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Identification of a novel human Rad51 variant that promotes DNA strand exchange

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ABSTRACT

Rad51 plays a key role in the repair of DNA double-strand breaks through homologous recombination, which is the central process in the maintenance of genomic integrity. Five paralogs of the human Rad51 gene (*hRad51*) have been identified to date, including *hRad51B*, *hRad51C*, *hRad51D*, *Xrcc2* and *Xrcc3*. In searches of additional *hRad51* paralogs, we identified a novel *hRad51* variant that lacked the sequence corresponding to exon 9 (*hRad51-Δex9*). The expected amino acid sequence of *hRad51-Δex9* showed a frame-shift at codon 259, which resulted in a truncated C-terminus. RT-PCR analysis revealed that both *hRad51* and *hRad51-Δex9* were prominently expressed in the testis, but that there were subtle differences in tissue specificity. The hRad51-Δex9 protein was detected as a 31-kDa protein in the testis and localized at the nucleus. In addition, the hRad51-Δex9 protein showed a DNA-strand exchange activity comparable to that of hRad51. Taken together, these results indicate that hRad51-Δex9 promotes homologous pairing and DNA strand exchange in the nucleus, suggesting that alternative pathways in hRad51- or hRad51-Δex9-dependent manners exist for DNA recombination and repair.

INTRODUCTION

Homologous recombination (HR) is a fundamental process conserved in all organisms, maintaining genomic stability through the repair of exogenous and endogenous DNA double-strand breaks. HR also contributes to genomic diversity in evolution through its pivotal roles in the exchange of chromatids during meiosis (1). In addition, dysregulation of HR may lead to aberrant genetic rearrangements and genomic instability, resulting in translocations, deletions, duplications or loss of heterozygosity (2). Precise control of the HR equilibrium is therefore essential for genetic stability because both HR stimulation and repression lead to genome instability (3).

Rad51, a eukaryotic ortholog of bacterial *RecA*, plays a central role in the repair of double-strand DNA breaks by mediating homologous pairing and strand exchange in recombinatory structures known as Rad51 foci in the nucleus (4). *Rad51* belongs to the *Rad52* epistasis group in *Saccharomyces cerevisiae*, which is comprised of a number of the key genes (*Rad50* to *Rad57*) involved in recombinational repair of double-strand DNA breaks (5). Among the members of the *Rad52* epistasis group, *Rad51* shows the highest degree of sequence conservation in evolution, with 83% amino acid sequence homology between yeast and human orthologs and 99% homology between mouse and human orthologs (6). The functional importance of Rad51 has been further emphasized by the findings that Rad51 interacts with the tumor suppressor protein, p53 (7,8), and the breast cancer-susceptibility proteins, BRCA1 and BRCA2 (9–11). Additionally, elevated levels of hRad51 have been observed in a variety of tumor cells (12–14), suggesting that strict regulation of this recombinase may be essential for maintaining genome integrity.

To date, five human *Rad51* (*hRad51*) paralogs, *Rad51B* (*Rad51L1*), *Rad51C* (*Rad51L2*), *Rad51D* (*Rad51L3*), *Xrcc2* and *Xrcc3*, have been identified. Each of these genes shows only a limited degree of sequence similarity to *hRad51*, however, they all contain the RecA domain for DNA recombination and the Walker A and B motifs for ATP binding and hydrolysis in the predicted amino acid sequences (15–18). These *hRad51* paralogs have presumably arisen through a series of gene duplications in the early stages of eukaryotic evolution (19). In addition, the five hRad51 paralogs have been reported to assist the DNA strand exchange activity of hRad51, forming two distinct complexes, Rad51B-Rad51C-Rad51D-Xrcc2 and

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hRad51C-Xrcc3 (20). Deficiency in any of the Rad51 paralogs has been shown to lead to increased sensitivity to DNA cross-linking agents and ionizing radiation in vertebrate cells (21–23).

In an attempt to identify additional *hRad51* paralogs in humans, we searched a human testis cDNA library. We report here a novel splice variant of *hRad51*, *hRad51-△ex9*, which lacks the sequence corresponding to exon 9. This novel variant was also found in the expressed sequence tag (EST)-databases. The hRad51-△ex9 protein was localized in the nucleus and detected as an expected molecular weight of 31 kDa in the testis. The hRad51-Δex9 protein showed DNA strand exchange activity that was comparable to that of hRad51, suggesting that this novel variant also functions as a recombinase. Additionally, using site-directed mutagenesis, we found that a short basic motif located in the C-terminus of hRad51-Δex9 may play a functional role in nuclear localization of this novel variant.

MATERIALS AND METHODS

Identification of *hRad51-Δex9*

A human testis 5′-stretch cDNA library (Clontech) was screened using a *hRad51* cDNA probe. The cDNA probe was P^{32}-labeled by random primer labeling, and hybridization was conducted in 50% formamide, 5× SSPE (1× SSPE: 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 10× Denhardt's solution, 2% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C for 16 h. The filters were washed twice in 2× SSC (1× SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.1% SDS at room temperature and then twice in 0.2 × SSC, 0.1% SDS at 42°C. Next, the filters were exposed to Kodak XAR film at –70°C for varying periods of time. The positive phage clones were then sequenced using an ABI 310 automated DNA sequencer. The human EST database was also searched for identification of *hRad51* paralogs using the BLASTN program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). The EST AI018041 clone was purchased from Open Biosystems. The nucleotide sequence reported in this paper will appear in the GenBank under accession number EU362635.

RT-PCR analysis in human tissues

Human Multiple Tissue cDNA panels (Clontech) were PCR-amplified using *ExTag* polymerase (Takara) with primers specific to both *hRad51* and *hRad51-Δex9* (forward: 5′-tttggagaattccgaactgg-3′; and reverse: 5′-aggaagac agggagagtcg-3′), which were derived from the flanking regions of exon 9. The reaction mixture was subjected to 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s with a predenaturation at 94°C for 4 min and a final extension at 72°C for 7 min. The amplified PCR products were then analyzed by electrophoresis on 2.0% agarose gels.

