Supporting Information

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SI Materials and Methods

Affinity Purification of Telomeric DNA-Binding Proteins. Nuclear matrix proteins responsible for maintaining chromosome territories were isolated as described (1) from rat liver. Telomere-binding proteins were isolated by affinity purification with biotin-labeled telomeric DNA oligonucleotides $(TTAGGG)_3$ conjugated to streptavidin-coated agarose beads. The purification was performed in the presence of yeast tRNA and sonicated salmon sperm DNA. Binding proteins were eluted from beads with 2 M NaCl. The affinity purified proteins were resolved on 12% SDS/PAGE. The band corresponding to a 28-kDa protein was excised from gel and subjected to tryptic digestion, followed by MALDI-TOF mass spectrometric analysis. The data obtained were submitted to the MS-Fit Web site (http://prospector.ucsf.edu/prospector/mshome.htm) to identify matching proteins.

hnRNP A2* cDNA Cloning and Protein Purification. DNase I–treated mRNA from rat liver was reverse-transcribed into cDNA using a poly-T primer. To specifically amplify the hnRNP A2* isoform, we used the exon exclusion method developed in our laboratory (2). An oligonucleotide (5′-GGTTTTGGAGATTCTCGAGGTGGCGGTGGCCC-3′) complementary to the sequence within exon 7 of hnRNP A2 was annealed to cDNA, and the annealed cDNA was cleaved by XhoI, which cuts at the double-stranded CTCGAG/GAGCTC site in the annealed exon 7 sequence. hnRNP A2* mRNA was then specifically amplified by RT-PCR with the primers 5′-TAGCTAGCATGGAGAGAGAAAAGGAA-3′ and 5′-AAGAGCTCTCAATATCGGCTCCTTCCA-3′ (2). The amplicon was cloned into the NheI/SacI site of pET-28b (Novagen) and transformed into the *E. coli* strain BL21 (DE3). Transformed cells were grown in LB medium at 37 °C for 4 h. After inducing protein expression with 1 mM isopropyl thiogalactoside for 2 h, the cells were harvested and lysed by sonication in one-pellet volume of buffer A [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 40 mM imidazole, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] at 4 °C. Affinity purification of His-hnRNP A2* was performed as instructed by manufacturer (HisTrap HP column; GE Healthcare). The N-terminal His tag was then cleaved with thrombin, and hnRNP A2* protein was repurified using HisTrap HP Column.

Preparation of rTR. The gene coding rTR was obtained by RT-PCR and cloned into pMD19-T plasmid (Takara Biotechnology). The plasmid was cleaved with Crf 13I, Rsa I, FspB I, and BamH I to produce rTR fragments with lengths of 96, 209, 269, and 419 nt, respectively. In vitro transcription was carried out using the T7 Transcription Kit (Fermentas). rTR fragments were then 5′ end labeled with T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP.

EMSA. ^{32}P-labeled oligonucleotides were incubated with 400 nM recombinant hnRNP A2* at 4 °C for 30 min in binding buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM KCl] and resolved on 8% (wt/vol) native PAGE gel. The gel was then visualized by phosphoimaging using a Typhoon PhosphorImager (Amersham Biosciences), and the binding activity was quantified using ImageQuant (Amersham Biosciences).

T4 Polymerase Hydrolysis Assay. ^{32}P-labeled oligonucleotides were incubated with 1.5 μM hnRNP A2* or POT1 at 4 °C for 15 min in the binding buffer. The digestion was performed at 37 °C for 1 min in the system containing 0.5 U of T4 polymerase (Takara Biotechnology) and 1× reaction buffer [330 mM Tris-acetate (pH 7.8), 100 mM $Mg(CH_3CO_2)_2$, 5 mM DTT]. The reaction was stopped by phenol-chloroform extraction, followed by ethanol precipitation. The products were resolved on 19% polyacrylamide gel with 7 M urea.

Preparation of Telomerase Extract. Cell lysate for telomerase assay was prepared as described (3).

