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Purification of the Human SMN−GEMIN2 Complex and Assessment of Its Stimulation of RAD51-Mediated DNA Recombination Reactions

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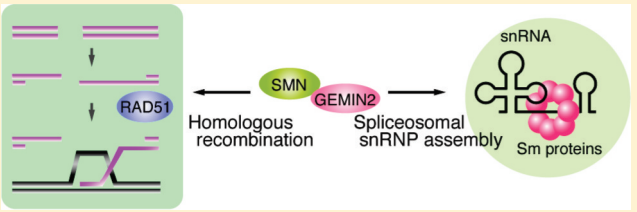
ABSTRACT: A deficiency in the SMN gene product causes the motor neuron degenerative disease spinal muscular atrophy. GEMIN2 was identified as an SMN-interacting protein, and the SMN−GEMIN2 complex constitutes part of the large SMN complex, which promotes the assembly of the spliceosomal small nuclear ribonucleoprotein (snRNP). In addition to its splicing function, we previously found that GEMIN2 alone stimulates RAD51-mediated recombination *in vitro*, and functions in DNA double-strand-break (DSB) repair through homologous recombination *in vivo*. However, the function of SMN in homologous recombination has not been reported. In the present study, we successfully purified the SMN−GEMIN2 complex as a fusion protein. The SMN−GEMIN2 fusion protein complemented the growth-defective phenotype of *GEMIN2*-knockout cells. The purified SMN−GEMIN2 fusion protein enhanced the RAD51-mediated homologous pairing much more efficiently than GEMIN2 alone. SMN−GEMIN2 possessed DNA-binding activity, which was not observed with the GEMIN2 protein, and significantly stimulated the secondary duplex DNA capture by the RAD51-single-stranded DNA complex during homologous pairing. These results provide the first evidence that the SMN−GEMIN2 complex plays a role in homologous recombination, in addition to spliceosomal snRNP assembly.



Double-strand breaks (DSBs) are a serious form of DNA damage induced by DNA damaging agents, such as ionizing radiation (IR), DNA cross-linkers, topoisomerase inhibitors, and replication errors.[1−4] In mitosis, two pathways—homologous recombination (HR) and nonhomologous DNA end-joining (NHEJ)—are mainly employed to repair the DSBs in cells.[5,6] NHEJ is an error-prone pathway and is considered to be required for the acute treatment of DSBs. On the other hand, HR is a more accurate pathway and is preferred for repair between sister chromatids, when DSBs occur as a consequence of replication blocks. HR also functions in meiosis. In contrast to mitotic HR, meiotic HR preferentially occurs between homologous chromosomes and ensures their correct segregation at meiosis I.[7,8]

RAD51, a eukaryotic recombinase, catalyzes the homologous-pairing and strand-exchange reactions *in vitro*.[9−12] *RAD51*$^{-/-}$ vertebrate cells display extensive chromosomal breakage in mitotic cells, which induces cell death,[13] indicating that RAD51 is an essential protein in the early stages of the HR pathway. In the yeast *Saccharomyces cerevisiae*, the *Rad51* gene is not essential for cell viability, but the *Rad51*-deletion strain displays high sensitivity for DSB-inducing agents, such as ionizing radiation.[14] For efficient HR in eukaryotes, RAD51-interacting proteins, which stimulate the RAD51-mediated recombinase activity, have been identified in *S. cerevisiae*.[14−17] Orthologs of these yeast Rad51-interacting proteins have been found in humans.[14−17] Human RAD52 directly interacts with human RAD51[18−21] and stimulates the RAD51-mediated recombination reaction *in vitro*.[22] Human RAD54 and RAD54B, which directly interact with RAD51,[23,24] also stimulate the RAD51-mediated recombinase activity.[25−27] Five RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) are required for RAD51 assembly onto the DSB sites *in vivo*,[28] and the RAD51B−RAD51C complex stimulates the RAD51-mediated strand-exchange reaction *in vitro*.[29] In addition to these orthologs of the yeast Rad51-interacting proteins, human BRCA2, RAD51AP1, EVL, and PSF, which are not conserved in *S. cerevisiae*, were identified as RAD51-interacting proteins and were also found to stimulate RAD51-mediated homologous pairing and strand exchange *in vitro*.[30−37]

We previously identified human GEMIN2 as a novel RAD51-interacting protein.[38] The purified GEMIN2 protein enhanced the RAD51-mediated homologous pairing and strand exchange reactions.[38] In agreement with these biochemical results, the depletion of GEMIN2 in the chicken B cell line DT40, as well as in human cells, significantly reduced the recruitment of RAD51 to DNA lesions.[38] GEMIN2 is ubiquitously expressed in various tissues[39] and was first identified as survival motor

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neuron (SMN) protein interacting protein 1 (formerly SIP1).[40] The homozygous *GEMIN2*-knockout caused early embryonic lethality in mouse.[41] GEMIN2 is a component of the SMN complex, which promotes the ATP-dependent assembly of the spliceosomal small nuclear ribonucleoprotein (snRNP).[42] In the SMN complex, GEMIN2 forms a subcomplex with SMN.[43] SMN deficiency causes the motor neuron degenerative disease spinal muscular atrophy.[44] However, the function of SMN in the HR pathway has not been reported yet.

In the present study, we successfully purified the SMN−GEMIN2 complex as a fusion protein. The SMN−GEMIN2 fusion protein was confirmed to be functional in the *GEMIN2*-knockout DT40 cells. The purified SMN−GEMIN2 protein possessed DNA-binding activity and significantly enhanced RAD51-mediated homologous pairing and strand exchange. These results provide the first evidence that the SMN−GEMIN2 complex plays a role in DSB repair through the HR pathway, in addition to spliceosomal snRNP assembly.