Expression and purification of the recombinant hRad51 and hRad51-Δex9 proteins

The full-coding sequences of *hRad51* and *hRad51-Δex9* were PCR-amplified from recombinant phage clones using *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. The sequences of the oligonucleotide primers are available upon request. A unique restriction site, either NotI or BamHI, was introduced into each primer to allow convenient subcloning. The PCR-amplified fragments were then gel-purified and ligated into pET28b (Novagen) or pET21c (Novagen) at the NotI and BamHI restriction sites in frame with the C-terminal hexahistidine tag. The resulting expression constructs were then confirmed to contain the desired sequences by DNA sequence analysis using the BigDye termination version 3.0 (ABI). Among the expression constructs, pET28b-hRad51 and pET21c-hRad51-Δex9 were used for expression of the hRad51 and hRad51-Δex9 proteins, respectively.

The *Escherichia coli* strain, BL21 (DE3) (Novagen), was used for transformation of the pET-derived expression constructs. The recombinant proteins were expressed and purified as previously described (24). However, the hRad51-Δex9 protein resulted in the formation of inclusion bodies. Denaturing and refolding of the hRad51-Δex9 protein into an enzymatically active form were done as previously published for other human proteins (25). Briefly, the inclusion bodies were precipitated by centrifugation at 8000 *g* for 20 min and then homogenized in 6 M urea, 10 mM K_2HPO_4, pH 8.2 and 3 mM β-mercaptoethanol. The solubilized recombinant proteins were then purified using Ni–NTA agarose resins (Qiagen). For refolding, the denatured hRad51-Δex9 protein was first dialyzed overnight against a buffer of 10 mM K_2HPO_4, pH 9.6, 200 μM $CuCl_2$ and 2% sodium *N*-lauroylsarcosinate and then against a buffer of 10 mM K_2HPO_4, pH 9.6 and 5 μM $CuCl_2$. Next, the proteins were further dialyzed twice against 10 mM K_2HPO_4, pH 7.0. The concentration of the dialyzed protein samples was then determined using a BCA Protein Assay Kit (Bio-Rad). All of the purification procedures were conducted at 4°C. The purity and size of the recombinant proteins were assessed by SDS–PAGE. The purified recombinant proteins were further confirmed by western blot analysis using a commercial hRad51 polyclonal antibody (Calbiochem).

DNA strand exchange assays

DNA strand exchange assays were done as previously described (26,27). Briefly, the recombinant hRad51 or hRad51-Δex9 protein (final concentration, 3.5 μM) was mixed with 125 ng (final concentration, 16.8 μM in nucleotides) of ϕX 174 viral DNA (New England Biolabs) in 20 μl buffer containing 20 mM HEPES, pH 6.5, 1 mM DTT, 6.6 mM $MgCl_2$, 3 mM ATP, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase and 50 μg/ml BSA. After 5 min of incubation at 37°C, 120 ng (final concentration, 8.4 μM in base pairs) of PstI-linearized ϕX 174 dsDNA (New England Biolabs) in 1 μl and 1 μl of 100 mM $MgCl_2$ were added to the reaction mixture. Following subsequent incubation for 15, 30, 60, 120 or 240 min at 37°C, 0.5% SDS and 0.5 mg/ml proteinase K were added to stop the exchange reaction. The incubated DNA samples were then run in 0.8% agarose gels. The gels were stained with 0.1 μg/ml of syber green (Molecular Probe) for 2 h and then

distained in ddH_2O for 2 h. Images were processed using Photoshop 7.0 (Adobe).

Generation of a hRad51-Δex9-specific polyclonal antibody

A synthetic peptide (EERKRGNQNLQNLRLS) was covalently conjugated to maleimide-activated keyhole limpet homocyanin. The peptide conjugate was then emulsified with an equal volume of complete Freund's adjuvant. Adult rabbits of 1.8–2.0 kg in weight were intramuscularly injected with 500 μg of the emulsified peptide conjugate four times at a 2-week interval. The rabbits were bled on Days 7 and 14 after the last injection, and the presence of antibodies was then evaluated using an ELISA assay. The antibodies were then purified using a Protein A Agarose Kit (KPL) according to the manufacturer's instructions.

Western blot analysis in human tissues

Human tissue specimens were homogenized in a lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM aprotinin and 1 mM chymostatin. The protein concentrations of the tissue extracts were determined using a BCA Protein Assay Kit (Bio-Rad). For western blot analysis, 100 μg of tissue extracts was subjected to 12.5% SDS–PAGE and then immunoblotted onto a nitrocellulose membrane (Amersham Bioscience). The membranes were then blocked in Tris-buffered saline Tween-20 (TBST) containing 5% skimmed milk for 1 h at room temperature, after which they were incubated with the hRad51-Δex9-specific antibody, a commercial hRad51 polyclonal antibody (Calbiochem), or preimmune serum for 1 h at room temperature. The protein bands were visualized using an ECL detection system (Amersham-Pharmacia Biotech), and GAPDH was used as an internal control.

Subcellular localization of hRad51-Δex9

Mammalian expression constructs of *hRad51* and *hRad51-Δex9* were generated by PCR-amplifying their full coding sequences from recombinant phage clones using *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. The sequences of the oligonucleotide primers are available upon request. A unique restriction site, either SacI or BamHI, was introduced into each primer for convenient subcloning. The PCR-amplified DNA fragments were then ligated into pEGFP-C1 (BD Biosciences) in frame with the N-terminal GFP tag. The resulting constructs were transiently transfected into COS-7 cells that were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin and 100 U/ml of penicillin. At 4–10 h posttransfection, the cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 5 min at room temperature. The fixed cells were rinsed twice with PBS, permeabilized by incubation in 0.2% Triton X-100 for 10 min and then rinsed three times with 0.1% BSA in PBS. Nuclei were stained with propidium iodide (1:1000) (Molecular Probes), and confocal microscopic analysis was performed using a Zeiss LSM510 laser-scanning microscope.

Mutagenesis of hRad51-Δex9

Site-directed mutagenesis was performed using a PCR-based DpnI-treatment method that has been previously described (28). Mutagenic primers were designed to create R264A, K265Q and Del264RK in the amino acid sequence of hRad51-Δex9. The sequences of the oligonucleotide primers are available upon request. Thermocycling was conducted using *Pfu* DNA polymerase (Stratagene) according to the manufacturer's suggestions. The creation of mutations *in the hRad51-Δex9* cDNA was confirmed by sequence analysis using the BigDye termination version 3.0 (BD Biosciences). To construct a C-terminal deletion mutant of *hRad51*, the sequence corresponding to codons 1 to 258 of *hRad51* was PCR-amplified using *Pfu* DNA polymerase (Stratagene) with the following PCR primers: forward, 5′-ccgagctcgaatggcaatgcagatgcagc-3′; and reverse, 5′-cgcggatcctcactcatcagcgagtcgcag-3′. A unique restriction site, either SacI or BamHI, was introduced into each primer to allow convenient subcloning. The PCR-amplified DNA fragments were then ligated into pEGFP-C1 (BD Biosciences) in frame with the N-terminal GFP tag.