Analysis of Telomerase Catalytic Activity. The analysis of catalytic activity of telomerase was carried out with TRAP (4) using either TS (5′-AATCCGTCGAGCAGAGTT-3′) or TSG4 (5′-GGGCTAGGGCTAGGGCTAGGGAGTT-3′) as a substrate. Telomerase extension was carried out in the absence or presence of different concentrations of hnRNP A2* or POT1. Substrates were extended by telomerase extract from 5,000 cells at 30 °C (TS) or 37 °C (TSG4) for 10 min. The reaction was stopped by heating the sample to 75 °C for 5 min. After phenol-chloroform extraction and ethanol precipitation extension products were amplified by PCR (33 cycles: 94 °C for 30 s; 59 °C for 30 s), in which TS and 5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′ were used to amplify the products from TS substrate, TS and 5′-GTGCCCTTACCCTTACCCTTACCTAA-3′ were used for TSG4 substrate. The PCR products were resolved on 12% native polyacrylamide gel, stained with ethidium bromide, and visualized on a ChemiImager 5500 (Alpha Innotech). Telomerase activity was calculated as described (4).

Analysis of Telomerase Processivity. A modified version of TRAP assay designed for analyzing telomerase processivity (5) was performed as described (6) using MTS as the substrate.

Preparation of Subcellular Fractions and Western Blot. Subcellular fractions were prepared as described (7) with minor modifications. Briefly, the cells were swelled at 4 °C for 10 min in hypotonic solution then lysed by passing through a 19.5-gauge needle five times. The nuclei were collected by centrifugation through a glycerol cushion. The collected nuclei were extracted with LIS buffer [10 mM LIS, 100 mM lithium acetate (LiAc), 1 mM EDTA, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, and 20 mM Hepes-KOH (pH 7.4)] for 10 min at room temperature, followed by centrifugation at 20,000 × *g* for 20 min. The supernatant and pellet were collected as nucleoplasmic and nuclear matrix fraction, respectively.

Telomerase Activity and mRNA Levels in Different Rat Tissues. Organs were taken immediately after male rats were killed. For telomerase activity assay, tissues were homogenized with a sterile Duall tissue grinder (Scientz, Ningbo, China) in CHAPS buffer [0.5% CHAPS, 10 mM Tris (pH 8.0), 1 mM $MgCl_2$, 0.1 mM PMSF (pH 8.0)] and incubated for 30 min on ice. The lysate was centrifuged at 12,000 × *g* for 30 min at 4 °C. The resulting supernatants were frozen in liquid nitrogen or stored at −80 °C until use. Protein concentration of the lysates was determined using Bradford assay. Total protein (200 ng) was used in each TRAP assay. Total RNA was isolated from tissues using an RNA purification kit (Promega), and 0.05 μg of RNA was used for RT-PCR.

Cell Culture. BRL-3A rat and HeLa cells were cultured at 37 °C under 5% CO_2 in DMEM (Gibco) medium supplemented with 10% FBS, penicillin G (100 units/mL), penicillin (100 units/mL), and streptomycin B (0.1 mg/mL).

Retroviral Vectors and Virus Production. KpnI and EcoRI sites were added to the ends of hnRNP A2* cDNA via PCR amplification and the amplified fragment was inserted into the KpnI/EcoRI sites of the pHM6 expression vector (Roche), resulting in a HA-hnRNP A2* fusion. The HA-hnRNP A2* segment was then amplified by the T7 promoter forward primer, 5′-TAATACGAGTCACTATAGGGA-3′, and the HM6 antisense primer, 5′-ATTTAGGTGACACTATAG-3′, and cloned into the pGEM-T vector (Promega). HA-hnRNP A2* could then be subcloned into the NotI-EcoRI sites of pQCXIN retroviral vector (for BRL-3A cells) or EcoRV-KpnI sites of pMSCV-IThy1-1 retroviral vector (for HeLa cells). The recombinant pQCXIN HA-hnRNP A2* vector was transfected into EcoPack 2-293 cells and pMSCV-IThy1-1 HA-hnRNP A2* was transfected together with pGag-pol and pMD.G-VSVG-env into 293T cells to produce retrovirus. Medium containing the virus was collected 48 h after transfection (8).

Stable Expression of HA-hnRNP A2* in Rat BRL-3A and HeLa Cells. BRL-3A and HeLa cells at about 70% confluence were exposed to a 1:1 mixture of virus-containing and fresh culture medium for 12 h. The cells were then cultured in fresh medium containing 800 μg/mL (for BRL-3A) or 300 μg/mL (for HeLa) G418 (Sigma). After 2 wk of selection, G418-resistant BRL-3A clones were isolated for continuous culturing, whereas the pool of G418-resistant HeLa cells was cultured under persistent selection stress (100 μg/mL G418) for about 2 mo (9).