MATERIALS AND METHODS

Protein Purification. The human *SMN−GEMIN2* fusion gene, in which the C-terminal end of SMN was fused to the N-terminal end of GEMIN2 by a linker peptide (Thr-Gly-Ser-Thr-Gly-Ser-Gly-Thr-Thr-Gly-Ser-Thr-Gly-Ser), was ligated into the *Nde*I site of the pET15b plasmid (Novagen, Darmstadt, Germany). The SUMO1 coding sequence was inserted just after the hexahistidine tag (His_6) sequence. The His_6-SUMO-tagged SMN−GEMIN2 fusion protein was overexpressed in the *Escherichia coli* strain BL21(DE3), which also carried an expression vector for the minor tRNAs (Codon(+)-RIL, Novagen, Darmstadt, Germany). The *E. coli* cells containing the SMN−GEMIN2 fusion plasmid were cultured at 30 °C. At the logarithmic phase of growth (A_{600} = 0.8), the SMN−GEMIN2 production was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration), and the cells were further cultured for 12 h at 18 °C. The cells were disrupted by sonication in buffer A (50 mM Tris-HCl (pH 7.5), 5 mM imidazole, 10% glycerol, 500 mM NaCl, and 5 mM 2-mercaptoethanol). The cell lysate was clarified by centrifugation, and the supernatant was mixed with 2 mL of ProBond resin (Invitrogen, Carlsbad, CA). The sample was gently mixed for 1 h at 4°C. The resin was packed in a column and washed with 100 mL of buffer A, containing 70 mM imidazole, and the protein was eluted by a linear gradient of imidazole from 70 to 500 mM. The ProBond fractions containing the His_6-SUMO-tagged SMN−GEMIN2 protein were collected, and the protein was treated with 2 unit/mg of PreScission protease (GE Healthcare Biosciences, Uppsala, Sweden) during dialysis against buffer B (20 mM Tris-HCl [pH 7.5], 10% glycerol, 100 mM NaCl, 0.25 mM EDTA, and 2 mM 2-mercaptoethanol). After the removal of the His_6-SUMO tag, the sample was loaded on a hydroxyapatite column (2 mL, Bio-Rad Laboratories, Hercules, CA), which was equilibrated with buffer B. The resin was washed with 60 mL of buffer C (20 mM potassium phosphate [pH 7.5], 10% glycerol, 100 mM NaCl, 0.25 mM EDTA, and 2 mM 2-mercaptoethanol), and SMN−GEMIN2 was eluted with a linear gradient of 20−500 mM potassium phosphate. The peak fractions containing SMN−GEMIN2 were dialyzed against buffer B and were subjected to MonoS column chromatography (GE Healthcare Biosciences, Uppsala, Sweden). The column was washed with 10 mL of buffer B, and SMN−GEMIN2 was eluted with a linear gradient of 100−1000 mM NaCl. The purified SMN−GEMIN2 was stored in 20 mM HEPES−NaOH buffer (pH 7.5), containing 10% glycerol, 200 mM NaCl, 0.1 mM EDTA, and 2 mM 2-mercaptoethanol, at −80 °C. Human GEMIN2 and RAD51 were purified as described previously.[38,45,46]

Pulldown Assay with SMN−GEMIN2 Beads. SMN−GEMIN2 (200 μg) was covalently conjugated to Affi-Gel 10 beads (100 μL, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and the Affi-Gel 10-protein matrix was adjusted to a 50% slurry. RAD51 (20 μg) was incubated with SMN−GEMIN2-conjugated Affi-Gel 10 beads in 120 μL of 20 mM HEPES buffer (pH 7.5), containing 10% glycerol, 200 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.05% Triton X-100. After incubation at room temperature for 2.5 h, the SMN−GEMIN2 beads were washed two times with the same buffer (100 μL). Proteins bound to the SMN−GEMIN2 beads were fractionated by a 15% SDS-PAGE. Bands were visualized by Coomassie Brilliant Blue staining.

DNA Substrates for *in Vitro* Reactions. Single-stranded ϕX174 viral (+) strand DNA and double-stranded ϕX174 replicative form I DNA were purchased from New England Biolabs (Ipswich, MA). The linear dsDNA was prepared from the ϕX174 replicative form I DNA by *Pst*I digestion. High-pressure liquid chromatography-purified oligodeoxyribonucleotides were purchased from Nihon Gene Research Laboratory. The DNA sequences used in this study are the following: 50-mer ssDNA: 5′-GGA ATT CGG TAT TCC CAG GCG GTC TCC CAT CCA AGT ACT AAC CGA GCC CT-3′; 63-mer ssDNA: 5′-TCC TTT TGA TAA GAG GTC ATT TTT GCG GAT GGC TTA GAG CTT AAT TGC TGA ATC TGG TGC TGT-3′; 32-mer dsDNA: 5′-CCA TCC GCA AAA ATG ACC TCT TAT CAA AAG GA-3′ and 5′-TCC TTT TGA TAA GAG GTC ATT TTT GCG GAT GG-3′; and 49-mer dsDNA: 5′-GTC CCA GGC CAT TAC AGA TCA ATC CTG AGC ATG TTT ACC AAG CGC ATT G-3′ and 5′-CAA TGC GCT TGG TAA ACA TGC TCA GGA TTG ATC TGT AAT GGC CTG GGA C-3′. All of the DNA concentrations are expressed in moles of nucleotides.

DNA-Binding Assay. The reaction mixtures (10 μL) contained 24 mM HEPES buffer (pH 7.5), 40 mM NaCl, 0.02 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM DTT, 100 μg/mL BSA, 1 mM $MgCl_2$, and 2% glycerol. The ϕX174 ssDNA (20 μM) or the linearized ϕX174 dsDNA (20 μM) was mixed with SMN−GEMIN2 or GEMIN2, and the mixtures were incubated at 37 °C for 10 min. After the incubation, the protein−DNA complexes were analyzed by 0.8% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA) at 3.3 V/cm for 2.5 h. The bands were visualized by ethidium bromide staining.