RESULTS

Identification of *hRad51-Δex9*

The *hRad51* gene is composed of 10 exons that encode a 339-amino acid polypeptide with a calculated molecular mass of 37 kDa. In an effort to identify additional *hRad51* paralogs in humans, we searched a human testis cDNA library using a *hRad51* cDNA probe with low stringency and obtained seven autoradiographically positive phage recombinants (data not shown). Sequence analysis of the recombinants revealed that all of the isolated clones were *hRad51* cDNAs. However, one clone that contained a 1661-bp insert showed an exon–intron structure distinct from that of *hRad51*, specifically lacking the sequence corresponding to exon 9 of *hRad51* (Figure 1). This novel splice variant of *hRad51*, termed *hRad51-Δex9*, was also identified in searches of the human EST databases (EST ID number: AI018041). We conducted complete sequencing of EST AI018041 that was obtained from a commercial source and subsequently confirmed that the *hRad51-Δex9* cDNA was identical to AI018041, with the

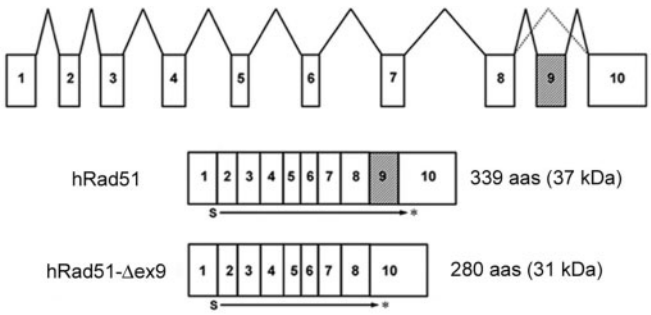

Figure 1. Schematic diagrams of the mRNA structures of *hRad51* and *hRad51-Δex9*. Exons are shown as numbered boxes, introns as bold lines. Hatched boxes indicate the deleted exon in the *hRad51-Δex9* mRNA. 'S' stands for the start codon, and 'Asterisk' for the stop codon.

exception that *hRad51-Δex9* contained longer 5′- and 3′UTR sequences than the EST AI018041 clone.

The 5′-UTR of *hRad51-Δex9* is at least 299 bp, the coding region is 843 bp and the 3′-UTR is 469 bp. The deletion of exon 9 causes a frame-shift at codon 259, which leads to premature termination at codon 281. The expected amino acid sequence of the hRad51-Δex9 protein consists of codons 1 to 258 of hRad51 and 22 'out of frame' codons from exon 10, containing the Walker A and B ATP-binding motifs at residues 127–135 and 218–222, respectively (Figure 2). In addition, a basic motif that is composed of one lysine and two arginine residues is located at residues 303–306 of hRad51, and a similar basic motif is found at residues 264–266 in the newly created C-terminus of hRad51-Δex9 (Figure 2).

RT-PCR ANALYSIS OF HRAD51-ΔEX9 IN HUMAN TISSUES

To determine the expression of *hRad51* and *hRad51-Δex9* in human tissues, RT-PCR analysis was conducted using primers derived from the flanking regions of exon 9. The RT-PCR analysis was expected to generate a 467-bp fragment for *hRad51-Δex9* and a 589-bp fragment for *hRad51*. DNA-amplicons of the expected sizes corresponding to both *hRad51* and *hRad51-Δex9* were most prominently detected in the testis (Figure 3). Both PCR amplicons were also detected, though to lesser extents, in the skeletal muscle, pancreas, thymus and ovary (Figure 3). Additionally, the *hRad51*-specific amplicon was detected in the placenta, lung, liver, kidney, spleen and colon tissues, however, the *hRad51-Δex9*-specific-amplicon was not

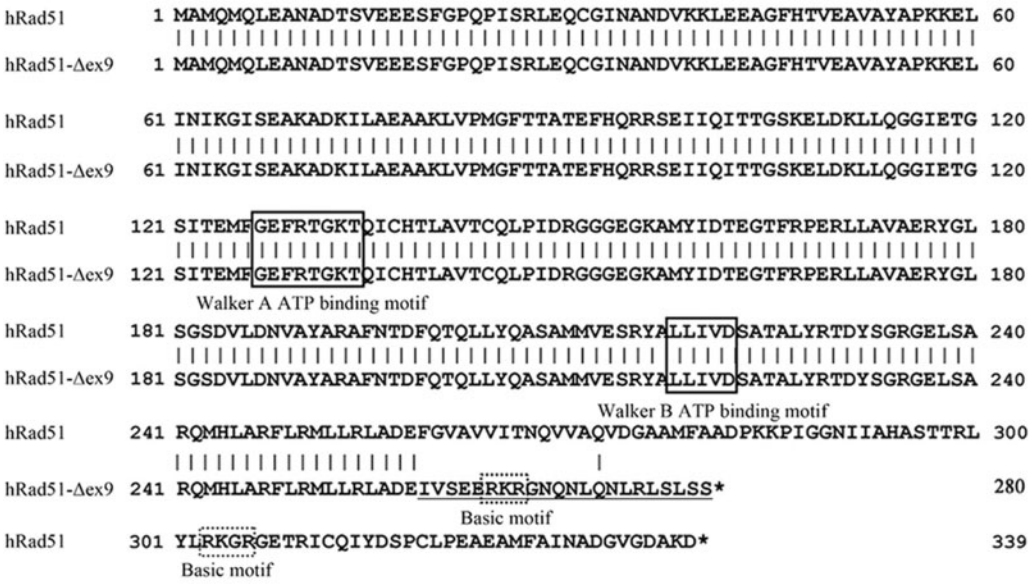

Figure 2. Alignment of the amino acid sequences of hRad51 and hRad51-Δex9. The hRad51 polypeptide sequence is aligned with the predicted amino acid sequence of hRad51-Δex9. The 22 'out of frame' codons are indicated with an underline in the amino acid sequence of hRad51-Δex9. Walker A and B ATP-binding motifs and basic motifs are also indicated.

