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Nuclear localization of Rad52 is pre-requisite for its sumoylation

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ABSTRACT

In *Saccharomyces cerevisiae*, Rad52 plays major roles in several types of homologous recombination. Here, we found that *rad52-K200R* mutation greatly reduced sumoylation of Rad52. The *rad52-K200R* mutant exhibited defects in various types of recombination, such as intrachromosomal recombination and mating-type switching. The K200 residue of Rad52 is part of the nuclear localization signal (NLS), which is important for transport into the nucleus. Indeed, the addition of a SV40 NLS to Rad52-K200R suppressed the sumoylation defect of Rad52-K200R. These findings indicate that nuclear localization of Rad52 is pre-requisite for its sumoylation.

Homologous recombination (HR) is an important mechanism for the maintenance of genome integrity through its ability to repair DNA double-strand breaks (DSBs) and single-strand gaps, and rescue collapsed replication forks. Among the members of the Rad52 epistasis group (*RAD50*, *-51*, *-52*, *-54*, *-55*, *-57*, *-59*, *MRE11*, and *XRS2*) in *Saccharomyces cerevisiae*, lack of *RAD52* activity produces the most severe phenotypes, reflecting the involvement of this gene in multiple HR pathways, including gene conversion, reciprocal exchange, single-strand annealing (SSA), and break-induced replication (BIR). Moreover, biochemical, and cytological analyses of Rad52 support early presynaptic, synaptic, and postsynaptic roles during recombination [1–3].

Comparisons of the primary structure of Rad52 protein shows that the region spanning amino acids 21–159 within the N-terminus is highly conserved during evolution, suggesting the existence of important functional residues in this region [4,5]. The highly conserved N-terminus of Rad52 contains domains that allow the protein to self-associate, bind DNA, and facilitate DNA annealing [1,2]. Rad52 forms ring structures by self-association [4,6–8] and mediates the exchange of RPA associated with single-stranded DNA for Rad51, resulting in the formation of Rad51 filaments [9–11].

Rad52 is modified by small ubiquitin-like modifier (SUMO) during meiosis or under damage-induced mitotic conditions [12]. SUMO (Smt3 in budding yeast) consists of approximately 100 amino acid residues, and is conjugated to substrate proteins by a series of reactions mediated by an E1 activating enzyme (Uba2/Aos1), an E2 conjugating enzyme (Ubc9), and, typically, a SUMO ligase (E3). Unlike ubiquitination, sumoylation of proteins does not lead to proteosomal degradation, but can affect protein functions, such as protein/DNA interactions, and/or enzymatic activity, as well as altering the sub-cellular localization of the protein [13]. Rad52 is sumoylated primarily at three sites (K10, K11, and K220) simultaneously [12]. A Rad52-3KR (K10R/K11R/K220R triple mutation) mutant shows the defect in the exclusion of Rad52 recombination foci from the nucleolus and rDNA hyper-recombination [14]. Moreover, we observed a moderate defect in damage-induced homologous recombination in the Rad52-3KR mutant [15]. In our previous study, we found that the K126R or R103A mutations in Rad52 also led to defective sumoylation due to defects in oligomerization and/or DNA-binding of the protein.

In this study, we characterized a new mutation, K200R, which severely affects sumoylation of Rad52. K200 resides near the recently recognized "pat7" type nuclear localization signal (NLS) in Rad52 [16]. We show here that the sumoylation defect of Rad52-K200R is due to a defect in nuclear localization and discuss the regulation of Rad52 sumoylation in the cell.

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Materials and methods

Yeast strains and plasmid construction. All of the yeast strains used in this study are listed in Supplemental information (Table S1). Strains in which a gene has been modified with an epitope tag or deletion were produced by transforming yeast with PCR reaction products. Transformants were selected using appropriate selective markers or by immunoblotting. The plasmids and constructs used are listed in Table S2.

Site-directed mutagenesis. Site-directed mutagenesis of Rad52 was performed using the Quik Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Two-hybrid assay. Cells were transformed with a prey plasmid (pGAD424; *GAL4*-activating domain fusion plasmid) and a bait plasmid (pGBD-C1; *GAL4*-binding domain fusion plasmid). Transformants that grew on synthetic complete medium (SC) plates lacking leucine and tryptophan were selected. These colonies were subsequently analyzed by a β-galactosidase filter assay.