Immunofluorescence Microscopy. BRL-3A rat cells grown on glass coverslips were fixed for 8 min at room temperature in PBS (pH 7.4) containing 2% paraformaldehyde and permeabilized for 8 min in PBST (0.5% Triton X-100 in PBS). Coverslips were then blocked with 10% serum (in PBS) from the same species as the secondary antibody for 20 min. Primary antibodies used included, goat (D-16; Santa Cruz) or rabbit (H-231; Santa Cruz) polyclonal anti-TERT, rabbit polyclonal anti-Coilin (H-300; Santa Cruz) (10), mouse monoclonal anti-HA (12CA5; Roche), rabbit polyclonal anti-hRAP1 (no. 765; gift from Titia de Lange, Rockefeller University, New York, NY) (11). Secondary antibodies included Cy3-conjugated donkey anti-goat, fluorescein-conjugated goat or donkey anti-mouse (Proteintech Group), and DyLight649-conjugated donkey anti-rabbit (Jackson ImmunoResearch) at the dilutions recommended by the manufacturers. The incubation time was 1 h for the primary antibody and 45 min for the secondary antibody at room temperature, followed by PBS washes (four times; 5 min each). Coverslips were mounted with VECTASHIELD Mounting Medium (Vector Laboratories) containing 0.5 μg/mL DAPI. Images were acquired with a Zeiss 510 META confocal microscope using a 100× or 63× oil objective (12).

Preparation of POT1. Recombinant human POT1 protein was prepared as described (13). The plasmid pET-14-POT1 (gift from Joachim Lingner, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) was transformed into the *E. coli* strain BL21 (DE3). The cells were grown in TB medium supplemented with 1% glucose and 0.05 mg/mL carbenicillin at 37 °C for 5 h. POT1 expression was induced with 5 μM isopropyl thiogalactoside for an additional 2 h at 25 °C. Cells were harvested and suspended in one pellet volume of buffer C [20 mM NaH_2PO_4 (pH 8.0), 200 mM NaCl, 0.2% Tween-20, 10 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol] containing 1 mg/mL of lysozyme. After sonication at 4 °C, the lysate was centrifuged at 20,000 × *g* for 20 min, and the supernatant was loaded on an affinity HisTrap HP columns (GE Healthcare). After washing with buffer C for 10-column volume, the His-POT1 was eluted with buffer C containing 400 mM imidazole. Purified proteins were dialyzed against buffer D [20 mM NaH_2PO_4 (pH 8.0), 50 mM NaCl, 0.2% Tween-20, 20% glycerol, 5 mM β-mercaptoethanol] and stored at −70 °C.

Analysis of G-Quadruplex Unfolding by hnRNP A2*. Oligonucleotide $GGG(TTAGGG)_3$ labeled with a fluorescein (FAM) (donor) at the 5′ end and a tetramethylrhodamine (TMR) (acceptor) at the 3′ end was mixed with 200 nM hnRNP A2* or 1 μM cDNA $CCC(TAACCC)_3$ in Tris-EDTA (TE) buffer (pH 8.0) containing 150 mM KCl. The kinetics of G-quadruplex opening upon addition of hnRNP A2* or cDNA was immediately monitored at 25 °C by recording the donor fluorescence at 515 nm using a slit of 5 nm.

PCR Amplification of hnRNP A2* in Rat, Mouse, and Human Cells. PCR was conducted in 25-μL volume with cDNA transcribed using a poly(T) primer from 0.2 μg of RNA of rat BRL-3A cells, HeLa cells, and mouse liver, respectively. Amplification was carried out with an initial denaturation at 94 °C for 2 min, followed by 32 cycles (29 cycles for rat samples) of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s.

Telomerase Pull-Down by hnRNP A2*. Telomerase pull-down was conducted as described (14) with minor modifications. BRL-3A cells (1×10^6) were lysed in 100 μL of CHAPS [0.5% CHAPS, 10 mM Tris (pH 8.0), 1 mM $MgCl_2$, 0.1 mM PMSF] buffer. A total of 30 μg of recombinant His-tagged hnRNP A2* was immobilized to 5 μL of nickel resin. The beads were blocked with 1% BSA at 4 °C for 1 h, followed by two washes with a cold CHAPS buffer, and 100 μL of cell lysate and 100 μL of CHAPS buffer were added and incubated at 4 °C for 2 h. After three washes with cold CHAPS buffer, the beads were used to detect telomerase activity using the TRAP method. Untreated beads and cell extract pretreated with 200 μg/mL RNase A were used as controls.