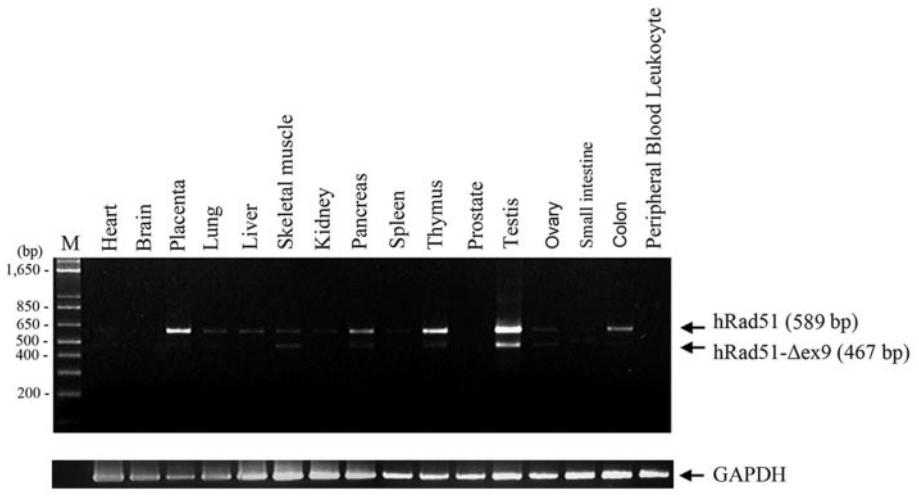

Figure 3. RT-PCR analysis of *hRad51* and *hRad51-Δex9* in human tissues. A typical example of RT-PCR analysis of *hRad51* and *hRad51-Δex9* using poly(A)$^+$ RNA obtained from 16 different human tissues. The analysis was repeated in triplicate, and *GAPDH* was used as an internal control.

detected in these tissues, suggesting that different tissue-specificities exist between *hRad51* and *hRad51-Δex9* (Figure 3).

The DNA strand exchange activity of the hRad51-Δex9 protein

In an effort to express and purify enzymatically active forms of the hRad51 and hRad51-Δex9 proteins, we expressed the full coding domain sequences of *hRad51* and *hRad51-Δex9* using an *E. coli* expression system. Upon induction with 1 mM IPTG at 37°C, the hexa-histidine tagged recombinant proteins of both hRad51 and hRad51-Δex9 were expressed at high levels. Fractionation of the cell lysates into different cellular compartments, such as cytoplasmic extracts, periplasmic extracts and inclusion body fractions, revealed that the recombinant hRad51 protein was present in the soluble fractions. However, the recombinant hRad51-Δex9 protein was expressed within the inclusion bodies. The insoluble hRad51-Δex9 protein was denatured by urea during purification and subsequently refolded by stepwise dialysis in the presence of *N*-lauroylsarcosinate and Cu^{2+}. The apparent sizes of the expressed recombinant proteins were in good agreement with the deduced molecular mass, which was 38 kDa for the recombinant Rad51 protein and 32 kDa for the recombinant hRad51-Δex9 protein. The purified recombinant proteins were confirmed by western blot analysis using a commercial human Rad51 antibody (Figure 4A).

To assess the DNA strand exchange activities of hRad51 and hRad51-Δex9, we used the purified recombinant proteins with circular single-strand DNA (ssDNA) and linear double-strand DNA (dsDNA) of bacteriophage ϕX174. In DNA strand exchange reactions, the circular ssDNA forms joint molecules with the linear dsDNA through homologous pairing, and then the joint molecules are converted into nicked circular forms (Figure 4B). Both the recombinant hRad51 and hRad51-Δex9 proteins showed the expected joint molecules and nicked circular forms of ϕX174 at each of the time-intervals tested. The intensities of the bands corresponding to the nicked circular form appeared approximately the same in the either reactions with hRad51 or hRad51-Δex9 (Figure 4C), suggesting that strand exchange activity of hRad51-Δex9 is approximately similar to that of hRad51 at least *in vitro*. However, the hRad51-Δex9 protein showed a significantly higher activity than hRad51 in homologous DNA pairing at all the time-intervals (Figure 4C). These results are comparable with the previous findings on C-terminal deletion mutants of the *E. coli* RecA protein, which also showed an enhanced activity in homologous DNA pairing (29–31).

Western blot analysis of hRad51-Δex9 in human tissues

To evaluate the expression of *hRad51-Δex9* at the protein level *in vivo*, we generated a polyclonal antibody against the peptide sequence specific to hRad51-Δex9. This hRad51-Δex9 polyclonal antibody reacted with the purified recombinant hRad51-Δex9 protein, but not with the recombinant hRad51 protein (data not shown). Human placenta, lung, testis and small intestine tissues were then tested by western blot analysis. A band with the expected molecular mass of 31 kDa for hRad51-Δex9 was prominently detected in the testis; however, this 31-kDa band was rarely detected in the other tissues tested (Figure 5A). We also investigated the expression of hRad51 and hRad51-Δex9 using a commercial antibody expected to react with both hRad51 and hRad51-Δex9. The 37-kDa hRad51 band was prominently detected in the testis, but at much lower levels in the placenta, lung and small intestine (Figure 5B). The 31-kDa band corresponding to hRad51-Δex9, however, was detected only in the testis (Figure 5A). These findings are consistent with those of the RT-PCR analysis that also showed prominent expression of *hRad51-Δex9* only in the testis.

Nuclear localization of hRad51-Δex9

To investigate the cellular localization of hRad51 and hRad51-Δex9, mammalian expression constructs containing the full coding sequence of *hRad51* or *hRad51-Δex9* in frame with the N-terminal GFP tag were transfected into COS-7 cells. Confocal microscopic analysis of the direct fluorescence of the fusion proteins displayed subcellular signals of hRad51 and hRad51-Δex9 in the nucleus (Figure 6Aa and b). In addition, both the hRad51 and hRad51-Δex9 proteins were co-localized with nucleus-specific propidium iodide staining, further confirming the