Immunoblotting. Cells were grown at 30 °C in YPAD medium to log phase. The cells were harvested by centrifugation, suspended in cold water, and then placed on ice for 15 min in cold 0.58 M NaOH containing 2.3% β-mercaptoethanol. Protein was precipitated by the addition of 60% trichloroacetic acid (TCA) at a final concentration of 11.25% and collected by centrifugation. The pellet was resuspended in HU-buffer (8 M urea, 5% SDS, 200 mM Tris–HCl, pH 6.8, 1 mM EDTA, pH 8.0, bromo-phenol blue) and heated at 65 °C for 10 min. The suspension was neutralized with NaOH. The suspension was mixed with 5x sample buffer and heated at 100 °C for 5 min. After removal of debris by centrifugation, proteins were separated on 10% polyacrylamide gels and analyzed by immunoblotting with anti-Penta.His and anti-Tetra.His (QIAGEN) to detect histidine tags.

Overexpression analysis. Cells were grown at 30 °C in SC medium containing 2% raffinose but lacking uracil to log phase before culturing in 2% galactose for 12 h at 30 °C. Protein samples were then prepared for immunoblotting, as described.

Assay to measure sister chromatid recombination (SCR) frequency. The strains constructed for detecting unequal SCR have been described previously [17,18]. Cells were inoculated onto SC plates lacking tryptophan and containing 0%, 0.0025%, or 0.005% MMS to monitor viability, or were inoculated on SC plates lacking tryptophan and histidine and containing 0%, 0.0025%, or 0.005% MMS to assay recombination. In both cases, the cells were incubated at 30 °C for 5 days. The number of His^+ colonies was scored for each of 11 plates and the mean number of His^+ colonies per plate was determined.

Detection of double-strand break (DSB) induction, strand invasion, and ligation. Cells were grown to log phase at 30 °C in SC medium containing 2% raffinose but lacking tryptophan. HO-endonuclease was induced in these cells by the addition of 20% galactose at a final concentration of 2% v/v for 1 h. Cells were then shifted to 2% glucose to repress expression of the endonuclease. Genomic DNA was isolated every hour thereafter for PCR analysis. The primers used for the detection of DSBs, strand invasion, and ligation were: 5′-CTTTAGTTTCAGCTTTCCG-3′ (pI) and 5′-ACTCTATAAGGCCAAATGTACAAAC-3′ (pJ); 5′-GCAGCACGGAATATGGGACT-3′ (pG) and 5′-ATGTGAACCGCATGGGCAGT-3′ (pH); and 5′-AGATGAGTTTAAATCCAGCATACTAG-3′ (pC) and 5′-TGTTGTCTCACTATCTTGCCAATAAG-3′ (pD), respectively. The primers used to detect the control, *ARG5,6* were 5′-CAAGGATCCAGCAAAGTTGGGTGAAGTATGGTA-3′ and 5′-GAAGGATCCAAATTTGTCTAGTGTGGGAACG-3′.

2D-gel analysis. Cell synchronization was performed by adding nocodazole (final concentration of 10 μg/ml) together with DMSO (final concentration of 1% v/v) for 2.5 h and evaluating the percentage of large budded cells in the culture. The release from nocodazole arrest was performed by centrifugation, followed by washing in YPD medium containing 1% v/v DMSO. Cells were suspended in SC medium lacking tryptophan and containing MMS at a final concentration of 0.033% v/v, and were cultured at 30 °C. Purification of DNA intermediates in the presence of CTAB, the 2D gel procedure, and hybridizations were carried out as previously described [19].

Results and discussion

Isolation of a new rad52 mutation affecting its sumoylation

To clarify the regulatory mechanism of Rad52 sumoylation, we searched for new mutants that affect Rad52 sumoylation using a Rad52-His overexpression plasmid. As reported previously, modification of overexpressed Rad52-His by SUMO is observed in

