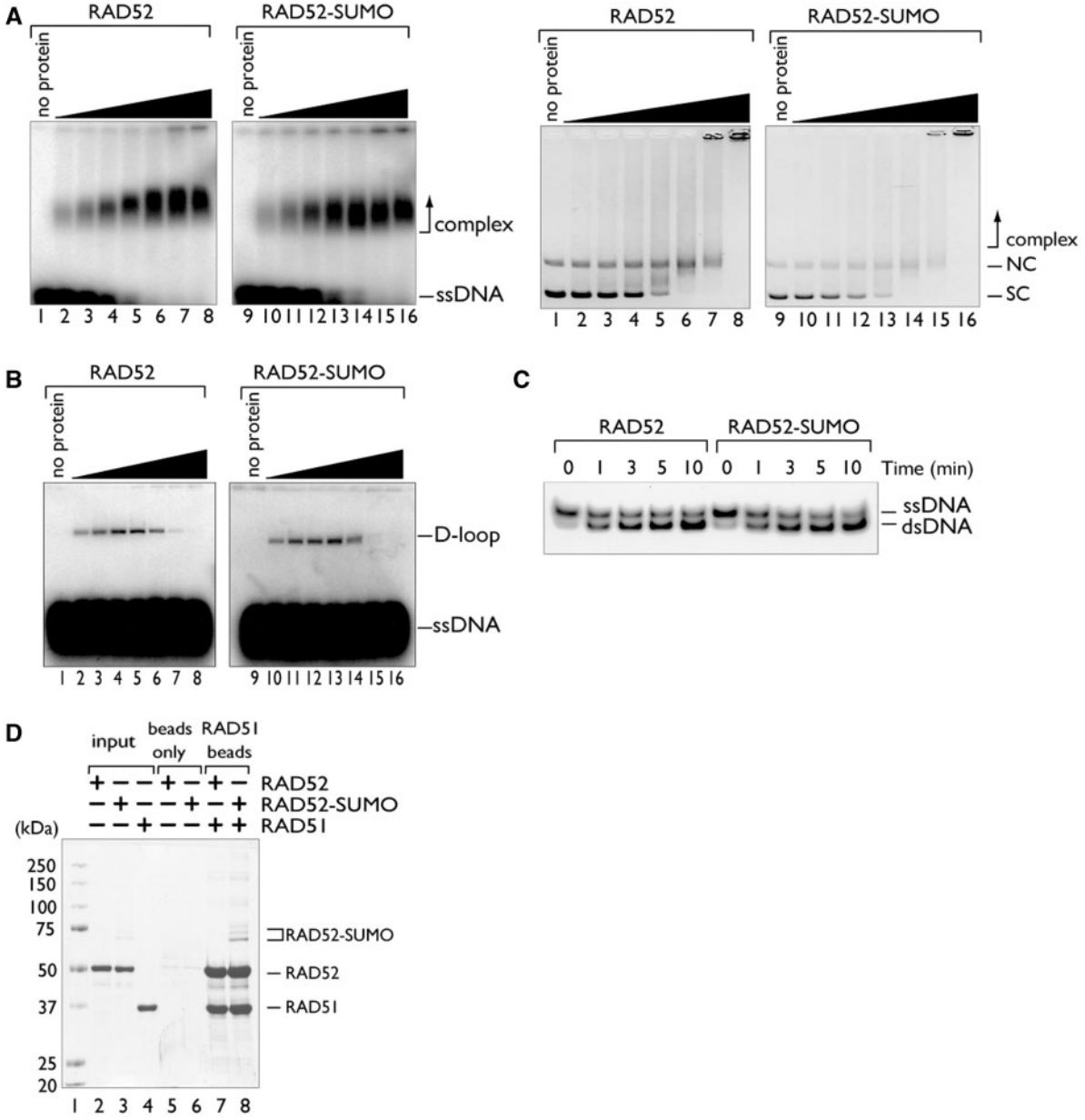

Fig. 6 Biochemical activities of the sumoylated RAD52 protein. (A) ssDNA- and dsDNA-binding activities. Increasing amounts of the proteins (0.03, 0.06, 0.12, 0.25, 0.5, 1 and 2 μM in lanes 2–8 and lanes 10–16, respectively) were incubated with either a ^{32}P-labelled ssDNA (sense SAT-1-50, 50-mer, 1 μM in nucleotides) or a negatively supercoiled plasmid DNA (pGsat4, 3.2 kb, 30 μM in nucleotides). Products were fractionated through 1% agarose gels. Lanes 1 and 9 indicate negative control experiments without protein. SC denotes supercoiled DNA, and NC denotes nicked circular DNA. (B) D-loop formation activities. Increasing amounts of RAD52 or RAD52–SUMO (0.03, 0.06, 0.12, 0.25, 0.5, 1 and 2 μM in lanes 2–8 and lanes 10–16, respectively) were incubated with a ^{32}P-labelled ssDNA (5S-repeat-10-1, 50-mer, 1 μM in nucleotides) at 37°C for 5 min, followed by the addition of a negatively supercoiled plasmid DNA (pB5Sarray, 5.3 kb, 30 μM in nucleotides). After deproteination, products were fractionated through 1% agarose gels. (C) ssDNA annealing was catalysed by RAD52 or RAD52–SUMO. Proteins (0.25 μM) were first complexed with a ^{32}P-labelled 1-μM ssDNA (sense SAT-1-50, 50-mer) followed by the addition of a complementary ssDNA (antisense