D-Loop Formation Assay. RAD51 was incubated with GEMIN2 or SMN−GEMIN2 at 37°C for 5 min in 8 μL of reaction buffer, containing 24 mM HEPES-NaOH (pH 7.5), 35 mM NaCl, 0.02 mM EDTA, 0.4 mM 2-mercaptoethanol, 2% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 2 mM $CaCl_2$, and 100 μg/mL BSA. After this incubation, 1 μL of a ^{32}P-labeled oligodeoxyribonucleotide 50-mer (1 μM, 5′-GGA ATT CGG TAT TCC CAG GCG GTC TCC CAT CCA AGT ACT AAC CGA GCC CT-3′) was added, and the samples were further incubated at 37 °C for 5 min. The reactions were then initiated by the addition of 1 μL of the pB5Sarray superhelical dsDNA (20 μM) and were continued at 37 °C for 30 min. The

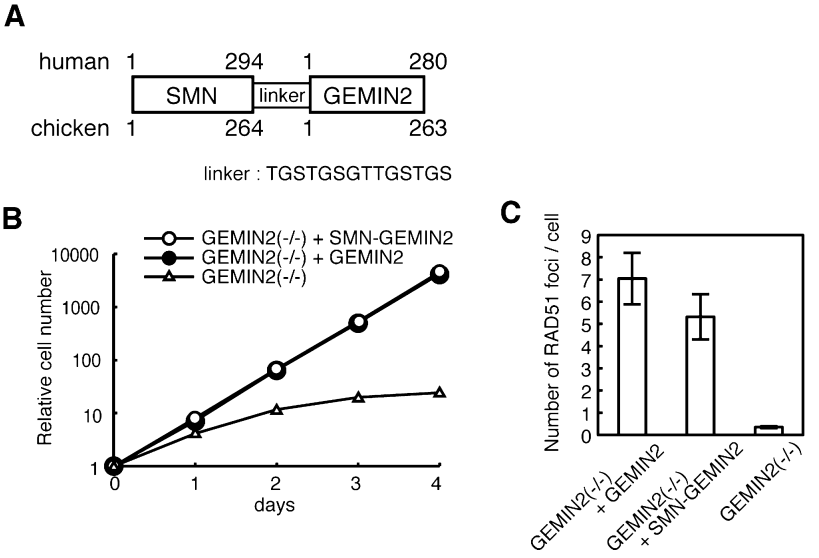

Figure 1. SMN−GEMIN2 fusion protein is functional *in vivo*. (A) Schematic representation of the SMN−GEMIN2 fusion protein. Boxes represent the SMN and GEMIN2 proteins. Numbers at the top and bottom of the boxes correspond to the amino acid residues of the human and chicken proteins, respectively. The amino acid sequence of the linker peptide is represented. (B) Growth curves of cells with the indicated genotypes. Tetracycline was added at time zero to repress the GEMIN2 transgene in the indicated GEMIN2 conditional mutant cells. The relative cell numbers are plotted for the *GEMIN2*$^{-/-}$ clones complemented by the SMN−GEMIN2 (open circles) and GEMIN2 (closed circles) production, respectively. Open triangles indicate the *GEMIN2*$^{-/-}$ clones without GEMIN2 or SMN−GEMIN2 production. (C) Quantification of RAD51 subnuclear foci in individual cells with the indicated genotypes. The cells were treated with tetracycline for 4 days, and the number of RAD51 foci was counted 3 h after irradiation with 4 Gy γ-rays. Data shown are the means of three experiments. Error bars indicate standard deviations.

pB5Sarray DNA contained 11 repeats of a sea urchin 5S rRNA gene (207 bp fragment) within the pBlueScript II SK(+) vector and was prepared by a method avoiding alkaline treatment of the cells harboring the plasmid DNA to prevent the dsDNA substrate from undergoing irreversible denaturation. The reactions were stopped by the addition of 0.1% SDS and 1.6 mg/mL proteinase K (Roche Applied Science, Basel, Switzerland) and were further incubated at 37 °C for 15 min. After adding 6-fold loading dye, the deproteinized reaction products were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 4 V/cm for 2 h. The gels were dried and were exposed to an imaging plate. The gel images were visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan), and the band intensities were quantitated.

Oligonucleotide-Based Homologous-Pairing Assay. In this assay, a 63-mer ssDNA and a 32-mer dsDNA, which were used in previous studies,[47,48] were employed. RAD51 (4 μM), RPA (1.5 μM), and the indicated amounts of SMN−GEMIN2 were incubated at 37 °C for 10 min. For the reaction without RPA, RAD51 (2.2 μM) and the indicated amounts of SMN−GEMIN2 were incubated at 37 °C for 10 min. A 63-mer ssDNA (15 μM) was added to the reaction mixture, and the samples were further incubated at 37 °C for 10 min in 9 μL of reaction buffer, containing 26 mM HEPES-NaOH (pH 7.5), 55 mM NaCl, 0.03 mM EDTA, 0.6 mM 2-mercaptoethanol, 3% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 2 mM $CaCl_2$, 20 mM creatine phosphate, 75 μg/mL creatine kinase, and 100 μg/mL BSA. The strand-exchange reaction was initiated by the addition of 1 μL of the 32-mer dsDNA (1.5 μM) and was continued at 37 °C for 30 min. The reactions were stopped by the addition of 0.1% SDS and 1.6 mg/mL proteinase K (Roche Applied Science, Basel, Switzerland) and were further incubated at 37 °C for 15 min. After adding 6-fold loading dye, the deproteinized reaction products were separated by 15% polyacrylamide gel electrophoresis in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) at 20 mA for 35 min. The gels were dried and were exposed to an imaging plate. The gel images were visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan), and the band intensities were quantitated.