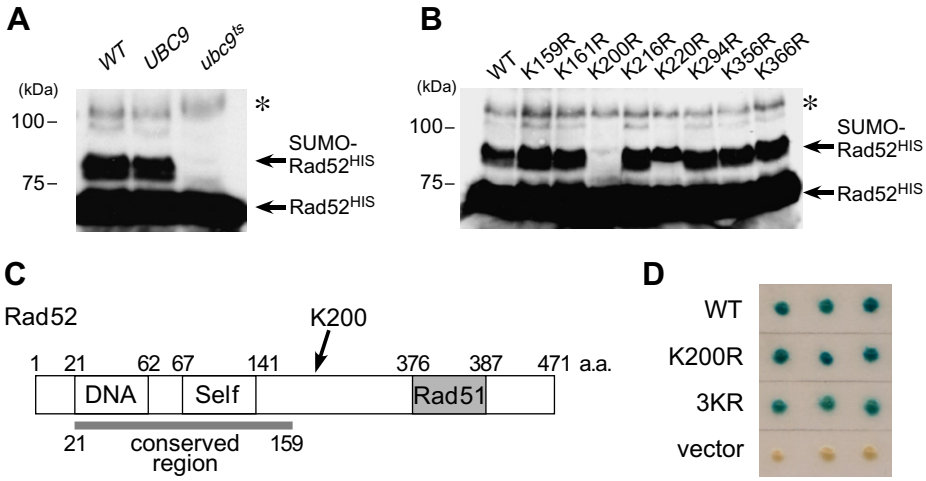

Fig. 1. Isolation of a new *rad52* mutation important for sumoylation. (A) Detection of sumoylation of Rad52. Rad52-His10 was overexpressed from the *GAL1*-promoter plasmid (pYES2) in wild-type (CB001 uA), *UBC9* (CB UBC9), or *ubc9ts* (CB ubc9ts) cells. Lysates prepared from these cells were analyzed by immunoblotting using anti-His antibodies. The asterisk denotes cross-reactive proteins. (B) Sumoylation of Rad52 mutants overexpressed from the *GAL1*-promoter plasmid (pYES2) in wild-type (CB001 uA) cells. Lysates prepared from the cells were analyzed by immunoblotting with anti-His. The asterisk denotes a cross-reactive protein. (C) The Rad52 primary structure is represented schematically. The protein contains DNA-binding, self-association, and Rad51-binding domains. The amino acid numbers are based on the open reading frame assignment of the SGD database (http://www.yeastgenome.org). (D) Yeast two-hybrid assays detect self-association. Interactions were detected by monitoring β-galactosidase activity, identified by the blue colony color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

wild-type but not in *ubc*9ts cells, which are defective in the SUMO-conjugating enzyme (Fig. 1A) [20]. Since lysine residues are targets of sumoylation, we searched primarily for new lysine to arginine point mutants of Rad52 that affected sumoylation. This search led to identification of a new *rad52* allele. Among the eight new Rad52 mutants, sumoylation of Rad52 was greatly reduced by the K200R mutation (Fig. 1B). A slight decrease in Rad52 sumoylation was observed in the Rad52-K220R mutant, which is one of the 3KR mutations, as reported previously [12].

A comparison of the primary structure of Rad52 among various eukaryotic species reveals a highly conserved N-terminal region spanning amino acids 21–159 [4,5] (Fig. 1C). This highly conserved region is involved in Rad52 self-association and/or DNA-binding [1,2]. We have previously reported that the decrease in sumoylation of Rad52-R103A and Rad52-K126R is due to a defect in self-association of Rad52 [15]. Although K200 lies outside of the conserved region, we examined whether lysine 200 of Rad52 is required for self-association of Rad52 using a yeast two-hybrid assay. Self-association of Rad52-K200R occurred normally (Fig. 1D), suggesting that Rad52-K200R diminishes sumoylation by a mechanism that does not affect self-association.