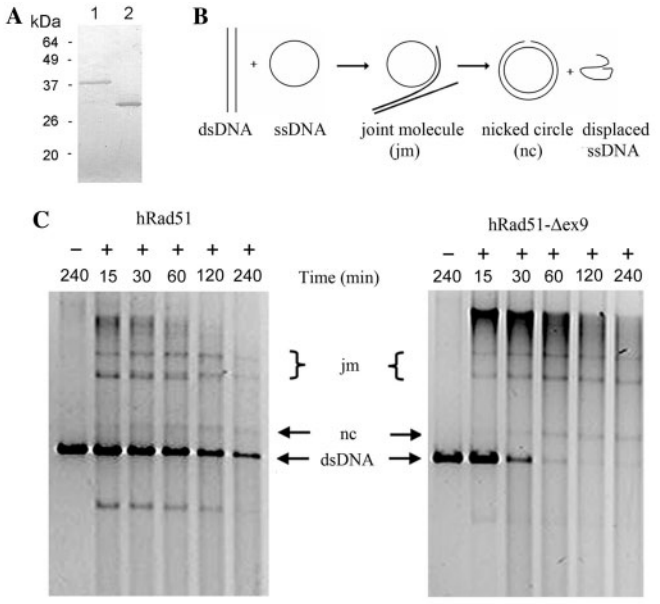

Figure 4. DNA strand exchange activity of hRad51 and hRad51-Δex9. (**A**) Western blot analysis of the purified recombinant hRad51 and hRad51-Δex9 protein using a commercial hRad51 antibody. Lane 1, hRad51; Lane 2, hRad51-Δex9. (**B**) Schematic diagram of DNA strand exchange between circular ssDNA and linear dsDNA of ϕX 174. (**C**) DNA strand exchange reactions mediated by the purified recombinant hRad51 and hRad51-Δex9 proteins. After incubation with 3.5 μM of either the hRad51 or hRad51-Δex9 protein for a series of time-intervals (15, 30, 60, 120 and 240 min), the DNA was analyzed by 0.8% agarose gel electrophoresis, followed by staining with Syber green. When the hRad51 or hRad51-Δex9 protein was not included in the strand-exchange reactions, no bands corresponding to the forms of joint molecules or nicked circles were detected at 240 min of incubation (the first lane in each panel).

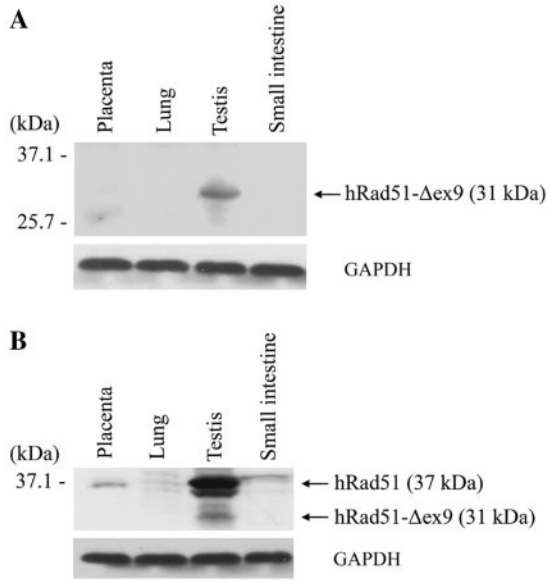

Figure 5. Detection of hRad51-Δex9 in human tissues by western blot analysis. Approximately 100 μg of human placenta, lung, testis and small intestine tissue extracts were subjected to western blot analysis using a hRad51-Δex9-specific antibody (**A**) or a commercial hRad51 antibody (**B**).

nuclear localization of these proteins in the transfected cells (data not shown). However, the mutated hRad51 protein that did not contain the C-terminal region from codons 259 to 339 was primarily detected in the cytoplasmic area (Figure 6Ac). Taken together, these results indicate that the signal for the nuclear localization of hRad51 may reside in the C-terminus and, furthermore, that the frame-shifted region of hRad51-Δex9 may regain the residues required for nuclear localization.

A basic motif containing a stretch of lysine and arginine residues was found at residues 264–266 (RKR) in the frame-shifted C-terminal region of hRad51-Δex9. Similar types of basic motifs have been known to act as a nuclear localization signal (NLS) in a number of nuclear proteins (32,33). To determine, therefore, if this basic motif in the C-terminus of hRad51-Δex9 could function as an NLS, we generated a series of mutant constructs that harbor a del254-256RK, R264A or K265Q mutation in the basic motif. In localization studies conducted using the mutant constructs, each of the mutated hRad51-Δex9 proteins was primarily detected in the cytoplasmic areas, but rarely in the nuclei (Figure 6Ba–c). These results strongly suggest that the basic motif located in the newly created C-terminal region of hRad51-Δex9 may function as a NLS in nuclear localization of this hRad51 variant.