Telomeric Restriction Fragment Measurement. The average length of telomeric restriction fragment (TRF) was measured as described (15).

siRNA Knockdown of TERT in Rat Cells. A short interfering (si)RNA duplex targeting rat TERT sequence (GCCAGCATGTTAGGAAGAA) was provided by RiboBio. Nontargeting siRNA (RiboBio) was used as a control for off-target effects caused by RNA interference. Subconfluent cells were incubated with 50 nM siRNA in 2 mL of medium containing Lipofectamine 2000 (Invitrogen) in six-well plates on coverslips. Cells were then examined for the expression of TERT protein using immunofluorescence microscope as described above.

1. Ma H, Siegel AJ, Berezney R (1999) Association of chromosome territories with the nuclear matrix. Disruption of human chromosome territories correlates with the release of a subset of nuclear matrix proteins. *J Cell Biol* 146(3):531–542.
2. Wang F, Zhao Y, Hao YH, Tan Z (2008) Identification of low-abundance alternatively spliced mRNA variants by exon exclusive reverse transcriptase polymerase chain reaction. *Anal Biochem* 383(2):307–310.
3. Kim NW, et al. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266(5193):2011–2015.
4. Kim NW, Wu F (1997) Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res* 25(13):2595–2597.
5. Szatmari I, Aradi J (2001) Telomeric repeat amplification, without shortening or lengthening of the telomerase products: A method to analyze the processivity of telomerase enzyme. *Nucleic Acids Res* 29(2):E3.
6. Xue Y, et al. (2007) Human telomeric DNA forms parallel-stranded intramolecular G-quadruplex in K+ solution under molecular crowding condition. *J Am Chem Soc* 129(36):11185–11191.
7. Ludérus ME, et al. (1996) Structure, subnuclear distribution, and nuclear matrix association of the mammalian telomeric complex. *J Cell Biol* 135(4):867–881.
8. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci USA* 102(37):13212–13217.

9. Moore MJ, et al. (2004) Retroviruses pseudotyped with the severe acute respiratory syndrome coronavirus spike protein efficiently infect cells expressing angiotensin-converting enzyme 2. *J Virol* 78(19):10628–10635.
10. Whittom AA, Xu H, Hebert MD (2008) Coilin levels and modifications influence artificial reporter splicing. *Cell Mol Life Sci* 65(7-8):1256–1271.
11. Scherthan H, et al. (2000) Mammalian meiotic telomeres: Protein composition and redistribution in relation to nuclear pores. *Mol Biol Cell* 11(12):4189–4203.
12. Tanaka H, et al. (2005) DNA damage-induced phosphorylation of the human telomere-associated protein TRF2. *Proc Natl Acad Sci USA* 102(43):15539–15544.
13. Kelleher C, Kurth I, Lingner J (2005) Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol Cell Biol* 25(2):808–818.
14. Eversole A, Maizels N (2000) In vitro properties of the conserved mammalian protein hnRNP D suggest a role in telomere maintenance. *Mol Cell Biol* 20(15):5425–5432.
15. Zhao Y, et al. (2009) Telomere extension occurs at most chromosome ends and is uncoupled from fill-in in human cancer cells. *Cell* 138(3):463–475.