Duplex DNA Capture Assay. RAD51 (0.6 μM) and either SMN−GEMIN2 (1 μM) or GEMIN2 (1 μM) were preincubated at 37 °C for 5 min. Magnetic streptavidin beads conjugated with 5′-biotinylated poly dT ssDNA (83-mer, 6 μM) were added to the reaction mixture, and the samples were further incubated at 37 °C for 5 min in 9 μL of a reaction solution, containing 24 mM HEPES−NaOH (pH 7.5), 60 mM NaCl, 0.02 mM EDTA, 0.4 mM 2-mercaptoethanol, 2% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 2 mM $CaCl_2$, 0.01% Nonidet P-40, and 100 μg/mL BSA. The reactions were initiated by the addition of 1 μL of ^{32}P-labeled heterologous dsDNA (49-mer, 1.5 μM) and were continued at 37 °C for 10 min. The supernatants were retained and were treated with 0.4% SDS and 1.8 mg/mL proteinase K at 37 °C for 15 min. The resulting ssDNA beads were washed twice with 10 μL of the reaction solution. The dsDNA captured by the ssDNA beads was recovered by a treatment with 0.4% SDS and 1.8 mg/mL proteinase K at 37 °C for 15 min. After adding 6-fold loading dye, the dsDNA was analyzed by 8% polyacrylamide gel electrophoresis in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). For reference, a quarter of each supernatant fraction was analyzed by 8% polyacrylamide gel electrophoresis. The gels were dried and were exposed to an imaging plate. The gel images were visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan), and the band intensities were quantitated.

Circular Dichroism (CD) Measurement. Circular dichroism spectra of SMN-GEMIN2 (10.5 μM) and GEMIN2 (10.5 μM) were measured on a JASCO J-820 spectropolarimeter (Japan

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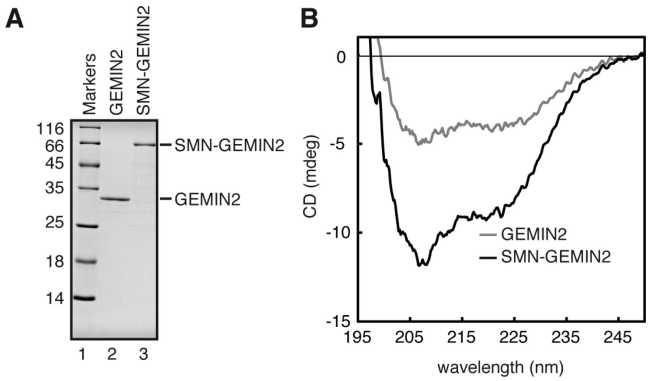

Figure 2. Purification and circular dichroism spectrum of the SMN−GEMIN2 fusion protein. (A) Purified human GEMIN2 and SMN−GEMIN2. GEMIN2 (0.5 μg, lane 2) and SMN−GEMIN2 (0.5 μg, lane 3) were analyzed by 15% SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1 indicates the molecular mass markers. (B) Circular dichroism spectra of SMN−GEMIN2 (10.5 μM) and GEMIN2 (10.5 μM).

Spectroscopic Co., Ltd., Tokyo, Japan), using a 1 cm path length quartz cell. The experiments were performed in 0.2 mM HEPES buffer (pH 7.5), containing 0.1% glycerol, 2 mM NaCl, 1 μM EDTA, and 0.02 mM 2-mercaptoethanol.

Generation of a Chimeric Gene Composed of Chicken SMN and GEMIN2. To isolate the SMN cDNA, we performed RT-PCR using the following primers: 5′-GGG AAT TCA TGG CGG GCA GGG TGC TGT TC-3′ (sense primer containing *Eco*RI at the 5′ end) and 5′-AAA CCG GTG GTG CCG CTG CCG GTG CTG CCG GTT TTT AGA TAG GCT TCT CTC TCT AG-3′ (reverse primer containing *Age*I at the 5′ end). Note that this procedure replaced the termination codon of the chicken SMN cDNA by the nucleotides encoding the linker amino acid sequence TGSTGSGTTGSTGS. To isolate the GEMIN2 cDNA, we performed RT-PCR using the following primers: 5′- AAA CCG GTA GCA CCG GCA GCA TGG AGC CGG CCG TGG AGG-3′ (sense primer containing *Age*I at the 5′ end) and 5′-GGG ATC CCT AGG AGG GTT CAT CAG CTA A-3′ (reverse primer containing *Bam*HI at the 5′ end). These two amplified cDNAs were ligated together at the *Age*I site, and the resultant fragment was cloned into the PCR2.1 vector. The resulting chimeric *SMN−GEMIN2* gene was excised by *Eco*RI plus *Bam*HI and inserted into pcDNA3.1(−), carrying the neomycin resistance gene (Invitrogen, Carlsbad, CA).

Cell Culture. DT40 cells were cultured at 39.5 °C in RPMI-1640 medium supplemented with 10^{-5} M β-mercaptoethanol, penicillin−streptomycin, 10% fetal calf serum (FCS), and 1% chicken serum (Sigma-Aldrich, St. Louis, MO).[49]

Generation of Chimeric SMN−GEMIN2 Gene Expressing Cells. The generation of conditional GEMIN2-deficient cells, carrying the chicken *GEMIN2* transgene under the control of a tetracycline-repressible promoter (*GEMIN2*$^{-/-}$*tetGEMIN2* cells), was described previously.[38,50] The *GEMIN2*$^{-/-}$*tetGEMIN2* cells were transfected with the *SMN−GEMIN2* expression plasmid and selected with G418. To repress the *tetGEMIN2* transgene, G418-resistant clones were treated with tetracycline, as described previously,[38] and the clones with a normal proliferation rate were subsequently subjected to a phenotypic analysis.