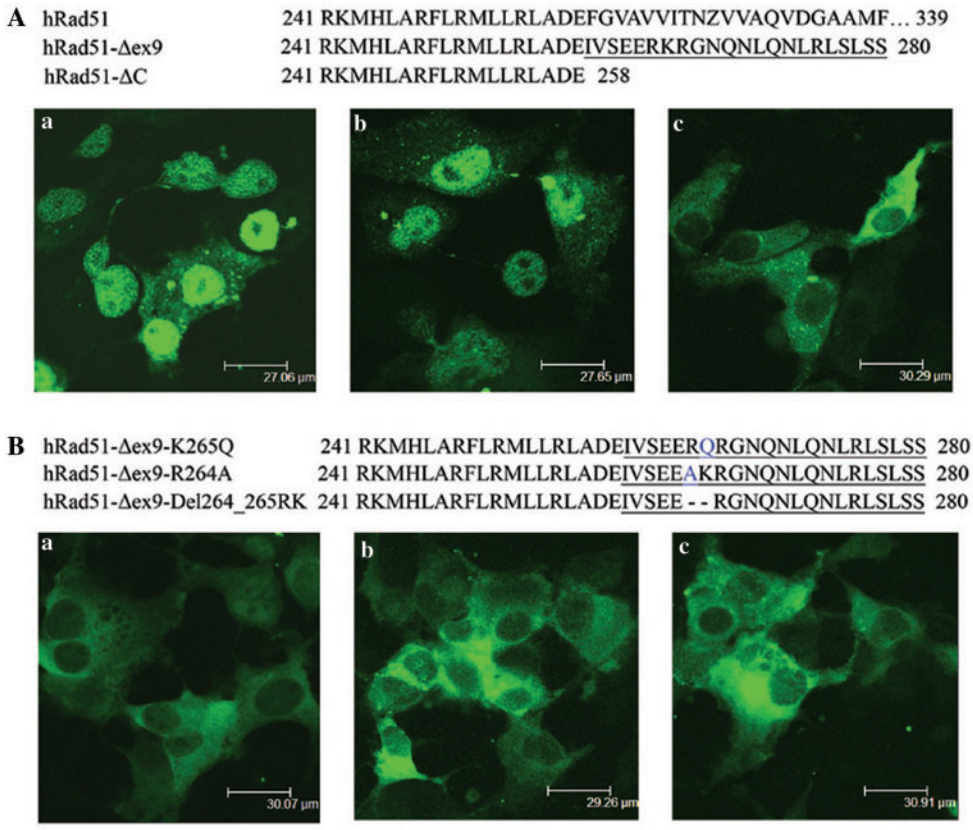

Figure 6. Nuclear localization of hRad51, hRad51-Δex9 and C-terminal mutants. (**A**) Direct fluorescence images of COS-7 cells transfected with hRad51 (a), hRad51-Δex9 (b), or hRad51-delC (c) at a magnification of ×1000. The amino acid sequences of hRad51 and hRad51-Δex9 are shown only from codons 241 to 280, and the frame-shifted region in hRad51-Δex9 is underlined. The hRad51-△C mutant does not contain the C-terminal sequence from codons 259 to 339. (**B**) Direct fluorescence images of COS-7 cells transfected with hRad51-Δex9-K265Q (a), hRad51-Δex9-R264A (b), or hRad51-Δex9-Del264_265RK (c) at a magnification of ×1000. The hRad51-Δex9-K265Q mutant harbors a substitution of Lys to Gln at codon 265, and the hRad51-Δex9-R264A mutant contains a substitution of Arg to Ala at codon 264. The residues mutated in hRad51-Δex9-K265Q and hRad51-Δex9-R264A are indicated in blue. In hRad51-Δex9-Del264_265RK, Arg-Lys residues at codons 264–265 are deleted.

DISCUSSION

Here we present a novel variant of *hRad51*, *hRad51-Δex9*, which aberrantly splices the *hRad51* mRNA from exon 8 to exon 10, skipping exon 9. The predicted amino acid sequence of this novel variant contains a truncated C-terminus of hRad51, however, it retains the RecA domain for DNA recombination and the Walker A and B motifs for ATP binding and hydrolysis. With a purified recombinant hRad51-Δex9 protein, we showed that this novel variant is capable of catalyzing DNA strand exchanges *in vitro*, although further biochemical characterization would be required to determine the precise enzymatic properties of this hRad51 variant. In expression studies, hRad51-Δex9 was predominantly detected in the testis at both the mRNA and protein levels and, further, the hRad51-Δex9 protein was localized in the nucleus. Taken together, these findings indicate that hRad51-Δex9 catalyzes homologous pairing and DNA-strand exchange in the nucleus, suggesting that alternative pathways involving either hRad51 or hRad51-Δex9 may exist for DNA repair and recombination.

Splice variants of other genes involved in DNA repair and recombination, including *Rad52*, *Rad51D* and *DMC1*, have been also reported (34–38). The murine and human *Rad52* mRNAs undergo alternative splicing, resulting in several variants with a truncated C-terminus (34,35). Rad52 is known to catalyze the replacement of replication protein A with Rad51 on ssDNA and to promote strand exchange between complementary ssDNA and dsDNA (39,40). The human Rad52 variants interacted with both ssDNA and dsDNA; however, they did not bind to the full-length human Rad52 due to deletion of the self-interaction domain (34). Furthermore, the murine Rad52 splice variants increased the frequency of sister chromatid repair in both mammalian cells and yeast, whereas the intact murine Rad52 was more likely involved in homology-directed repair (35). Alternatively spliced forms of *Rad51D* and *DMC1* in both humans and mice have been also identified, but their functional significance has not been evaluated (36–38). However, the presence of these variants of the proteins involved in HR further implies the presence of alternative pathways for the control of recombinational repair of dsDNA breaks.

Rad51 and its paralogs are found in the nucleus, however, it has not yet been determined if they are transported independently into the nucleus or through interactions with other proteins. BRCA2 has been known to play a critical role in the nuclear transport and foci formation of Rad51 upon exposure to exogenous damage (9–11). However, without any exogenous DNA damage, replication-associated formation of Rad51 foci occurred in a BRCA2-independent manner in CAPAN-1 cells that carry a BRCA2 truncation (41), suggesting that distinct mechanisms may be responsible for the nuclear localization and focus formation of Rad51 in the presence or absence of exogenous DNA-damaging agents. Further, several hRad51 paralogs have been shown to translocate into nucleus in a BRCA2-independent manner, using a basic motif composed of lysines and arginines as a NLS (42,43). hRad51C contains a basic motif composed of a short stretch of lysine and arginine residues at the C-terminus. Using a deletion construct of the C-terminal region, the basic motif of hRad51C was shown to function as a NLS for nuclear transport of hRad51C in mammalian cells (42). In addition, hRad51B that contains a basic motif at the N-terminus was shown to translocate into the nucleus in a BRCA2-independent manner (43). hRad51 also contains a basic motif at residues 303–306 (RKGR) in the C-terminus. This basic motif is deleted in hRad51-Δex9 due to the translational frame-shift. However, in the frame-shifted C-terminus of hRad51-Δex9, a similar basic motif reappears at residues 264–266 (RKR). Our studies with oligonucleotide-directed mutagenesis of the RKR motif in hRad51-Δex9 demonstrated that this short basic motif is required for the nuclear localization of hRad51-Δex9, suggesting that nuclear localization of hRad51-Δex9 may be independent of BRCA2 in the absence of any DNA-damaging agents, at least in the cultured cells tested.

Rad51 has been reported to interact with p53 and BRCA2, both of which play pivotal roles in maintaining genome integrity. In response to DNA damage, p53 modulates HR through physical interaction with several proteins implicated in recombination, including Rad51, Rad54, BLM and WRN (44,45). Using *in vitro* binding assays, p53 was reported to interact with the region between codons 125 and 220 of hRad51 (8). The p53-interactive region in hRad51 corresponds to the homo-oligomerization region that is critical for formation of the functional hRad51 nucleoprotein filaments (46). The conservation of the p53-interactive region in hRad51-Δex9 suggests that this novel variant also interacts with p53, unless the absence of the C-terminal region in hRad51-Δex9 affects the physical interaction with p53. BRCA2 interacts with Rad51 through the eight conserved BRC repeats (47,48), and mutations within these repeats are associated with an increased risk of breast cancer (49,50). Electron microscopy studies showed that the BRC repeat 4 interacts with the nucleotide-binding core of Rad51, whereas the BRC repeat 3 interacts with the N-terminal region of Rad51, suggesting that the BRC repeats bind to distinct regions of Rad51 (51). The BRCA2-interactive region in hRad51 was studied using yeast two-hybrid and *in vitro* binding assays, which revealed that the C-terminus of hRad51 (codons 98–339) is crucial for interaction with BRCA2 (47). Our finding that the C-terminal region (codons 280–389) of hRad51 is deleted in hRad51-Δex9 suggests that this novel variant may have a different binding property from hRad51 in interaction with BRCA2. Further characterization of the interactive profile of hRad51-Δex9, particularly with p53 and BRCA2, will be necessary to determine the functional roles that this novel recombinase may play in the maintenance of genome stability and the elimination of DNA double-strand breaks.