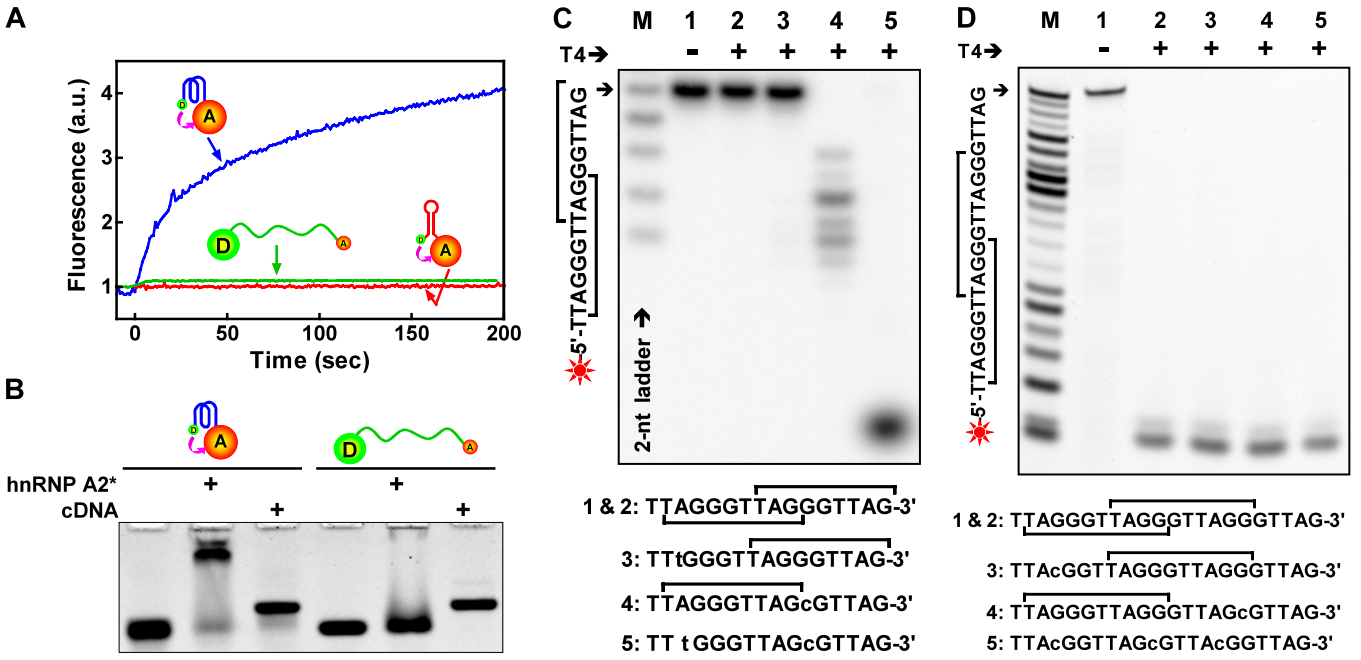

Fig. S1. hnRNP A2* unfolds telomere G-quadruplex specifically. (*A*) Changes in fluorescence resonance energy transfer (FRET) in G-quadruplex (blue), relaxed linear random DNA (green), and hairpin structure (red) upon addition of hnRNP A2*. (*B*) Electrophoresis mobility shift of G-quadruplex (blue) and relaxed linear random (green) DNA upon binding with hnRNP A2* or cDNA (cDNA). (*C*) POT1 binds to the MBS at the very 3′ end of telomere DNA and masks the end. The 5′ ^{32}P-labeled DNA was incubated with POT1 before digestion with T4 polymerase (T4), which trims off nucleotides from the 3′ end. Lowercase letters indicate mutations introduced to manipulate the number and position of MBS. Square bracket indicates MBS. (*D*) T4 polymerase (T4) fully digests telomeric DNA in the absence of hnRNP A2*. The first lane shows 1-nt ladder marker obtained by digesting TTAGGGTTAGGGTTAGGGTTAG with DNase I. Other conditions were the same as in Fig. 3*B*.