SAT-1-50, 50-mer) to initiate the reaction. Products were fractionated by PAGE on a 20% gel in 0.5× Tris-Borate-EDTA (TBE) buffer. (D) RAD51-binding activities. RAD52 or RAD52–SUMO (30 μg) was mixed with RAD51 (~20 μg) covalently conjugated to an Affi-Gel 15 matrix. After incubation for 90 min at room temperature, the Affi-Gel matrix was washed and was mixed with a denaturing buffer. The buffer was boiled at 95°C for 5 min and was fractionated by 10% SDS–PAGE. Lanes 2–4 show the purified proteins used for the binding assay. Lanes 5 and 6 are the negative controls using the Affi-Gel 15 matrices with active ester sites blocked by ethanolamine.

One of the key functions of sumoylation is the targeting of proteins to a particular location in the cell. Studies by Torres-Rosell *et al.* (*29*) have shown that sumoylation of the *S. cerevisiae* RAD52 protein regulates recombinational repair at the ribosomal gene focus. Mutations that disrupt RAD52 sumoylation result in RAD52 foci formation in the nucleolus and cause rDNA hyper-recombination. It has also been demonstrated that nuclear localization is a prerequisite for the sumoylation of the *S. cerevisiae* RAD52 protein (*47*). These observations suggest that SUMO plays a vital role in regulating the recombination activities of