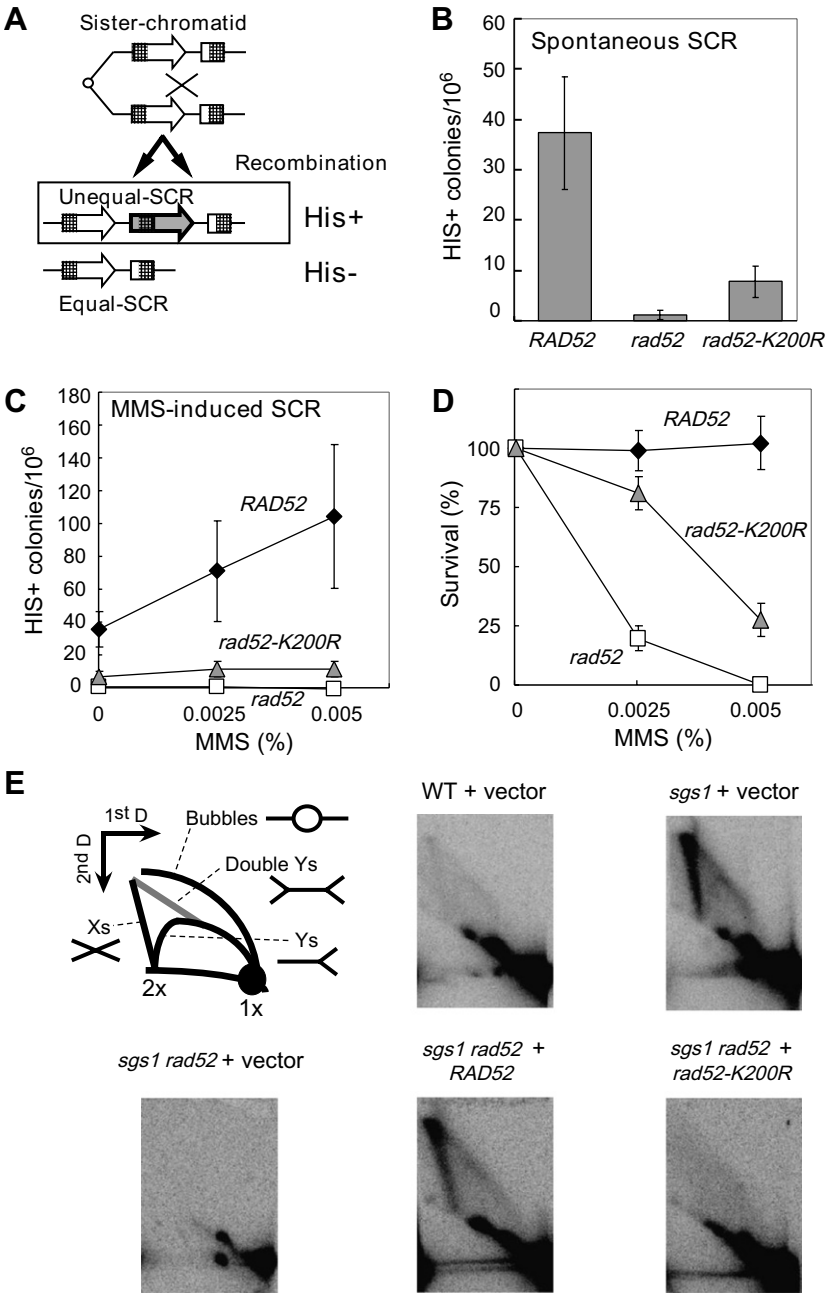

Fig. 2. Sister chromatid recombination is defective in *rad52-K200R* cells. (A) Schematic representation of the process and outcome of unequal sister chromatid recombination. See Materials and methods for details. The *rad52* cells (SCR rad52) that were transformed with pRS314-RAD52 (*RAD52*), pRS314 (*rad52*), or pRS314-rad52-K200R (*rad52-K200R*) were analyzed for: (B) Spontaneous recombination; (C) MMS-induced recombination; and (D), viability. The results are the average of three independent experiments in which a total of 11 plates were scored for each experimental condition. Error bars indicate the standard deviation. (E) X-molecule accumulation. Wild-type (W303-1A), *sgs1* (CY2570), and *sgs1 rad52* (CY5412) cells transformed with pRS314-RAD52 (*RAD52*), pRS314 (vector) or pRS314-rad52-K200R (*rad52-K200R*) were grown to log phase in SC medium lacking tryptophan at 25 °C. The cells were pelleted, synchronized with nocodazole, washed and released into SC medium lacking tryptophan and containing 0.033% MMS at 30 °C. The replication intermediates formed at the ARS305 origin of replication are shown for the 240 min.