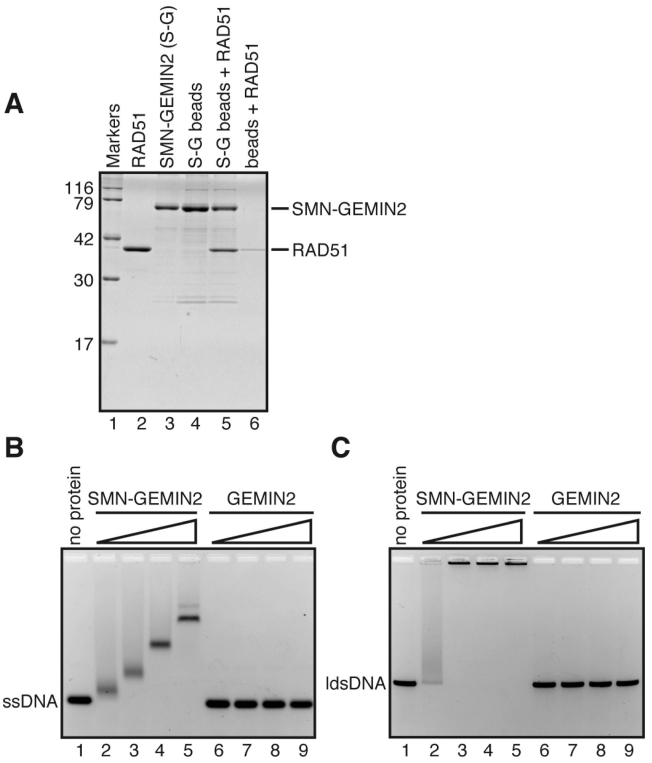

Figure 3. RAD51-binding and DNA-binding activities of SMN−GEMIN2. (A) RAD51 binding assay. RAD51 (20 μg) was incubated with the SMN−GEMIN2-conjugated beads. The SMN−GEMIN2 beads were then isolated, and the RAD51 bound to the beads was analyzed by 15% SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1 indicates the molecular mass markers. Lanes 2 and 3 represent purified RAD51 and SMN−GEMIN2, respectively. Lane 5 indicates the experiment with RAD51 and SMN−GEMIN2 beads. Lanes 4 and 6 indicate negative control experiments in the absence of RAD51 and SMN−GEMIN2, respectively. (B) DNA binding assay. The ϕX174 circular ssDNA (20 μM) was mixed with SMN−GEMIN2 (lanes 2−5) or GEMIN2 (lanes 6−9), and the reactions were conducted at 37 °C for 10 min. Samples were then analyzed by 0.8% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 2.5 h. The bands were visualized by ethidium bromide staining. The protein concentrations were 0.25 μM (lanes 2 and 6), 0.5 μM (lanes 3 and 7), 1 μM (lanes 4 and 8), and 2 μM (lanes 5 and 9). Lane 1 indicates a negative control experiment without the proteins. (C) The linear ϕX174 dsDNA (20 μM) was used instead of the ssDNA.

RAD51 Focus Detection. Cells were harvested at 3 h after 4Gy ^{137}Cs irradiation and were spun onto slides, using a Shandon Cytospin 3 centrifuge (Shandon, Pittsburgh, PA). Staining and visualization of RAD51 foci were performed as previously described,[51] using a rabbit polyclonal antibody that recognizes human RAD51, at a dilution of 1:500 (Calbiochem, San Diego, CA), and an Alexa Fluor 488 goat anti-human IgG antibody at a dilution of 1:1000 (Molecular Probes Inc., Eugene, OR). Fluorescence images were obtained and processed using an IX81 fluorescent microscope (Olympus, Tokyo, Japan).

RESULTS

SMN−GEMIN2 Fusion Protein Is Functional in GEMIN2$^{-/-}$ Cells. We previously reported that GEMIN2 is a novel HR factor, which stimulates RAD51 accumulation at DSB sites *in vivo* and enhances RAD51-mediated recombination reactions *in vitro*.[38] However, in cells, GEMIN2 forms a stable complex

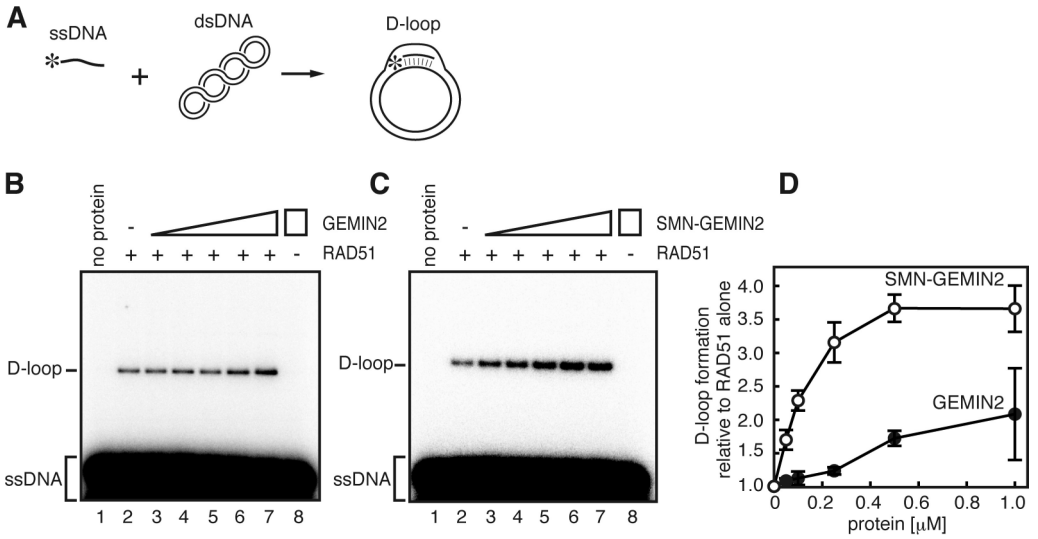

Figure 4. SMN−GEMIN2 significantly stimulates RAD51-mediated homologous pairing. (A) Schematic representation of the D-loop assay. Asterisks indicate the ^{32}P-labeled 5′-end of the ssDNA. (B, C) RAD51 (0.1 μM) and either GEMIN2 (panel B) or SMN−GEMIN2 (panel C) were incubated with a ^{32}P-labeled 50-mer oligonucleotide (1 μM) at 37°C for 5 min. The GEMIN2 (panel B) and SMN−GEMIN2 (panel C) concentrations were 0 μM (lane 2), 0.05 μM (lane 3), 0.1 μM (lane 4), 0.25 μM (lane 5), 0.5 μM (lane 6), and 1 μM (lane 7). The reactions were then initiated by the addition of the superhelical dsDNA (20 μM) and were continued at 37 °C for 30 min. Lane 1 indicates a negative control reaction without the proteins. Lane 8 in panels B and C indicates a control experiment with GEMIN2 (1 μM) and SMN−GEMIN2 (1 μM), respectively, in the absence of RAD51. The reactions were stopped by the addition of SDS, and the samples were treated with proteinase K. The reaction products were fractionated by 1% agarose gel electrophoresis in 1× TAE buffer. The gels were dried, exposed to an imaging plate, and visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan). (D) Graphic representation of the experiments shown in panels B and C. The average values of three independent experiments are shown with the SD values. Open and closed circles represent the experiments with SMN−GEMIN2 and GEMIN2, respectively.

with SMN,[42,43] which is the product of a gene responsible for a neurodegenerative disease, spinal muscular atrophy. To test the roles of SMN in HR, we tried to purify human SMN as a recombinant protein, but it was only recovered in the insoluble fraction. The coproduction of SMN and GEMIN2 was also unsuccessful.