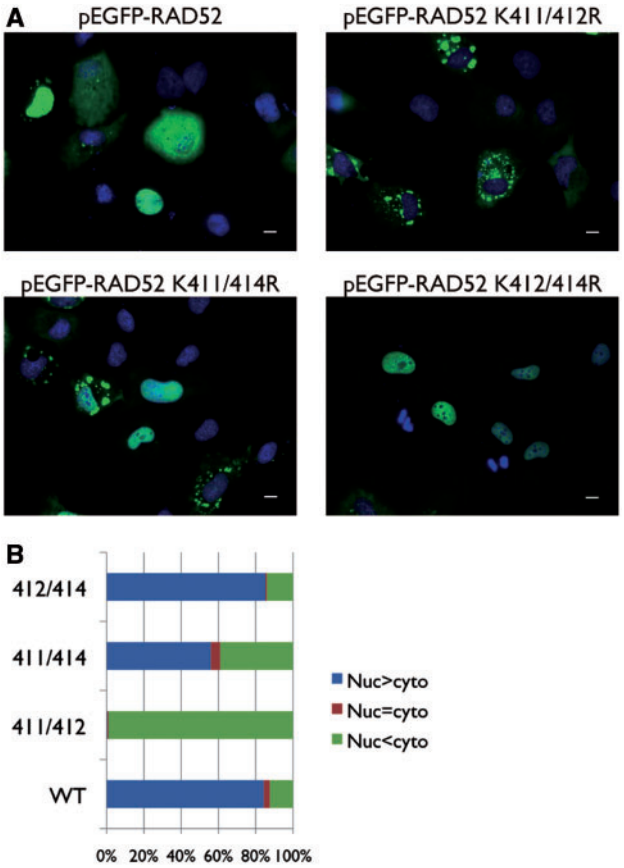

Fig. 7 Subcellular localization of GFP-tagged RAD52 wild-type and lysine mutants in GM0637 cells. (A) GM0637 cells were transfected with either GFP-tagged wild-type RAD52, K411/412R (RAD52K411/412R), K411/414R (RAD52K411/414R) or K412/414R (RAD52K412/414R) mutants expressing vectors. GFP signals and DNA are visualized in green and blue, respectively. Scale bars = 10 μm. (B) Bar plots of the percentages of cells expressing the indicated proteins show the different types of protein distribution: predominantly cytoplasmic distribution (Nuc < Cyto), nuclear distribution (Nuc > Cyto) and diffuse distribution (Nuc = Cyto). The number of cells examined were as follows: wild-type RAD52 (243), RAD52K411/412R (217), RAD52K411/414R (220) and RAD52K412/414R (236).

RAD52, by altering its localization within the nucleus. Our studies suggest the possibility that SUMO may also regulate the nuclear–cytosolic transport of RAD52. A similar regulation has been observed for the human poly(A) polymerase (*48*). However, because the presently identified sumoylation sites overlap with the putative NLS, it is difficult to ascertain whether the mutations introduced in the sumoylation sites affected the sumoylation process, and not the nuclear transport process, *in vivo*. Nevertheless, we note that all target lysine residues were mutated to arginine, which is one of the amino acid residues that constitute the consensus nuclear localization sequence. Therefore, the arginine-substituted NLS may still render it functional in the nuclear transport process, but non-functional in the sumoylation process. Interestingly, studies by Plate *et al.* (*49*) showed that self-association of the *S. cerevisiae* RAD52 protein is important for its nuclear localization, and the additive effect of multiple NLSs in the ring structure ensures efficient nuclear localization. It is of significant interest whether SUMO plays a role in this process.

SUMO is also known to covalently interact with SUMO-interacting motifs of target proteins and alter their functions. A potential role for SUMO-interacting motifs in DNA replication, repair, transcription and nuclear PML body formation has been suggested (*50*). A previous study showed that non-conjugated SUMO forms a complex with RAD51 and RAD52 (*51*). Therefore, while our results suggest that the DNA-binding and RAD51-binding activities of RAD52 are not regulated by sumoylation at the putative NLS, it remains possible that SUMO may regulate RAD52 functions, such as RAD51 binding, via non-covalent interactions.

Finally, an important issue is whether the yeast and human RAD52 proteins share similar regulatory mechanisms by the SUMO modification. In the case of the *S. cerevisiae* Rad52 protein, several sumoylation sites have been found, and both mono- and di-sumoylated RAD52 have been detected in yeast cell extracts (*28*). In contrast, the sumoylation pattern of the human RAD52 protein is clearly different from those of the *S. cerevisiae* RAD52 protein *in vivo*. Furthermore, the location of the sumoylation sites is not conserved between yeast and human RAD52 proteins. These observations suggest that sumoylation may have different regulatory roles in yeasts and humans. Humans have multiple SUMO isoforms, SUMO-2, SUMO-3 and SUMO-4 (*52*, *53*), in addition to the presently studied SUMO-1, while yeast has only one SUMO homolog, Smt3. Thus, RAD52 may also contain specific sumoylation sites for these isoforms. The determination of whether RAD52 has target sites for these SUMO isoforms and whether the regulatory mechanisms facilitated by sumoylation in yeast RAD52 are conserved in higher eukaryotes, including human, will await further investigation.

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Conflict of interest
None declared.

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