The rad52-K200R mutant is defective in sister chromatid recombination

To examine whether damage-induced homologous recombination was impaired in *rad52-K200R* cells, we assayed damage-induced unequal sister chromatid recombination (SCR) in haploid cells using the assay system developed by Kadyk and Hartwell to genetically detect unequal SCR (uSCR) using short repeated sequences [17]. They proposed that uSCR occurs when replication forks bypass lesions that would obstruct the passage of the replication fork [17,18]. Fig. 2A shows the structure of the substrate used to monitor unequal SCR. The spontaneous unequal SCR frequency of *rad52-K200R* cells was significantly lower than that of wild-type cells (Fig. 2B). In addition, the frequency of methyl methanesulfonate (MMS)-induced uSCR was very low in *rad52-K200R* cells compared to wild-type cells (Fig. 2C). We next examined DNA repair activity and damage-tolerance in cells exposed to MMS. The *rad52-K200R* cells showed higher sensitivity to MMS compared to wild-type cells (Fig. 2D).

Homologous recombination–related intermediates are often detected at replication forks because cells can overcome DNA lesions through replication-coupled recombination processes [21]. In *sgs1* cells, a recombination-dependent cruciform structure, termed the X-molecule, accumulates at damaged forks, with accumulation requiring both Rad51 and Rad52 functions [21]. Thus, we examined the influence of *rad52-K200R* mutation on the accumulation of the X-molecule in a *sgs1* background. As shown Fig. 2E, similar to the null mutation, the *rad52-K200R* mutation abolished the accumulation of X-molecules in MMS-treated *sgs1* cells. Taken together, these data indicate that Rad52-K200R has lost some Rad52 homologous recombination related functions.

Mutation of lysine 200 to arginine in Rad52 influences HO-induced intrachromosomal recombination

One of the most thoroughly characterized homologous recombination events is HO-endonuclease-induced *MAT* switching, using *HML* as the donor template. The donor DNA is the *HML* sequence located on the same chromosome as the HO break. Introduction of a galactose-inducible *GAL::HO* gene into cells provides the means to induce double-strand breaks (DSBs) synchronously in all cells of the population. The DSB end of *MAT* DNA created by HO-endonuclease is resected by 5′ to 3′ exonucleases to make a 3′-ended single-stranded DNA (ssDNA). The ssDNA recruits the Rad51 strand exchange protein, and the DNA–protein complex invades the donor DNA [22,23]. We monitored the formation of DSBs at the HO-endonuclease recognition site, primer extension following strand invasion on the donor strand, and ligation of synthesized DNA by PCR using the primers shown in Fig. 3A. As shown in Fig. 3B, DSB formation was induced equally well in wild-type, *rad52-K200R,* and *rad52* null mutants. Strand invasion was almost entirely abolished in *rad52* cells, as expected (Fig. 3C), and strand invasion and ligation were severely reduced but still detectable in *rad52-K200R* cells, suggesting that Rad52-K200R maintains residual activity (Fig. 3D).

Nuclear localization of Rad52 is pre-requisite for its sumoylation

During the course of our study, Plate et al. reported that Rad52 from *S. cerevisiae* contains a pat7 type NLS essential for its nuclear localization and that the NLS is centrally located in Rad52 [16]. In fact, both Rad52-KRR200-202AAA-YFP and Rad52-R201A-YFP (KRR233-235AAA and R234A in their report) remain localized primarily to the cytoplasm. The C-terminal addition of NLS_{SV40} to Rad52-R201A-YFP restores its nuclear localization and repair function in the cell. Since K200 resides in the NLS of Rad52, these observations suggest that the sumoylation defect of Rad52-K200R may be an indirect consequence of perturbing its sub-cellular localization. To test this hypothesis, we examined the effect of a *rad52-R201A* mutation on Rad52 sumoylation. As shown in Fig. 4A, the amount of sumoylated Rad52 was considerably reduced in the Rad52-R201A and Rad52-K200R mutants. Next, we added a C-terminal NLS_{SV40} to Rad52-K200R-His or Rad52-R201A-His. The addition of the NLS_{SV40} suppressed the sumoylation defect of Rad52-K200R-His and Rad52-R201A-His (Fig. 4A). Taken together, these data indicate that nuclear localization of Rad52 is pre-requisite for sumoylation and that the defects in spontaneous and damage-induced uSCR and mating-type switching in *rad52-K200R*