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REFERENCES

1. Haber,J.E. (1999) DNA recombination: the replication connection. *Trends Biochem. Sci.*, **24**, 271–275.
2. West,S.C. (2003) Molecular views of recombination proteins and their control. *Nat. Rev. Mol. Cell Biol.*, **4**, 435–445.
3. Bertrand,P., Saintigny,Y. and Lopez,B.S. (2004) p53's double life: transactivation-independent repression of homologous recombination. *Trends Genet.*, **20**, 235–243.
4. Baumann,P. and West,S.C. (1998) Role of the human RAD51 protein in homologous recombination and doublestranded-break repair. *Trends Biochem. Sci.*, **23**, 247–251.
5. Game,J.C. (1993) DNA double-strand breaks and the RAD50-RAD57 genes in Saccharomyces. *Semin. Cancer Biol.*, **4**, 73–83.
6. Shinohara,A., Ogawa,H., Matsuda,Y., Ushio,N., Ikeo,K. and Ogawa,T. (1993) Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and *recA*. *Nat. Genet.*, **4**, 239–243.
7. Sturzbecher,H.W., Donzelmann,B., Henning,W., Knippschild,U. and Buchhop,S. (1996) p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. *EMBO J.*, **15**, 1992–2002.
8. Buchhop,S., Gibson,M.K., Wang,X.W., Wagner,P., Sturzbecher,H.W. and Harris,C.C. (1997) Interaction of p53 with the human Rad51 protein. *Nucleic Acids Res.*, **25**, 3868–3874.
9. Sharan,S.K., Morimatsu,M., Albrecht,U., Lim,D.S., Regel,E., Dinh,C., Sands,A., Eichele,G., Hasty,P. and Bradley,A. (1997) Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature*, **386**, 804–810.
10. Scully,R., Chen,J., Ochs,R.L., Keegan,K., Hoekstra,M., Feunteun,J. and Livingston,D.M. (1997) Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell*, **90**, 425–435.
11. Yuan,S.S., Lee,S.Y., Chen,G., Song,M., Tomlinson,G.E. and Lee,E.Y. (1999) BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex *in vivo*. *Cancer Res.*, **59**, 3547–3551.
12. Han,H., Bearss,D.J., Browne,L.W., Calaluce,R., Nagle,R.B. and Von Hoff,D.D. (2002) Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. *Cancer Res.*, **62**, 2890–2896.
13. Raderschall,E., Stout,K., Freier,S., Suckow,V., Schweiger,S. and Haaf,T.E. (2002) Elevated levels of Rad51 recombination protein in tumor cells. *Cancer Res.*, **62**, 219–225.
14. Xia,S.J., Shammas,M.A. and Shmookler Reis,R.J. (1997) Elevated recombination in immortal human cells is mediated by HsRAD51 recombinase. *Mol. Cell. Biol.*, **17**, 7151–7158.
15. Albala,J.S., Thelen,M.P., Prange,C., Fan,W., Christensen,M., Thompson,L.H. and Lennon,G.G. (1997) Identification of a novel human RAD51 homolog, RAD51B. *Genomics*, **46**, 476–479.
16. Dosanjh,M.K., Collins,D.W., Fan,W., Lennon,G.G., Albala,J.S., Shen,Z. and Schild,D. (1998) Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes. *Nucleic Acids Res.*, **26**, 1179–1184.
17. Liu,N., Lamerdin,J.E., Tebbs,R.S., Schild,D., Tucker,J.D., Shen,M.R., Brookman,K.W., Siciliano,M.J., Walter,C.A., Fan,W. *et al.* (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol. Cell*, **1**, 783–793.
18. Pittman,D.L., Weinberg,L.R. and Schimenti,J.C. (1998) Identification, characterization, and genetic mapping of Rad51d, a new mouse and human RAD51/RecA-related gene. *Genomics*, **49**, 103–111.
19. Lin,Z., Kong,H., Nei,M. and Ma,H. (2006) Origins and evolution of the *recA/RAD51* gene family: Evidence for ancient gene duplication and endosymbiotic gene transfer. *Proc. Natl Acad. Sci. USA*, **103**, 10328–10333.
20. Miller,K.A., Sawicka,D., Barsky,D. and Albala,J.S. (2004) Domain mapping of the Rad51 paralog protein complexes. *Nucleic Acids Res.*, **32**, 169–178.
21. Griffin,C.S., Simpson,P.J., Wilson,C.R. and Thacker,J. (2000) Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation. *Nat. Cell Biol.*, **2**, 757–761.
22. Takata,M., Sasaki,M.S., Tachiiri,S., Fukushima,T., Sonoda,E., Schild,D., Thompson,L.H. and Takeda,S. (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Mol. Cell. Biol.*, **21**, 2858–2566.
23. Drexler,G.A., Rogge,S., Beisker,W., Wckardt-Schupp,F., Zdzienicka,M.Z. and Fritz,E. (2004) Spontaneous homologous recombination is decreased in Rad51C-deficient hamster cells. *DNA Repair*, **3**, 1335–1343.
24. Kurumizaka,H., Aihara,H., Kagawa,W., Shibata,T. and Yokoyama,S. (1999) Human Rad51 amino acid residues required for Rad52 binding. *J. Mol. Biol.*, **291**, 537–548.
25. Kim,M.S., Kim,S.S., Jung,S.T., Park,J.Y., Yoo,H.W., Ko,J., Csiszar,K., Choi,S.Y., and Kim,Y. (2003) Expression and purification of enzymatically active forms of the human lysyl oxidase-like protein 4. *J. Biol. Chem.*, **278**, 52071–52074.
26. Sung,P. and Robberson,D.L. (1995) DNA strand exchange mediated by a Rad51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell*, **82**, 453–461.
27. Shinohara,A. and Ogawa,T. (1998) Stimulation by Rad52 of yeast Rad51 mediated recombination. *Nature*, **391**, 404–407.
28. Li,S. and Wilkinson,M.F. (1997) Site-directed mutagenesis: a two-step method using PCR and DpnI. *Biotechniques*, **23**, 588–590.
29. Benedict,R.C. and Kowalczykowski,S.C. (1988) Increase of the DNA strand assimilation activity of recA protein by removal of the C terminus and structure-function studies of the resulting protein fragment. *J. Biol. Chem.*, **263**, 15513–15520.
30. Tateishi,S., Horii,T.,, Ogawa,T. and Ogawa,H., (1992) C-terminal truncated *Escherichia coli* RecA protein RecA5327 has enhanced binding affinities to single- and double-stranded DNAs. *J. Mol. Biol.*, **223**, 115–129.
31. Lusetti,S.L., Wood,E.A., Fleming,C.D., Modica,M.J., Korth,J., Abbott,L., Dwyer,D.W., Roca,A.I., Inman,R.B. and Cox,M.M. (2003) C-terminal deletions of the Escherichia coli RecA protein. Characterization of in vivo and in vitro effects. *J. Biol. Chem.*, **278**, 16372–16380.
32. Subramaniam,P.S., Mujtaba,M.G., Paddy,M.R. and Johnson,H.M. (1999) The carboxyl terminus of interferon-gamma contains a functional polybasic nuclear localization sequence. *J. Biol. Chem.*, **274**, 403–407.
33. Xiao,Z., Latek,R. and Lodish,H.F. (2003) An extended bipartite nuclear localization signal in Smad4 is required for its nuclear import and transcriptional activity. *Oncogene*, **20**, 1057–1069.
34. Habu,T., Taki,T., West,A., Nishimune,Y. and Morita,T. (1996) The mouse and human homologs of DMC1, the yeast meiosis-specific homologous recombination gene, have a common unique form of exon-skipped transcript in meiosis. *Nucleic Acids Res.*, **24**, 470–477.
35. Kito,K., Wada,H., Yeh,E.T. and Kamitani,T. (1999) Identification of novel isoforms of human RAD52. *Biochim. Biophys. Acta*, **1489**, 303–314.
36. Kawabata,M. and Saeki,K. (1999) Multiple alternative transcripts of the human homologue of the mouse TRAD/R51H3/RAD51D gene, a member of the rec A/RAD51 gene family. *Biochem. Biophys. Res. Commun.*, **257**, 156–162.
37. Kawabata,M., Akiyama,K. and Kawabata,T. (2004) Genomic structure and multiple alternative transcripts of the mouse TRAD/RAD51L3/RAD51D gene, a member of the recA/RAD51 gene family. *Biochim. Biophys. Acta*, **1679**, 107–116.
38. Thorpe,P.H., Marrero,V.A., Savitzky,M.H., Sunjevaric,I., Freeman,T.C. and Rothstein,R. (2006) Cells expressing murine RAD52 splice variants favor sister chromatid repair. *Mol. Cell. Biol.*, **26**, 3752–3763.