We then designed an SMN−GEMIN2 fusion protein, in which SMN and GEMIN2 were connected with a short linker peptide, containing Thr-Gly-Ser-Thr-Gly-Ser-Gly-Thr-Thr-Gly-Ser-Thr-Gly-Ser (Figure 1A). We first tested whether the physical connection between SMN and GEMIN2 by the linker peptide inhibited the GEMIN2 function *in vivo*. To do so, the chicken SMN−GEMIN2 fusion protein was expressed in the *GEMIN2*$^{-/-}$ chicken DT40 cells. *GEMIN2* is essential for cellular proliferation.[38] Therefore, we conditionally disrupted the *GEMIN2* gene, using a chicken *GEMIN2* or *SMN−GEMIN2* fusion transgene under the control of a tetracycline (doxycycline)-repressible promoter (*tetGEMIN2* transgene). As shown in Figure 1B, the SMN−GEMIN2 fusion alleviated the growth defect of the *GEMIN2*$^{-/-}$ cells as efficiently as GEMIN2 (Figure 1B). SMN−GEMIN2 also complemented the defective RAD51 foci formation in the *GEMIN2*$^{-/-}$ cells after DSB induction by γ-rays (Figure 1C). These results strongly suggested that the SMN−GEMIN2 fusion protein (SMN−GEMIN2) is functional *in vivo*.

Preparation of the SMN−GEMIN2 Fusion Protein. We next purified the SMN−GEMIN2 fusion protein, as a recombinant protein. To this end, we selected the human SMN and GEMIN2 proteins because human GEMIN2 alone stimulates RAD51-mediated recombination reactions *in vitro*.[38] Human SMN−GEMIN2 was expressed in *Escherichia coli* cells as an N-terminal His_6- and SUMO-tagged (His_6-SUMO tag) protein. The His_6-SUMO-tagged SMN−GEMIN2 protein was purified by Ni^{2+} chelating column chromatography. The His_6-SUMO tag was uncoupled with PreScission protease from the SMN−GEMIN2 portion, and SMN−GEMIN2 was further purified by hydroxyapatite column chromatography and MonoS column chromatography (Figure 2A, lane 3).

The purified SMN−GEMIN2 protein generated a circular dichroism (CD) spectrum with negative peaks at 208 and 222 nm, suggesting that SMN−GEMIN2 was folded into a higher-ordered structure (Figure 2B). We compared the CD spectra of SMN−GEMIN2 and GEMIN2 at the same molar concentration and found that the negative peak intensities of the SMN−GEMIN2 spectrum were about twice as strong as those of GEMIN2 alone. These results suggested that the SMN portion of SMN−GEMIN2 folded into a higher-ordered structure with the GEMIN2 portion (Figure 2B).

SMN−GEMIN2 Binds to RAD51 and DNA. We previously reported that GEMIN2 alone binds to RAD51.[38] Our pulldown assay, using beads conjugated with purified SMN−GEMIN2, revealed that RAD51 copelleted with the SMN−GEMIN2 beads (Figure 3A). This result indicated that SMN−GEMIN2 directly binds to RAD51. We next tested the DNA-binding activity of SMN−GEMIN2. Consistent with the previous results,[38] GEMIN2 alone bound to neither ssDNA nor dsDNA (Figure 3B,C, lanes 6−9). In contrast, the SMN−GEMIN2−ssDNA and SMN−GEMIN2−dsDNA complexes were clearly detected as slowly migrating bands on the agarose gel (Figure 3B,C, lanes 2−5), indicating that SMN−GEMIN2 bound to DNA. Therefore, the SMN and GEMIN2 portions of the complex may be responsible for the DNA-binding and RAD51-binding activities, respectively.