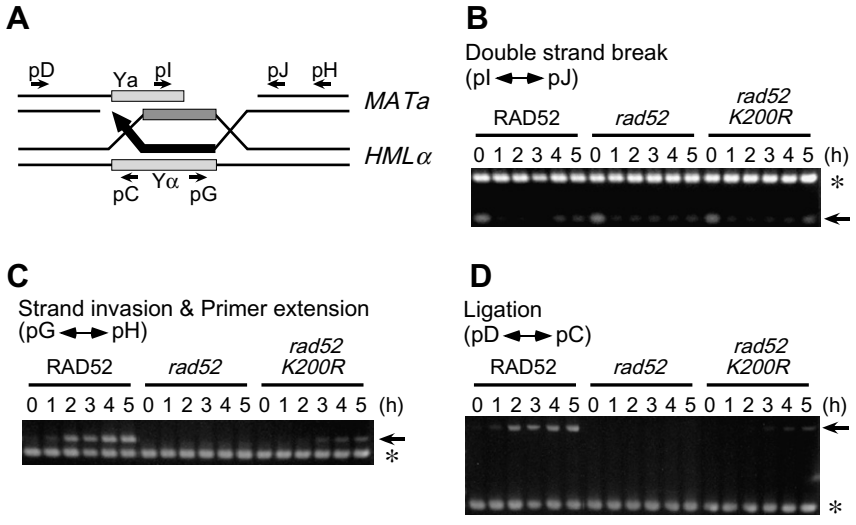

Fig. 3. Rad52-K200R is defective in mating-type switching. (A) The approximate location of the primers used to monitor DSB (pI and pJ; MATa primers), strand invasion and primer extension (pG and pH) and completion of repair (pC and pD; ligation primers) are shown. The *rad52* cells (JKM161 rad52) carrying the *HML* donor sequence were transformed with pRS314-RAD52 (*RAD52*), pRS314 (*rad52*), or pRS314-rad52-K200R (*rad52-K200R*), and DSBs were induced by HO-endonuclease. Genomic DNA was isolated at the indicated times and DSB formation (B), strand invasion (C), and ligation (D) were monitored by PCR. PCR products were separated on agarose gels and stained with ethidium bromide. The amount of input DNA was monitored by PCR amplification of the *ARG5,6* gene, indicated by the asterisks. The arrows indicate DNA amplified by each primer set.

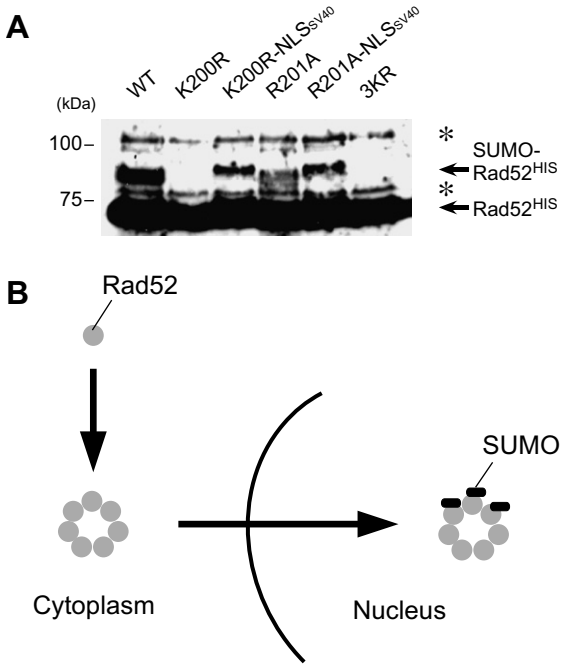

Fig. 4. Nuclear localization of Rad52 is important for its sumoylation. (A) Sumoylation of various Rad52 mutant proteins overexpressed from the *GAL1*-promoter plasmid (pYES2) in wild-type (CB001 uA) cells. Lysates prepared from the cells were analyzed by immunoblotting with anti-His. The asterisks denote cross-reactive proteins. (B) A model illustrating Rad52 sumoylation, which requires oligomerization of Rad52 and translocation into the nucleus.

result from the inability of Rad52 to enter the nucleus. Previously, a study by our lab showed that a defect in Rad52 self-association, such as in Rad52-K103A and -K126R mutants, reduces their sumoylation. However, Rad52-K200R self-associates to an extent similar to the wild-type Rad52. These results suggest that Rad52 oligomerizes in the cytoplasm and the multimeric Rad52 is transported into nucleus, where sumoylation of oligomerized Rad52 occurs (Fig. 4B).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.020.

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