39. New,J.H., Sugiyama,T., Zaitseva,E. and Kowalczykowski,S.C. (1998) Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A. *Nature*, **391**, 407–410.
40. Kumar,J.K. and Gupta,R.C. (2004) Strand exchange activity of human recombination protein Rad52. *Proc. Natl Acad. Sci. USA*, **101**, 9562–9567.
41. Tarsounas,M., Davies,D. and West,S.C. (2003) BRCA2-dependent and independent formation of RAD51 nuclear foci. *Oncogene*, **27**, 1115–1123.
42. French,K.A., Tambini,C.E. and Thacker,J. (2003) Identification of functional domains in the RAD51L2 (RAD51C) protein and its requirement for gene conversion. *J. Biol. Chem.*, **278**, 45445–45450.
43. Miller,K.A., Hinz,J.M., Yamada,N.A., Thompson,L.H. and Albala,J.S. (2005) Nuclear localization of Rad51B is independent of Rad51C and BRCA2. *Mutagenesis*, **20**, 57–63.
44. Saintigny,Y., Rouillard,D., Chaput,B., Soussi,T. and Lopez,B.S. (1999) Mutant p53 proteins stimulate spontaneous and radiation-induced intrachromosomal homologous recombination independently of the alteration of the transactivation activity and of the G1 checkpoint. *Oncogene*, **18**, 3553–3563.
45. Linke,S.P., Sengupta,S., Khabie,N., Jeffries,B.A., Buchhop,S., Miska,S., Henning,W., Pedeux,R., Wang,X.W., Hofseth,L.J. *et al.* (2003) p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. *Cancer Res.*, **63**, 2596–2605.
46. Benson,F.E., Stasiak,A. and West,S.C. (1994) Purification and characterization of the human Rad51 protein, an analogue of *E. coli* RecA. *EMBO J.*, **13**, 5764–5771.
47. Wong,A.K., Pero,R., Ormonde,P.A., Tavtigian,S.V. and Bartel,P.L. (1997) RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J. Biol. Chem.*, **272**, 31941–31944.
48. Chen,J., Silver,D.P., Walpita,D., Cantor,S.B., Gazdar,A.F., Tomlinson,G., Couch,F.J., Weber,B.L., Ashley,T., Livingston,D.M. *et al.* (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell*, **2**, 317–328.
49. Wooster,R., Bignell,G., Lancaster,J., Swift,S., Seal,S., Mangion,J., Collins,N., Gregory,S., Gumbs,C., Micklem,G. *et al.* (1995) Identification of the breast cancer susceptibility gene *BRCA2*. *Nature*, **378**, 789–792.
50. Tavtigian,S.V., Simard,J., Rommens,J., Couch,F., Shattuckeidens,D., Neuhausen,S., Merajver,S., Thorlacius,S., Offit,K., Stoppalyonnet,D. *et al.* (1996) The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds. *Nat. Genet.*, **12**, 333–337.
51. Galkin,V.E., Esashi,F., Yu,X., Yang,S., West,S.C. and Egelman,E.H. (2005) BRCA2 BRC motifs bind RAD51-DNA filaments. *Proc. Natl Acad. Sci. USA.*, **102**, 8537–8542.