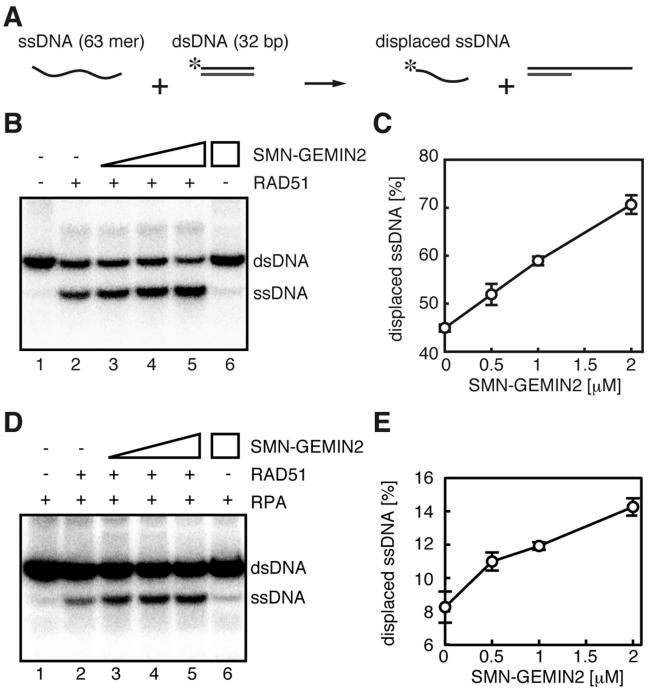

Figure 5. SMN−GEMIN2 stimulates RAD51-mediated homologous pairing in the presence of RPA. (A) Schematic representation of the oligonucleotide-based assay. Asterisks indicate the ^{32}P-labeled 5′-end of the DNA strand. (B) RAD51 (2.2 μM) and SMN−GEMIN2 (0, 0.5, 1, and 2 μM, lanes 2−5) were incubated with the 63-mer ssDNA (15 μM) at 37 °C for 10 min. The reactions were then initiated by the addition of the ^{32}P-labeled 32-mer dsDNA (1.5 μM) and were continued at 37 °C for 30 min. The reactions were stopped by the addition of SDS, and the samples were treated with proteinase K. The reaction products were separated by 15% polyacrylamide gel electrophoresis in 0.5× TBE buffer. The gels were dried, exposed to an imaging plate, and visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan). Lane 1 indicates a negative control reaction without the proteins. Lane 6 indicates the experiment with SMN-GEMIN2 (2 μM) in the absence of RAD51. (C) Graphic representation of the experiments shown in panel B. The average values of three independent experiments are shown with the SD values. (D) RAD51 (4 μM) and SMN−GEMIN2 (0, 0.5, 1, and 2 μM, lanes 2−5) were preincubated with RPA (1.5 μM) at 37 °C for 10 min. The reactions were performed in the same manner as in panel B. (E) Graphic representation of the experiments shown in panel D. The average values of three independent experiments are shown with the SD values.

SMN−GEMIN2 Stimulates RAD51-Mediated Homologous Pairing *in Vitro*. We next tested the effect of SMN−GEMIN2 in a D-loop formation assay (Figure 4A), which is a standard assay to detect the homologous-pairing activity of bacterial and eukaryotic recombinases. In this assay, a ^{32}P-labeled 50-mer oligonucleotide and superhelical dsDNA were used as substrates, and the D-loop structure, in which the ^{32}P-labeled 50-mer oligonucleotide forms new Watson−Crick base pairs with a complementary strand of the dsDNA, was detected by agarose gel electrophoresis. Consistent with a previous report,[38] GEMIN2 alone increased the amount of D-loops formed by RAD51 (Figure 4B, lanes 2−7, and Figure 4D). Intriguingly, SMN−GEMIN2 exhibited significantly higher stimulation activity for the RAD51-mediated D-loop formation, as compared to GEMIN2 alone (Figure 4C, lanes 2−7, and Figure 4D). SMN−GEMIN2 alone did not promote D-loop formation (Figure 4C, lane 8). Therefore, SMN−GEMIN2 stimulates RAD51-mediated homologous pairing more efficiently than GEMIN2 alone.

SMN−GEMIN2 Stimulates RAD51-Mediated Homologous Pairing in the Presence of RPA. We next performed the oligonucleotide-based assay,[47,48] in which the dsDNA 32-mer and the ssDNA 63-mer were used as substrates for homologous pairing (Figure 5A). The ^{32}P-labeled strand of the dsDNA was replaced with the incoming ssDNA by the RAD51-mediated, successive homologous-pairing and strand-exchange reactions. The displaced ^{32}P-labeled strand was detected as a reaction product by nondenaturing polyacrylamide gel electrophoresis (Figure 5A). Consistent with the results from the D-loop formation assay, SMN−GEMIN2 significantly stimulated the RAD51-mediated homologous-pairing reaction, in a concentration-dependent manner (Figure 5B, lanes 2−5, and Figure 5C).

RPA is known to accumulate on DSB sites together with RAD51 during HR repair, but *in vitro*, RPA is inhibitory for the RAD51-filament formation if incubated with ssDNA together with RAD51. On the other hand, RPA addition after RAD51-ssDNA filament formation stimulates RAD51-dependent strand exchange.[16,17] We then tested whether SMN−GEMIN2 stimulates the RAD51-mediated homologous pairing in the presence of RPA. Consistent with the previous studies, the addition of RPA inhibited the RAD51-mediated homologous pairing (Figure 5D,E), as compared to the reactions in the absence of RPA (Figure 5B,C). However, SMN−GEMIN2 efficiently stimulated the RAD51-mediated homologous pairing in the presence of RPA (Figure 5D,E). These results indicated that SMN−GEMIN2 could stimulate the RAD51-mediated homologous pairing in the natural context containing RPA.

SMN−GEMIN2 Stimulates dsDNA Capture during RAD51-Mediated Homologous Pairing. During homologous pairing, the RAD51−ssDNA complex binds to dsDNA (secondary dsDNA capture) and forms the three-component complex, containing RAD51, ssDNA, and dsDNA (synaptic complex). In this synaptic complex, the homologous sequences between ssDNA and dsDNA are aligned. We then tested whether SMN−GEMIN2 stimulates the secondary dsDNA capture by the RAD51−ssDNA complex. The RAD51−ssDNA complexes are formed on the ssDNA conjugated to the beads, and the dsDNA captured by the RAD51−ssDNA complexes was detected by the pulldown assay (Figure 6A).[52] As shown in Figure 6B,C (lane 4), we found that SMN−GEMIN2 drastically enhanced the secondary dsDNA capturing activity of the RAD51−ssDNA complex. Although GEMIN2 moderately enhanced the RAD51-mediated homologous pairing (Figure 4), it minimally enhanced the secondary dsDNA capture (Figure 6B,C, lane 3). Therefore, we concluded that the SMN−GEMIN2 complex may be the *bona fide* functional form that stimulates the RAD51-dependent recombination reactions.

DISCUSSION

SMN is the product of the survival motor neuron (*SMN*) gene. Reduced SMN production in human cells causes spinal muscular atrophy (SMA), which is an autosomal recessive neurodegenerative disease characterized by the loss of spinal cord motor neurons.[44] SMN forms a subcomplex with GEMIN2, as part of a large multiprotein complex (the SMN complex) that functions in the assembly of spliceosomal snRNP.[42,43] We previously found that GEMIN2 interacts with RAD51, which is an essential protein for DSB repair through the HR pathway, and reported that GEMIN2 actually functions in the HR repair

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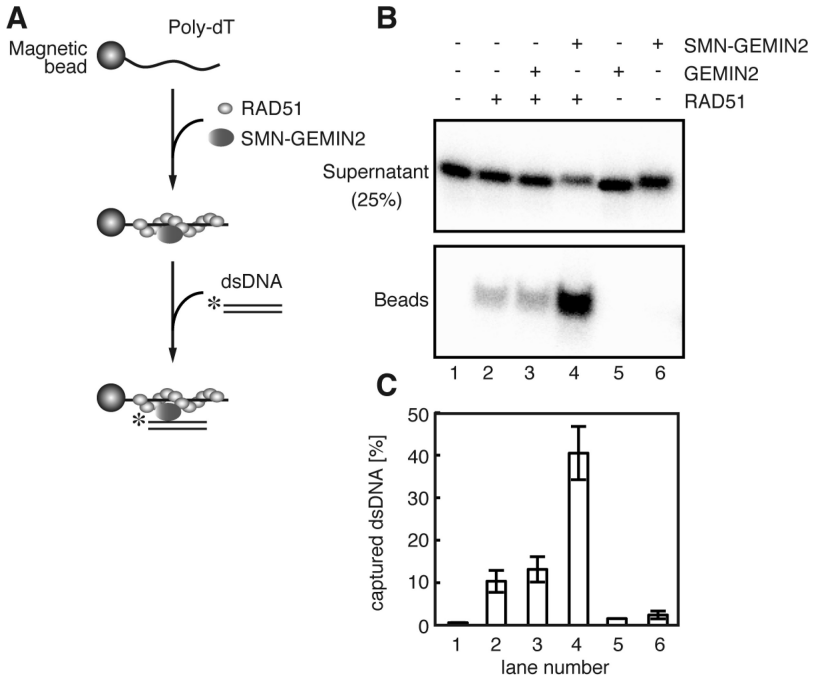

Figure 6. SMN−GEMIN2 enhances the secondary dsDNA capture by the RAD51−ssDNA complex. (A) Schematic representation of the secondary dsDNA capturing assay. (B) RAD51 (0.6 μM) was preincubated with GEMIN2 (1 μM) or SMN−GEMIN2 (1 μM) at 37 °C for 5 min. The samples were mixed with magnetic beads containing 83-mer poly dT ssDNA (6 μM) at 37 °C for 5 min. The reactions were then initiated by the addition of the ^{32}P-labeled dsDNA 49-mer (1.5 μM) and were continued at 37 °C for 10 min. The dsDNA captured by the RAD51−ssDNA complex was detected. Lane 1 indicates a negative control reaction without the proteins. Lanes 2−4 indicate the experiments with RAD51, RAD51 and GEMIN2, and RAD51 and SMN−GEMIN2, respectively. Lanes 5 and 6 indicate control experiments with GEMIN2 (1 μM) and SMN−GEMIN2 (1 μM), respectively, in the absence of RAD51. (C) Graphic representation of the experiments shown in panel B. The average values of three independent experiments are shown with the SD values.

pathway both *in vitro* and *in vivo*.[38] These findings suggested that GEMIN2 functions not only in spliceosomal snRNP assembly but also in HR. However, the role of SMN in this HR pathway has not been characterized.

In the present study, we successfully purified the SMN−GEMIN2 complex, in which SMN and GEMIN2 were physically connected by a linker peptide. We previously purified GEMIN2 alone[38] but were unable to purify SMN. Intriguingly, the SMN−GEMIN2 fusion protein efficiently complemented the growth-defective phenotype of the *GEMIN2*$^{-/-}$ cells, indicating that the SMN−GEMIN2 fusion is functional *in vivo*. Therefore, SMN−GEMIN2 may be a functional unit for DSB repair through the HR pathway.

We previously reported that purified GEMIN2 stimulates RAD51-mediated homologous pairing *in vitro*.[38] In the present study, we found that SMN−GEMIN2 also significantly stimulated the RAD51-mediated homologous pairing, to a much higher degree than that by GEMIN2 alone. SMN−GEMIN2 also enhanced the RAD51-mediated homologous pairing in the presence of RPA, which functions with RAD51 in HR repair *in vivo*. These results strongly suggested that SMN−GEMIN2 may be a novel complex that functions in the RAD51-mediated HR pathway for DSB repair. The enhanced RAD51-stimulation activity of SMN−GEMIN2 may be a consequence of the DNA-binding activity of SMN−GEMIN2. In fact, we found that SMN−GEMIN2 significantly enhances the secondary dsDNA capture by the RAD51−ssDNA complex, but GEMIN2 alone does not. Our previous study indicated that GEMIN2 alone binds to RAD51, but not to DNA.[38] SMN and GEMIN2 may exert the DNA-binding and RAD51-binding activities, respectively, in the SMN−GEMIN2 complex. GEMIN2 alone reportedly enhances RAD51−ssDNA complex formation,[38] and this may facilitate the RAD51-mediated homologous pairing to some extent. Therefore, during the homologous-pairing and strand-exchange processes, SMN−GEMIN2 may function in both stabilizing the RAD51 filament and facilitating the secondary dsDNA capture by the RAD51−ssDNA complex, through its RAD51-binding and DNA-binding activities.

In higher eukaryotes, a large number of RAD51-interacting proteins that exhibit mediator and/or activator functions in RAD51-mediated recombination reactions *in vitro* have been reported.[14−17] These factors may work together or have specific functions. In the present study, we found that the SMN−GEMIN2 complex is a novel RAD51-interacting factor, which functions as the activator in homologous pairing. Further studies will be required to elucidate the common and specific roles, such as the specificity for tissues, chromosomal loci, and cell cycle stages, of these RAD51 mediators and activators in the homologous recombinational repair pathway.

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■ ABBREVIATIONS

SMN, survival motor neuron; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; snRNP, small nuclear ribonucleoprotein; DSB, DNA double-strand break; IR, ionizing radiation; HR, homologous recombination; NHEJ, nonhomologous DNA end-joining; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; TAE, Tris-acetate-EDTA; TBE, Tris-boric acid-EDTA; BSA, bovine serum albumin; RPA, replication protein A; CD, circular dichroism; RT-PCR, reverse transcription polymerase chain reaction; RPMI, Roswell Park Memorial Institute.

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