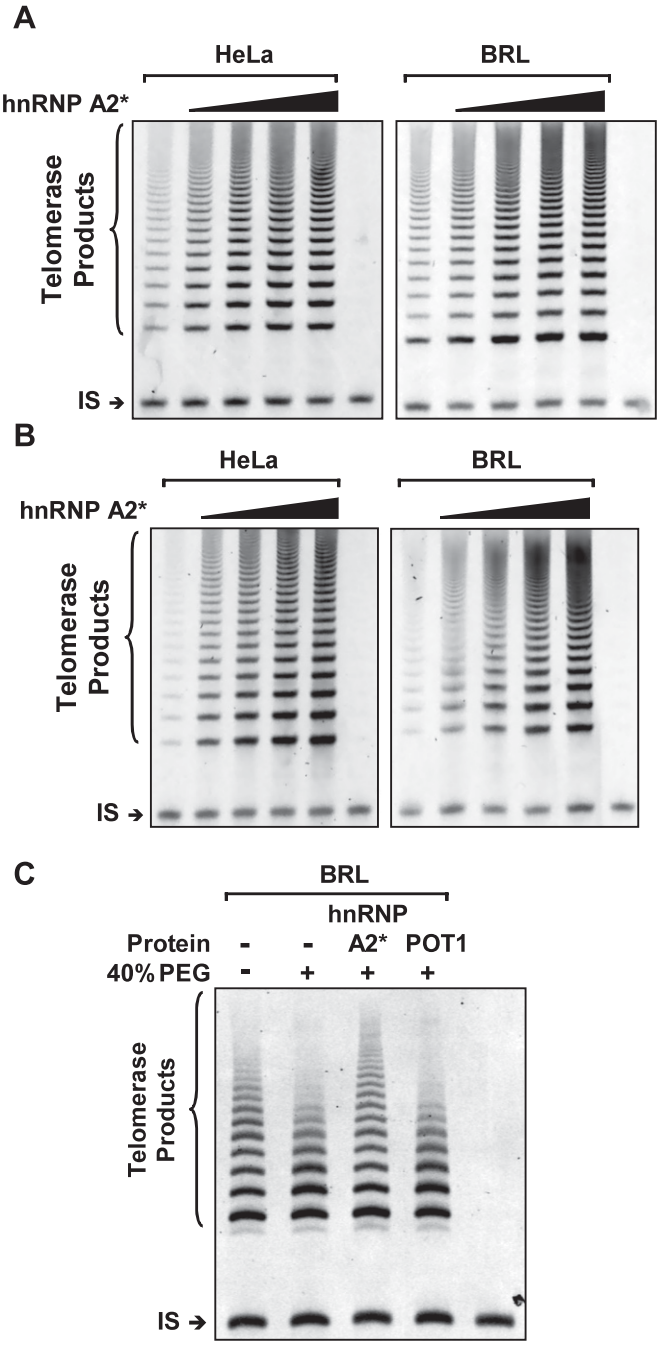

Fig. S2. (*A*) hnRNP A2* enhances the catalytic activity of telomerase from HeLa and BRL rat cells. Activity assayed by TRAP using a nontelomeric TS substrate. See Fig. 3*D* for hnRNP A2* concentrations. Last lane, telomerase-negative. (*B*) hnRNP A2* enhances the catalytic activity of telomerase from HeLa and BRL rat cells. Activity was assayed by TRAP using TSG4 substrate that forms G-quadruplex structure before being extended. See Fig. 3*E* for hnRNP A2* concentrations. Last lane, telomerase-negative. (*C*) hnRNP A2* enhances the extension processivity of telomerase from BRL rat cells. Processivity was assayed by a modified TRAP method using nontelomeric MTS substrate in the presence of 40% (wt/vol) PEG 200. Last lane, telomerase-negative. IS, internal standard.

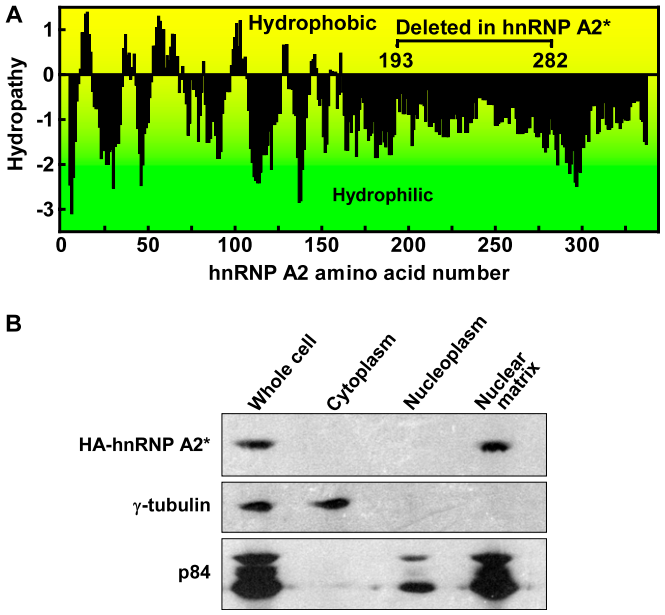

Fig. S3. (*A*) Hydropathy plot of hnRNP A2 showing region deleted in hnRNP A2*. (*B*) Western blot of hnRNP A2* in different subcellular fractions of rat cells. HA-hnRNP A2* was expressed in rat cells and detected with anti-HA antibody. γ-Tubulin and p84 are markers for cytosol (1) and nuclear matrix (2), respectively.

1. Moudjou M, Bordes N, Paintrand M, Bornens M (1996) gamma-Tubulin in mammalian cells: The centrosomal and the cytosolic forms. *J Cell Sci* 109(Pt 4):875–887.
2. Durfee T, Mancini MA, Jones D, Elledge SJ, Lee WH (1994) The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. *J Cell Biol* 127(3):609–622.

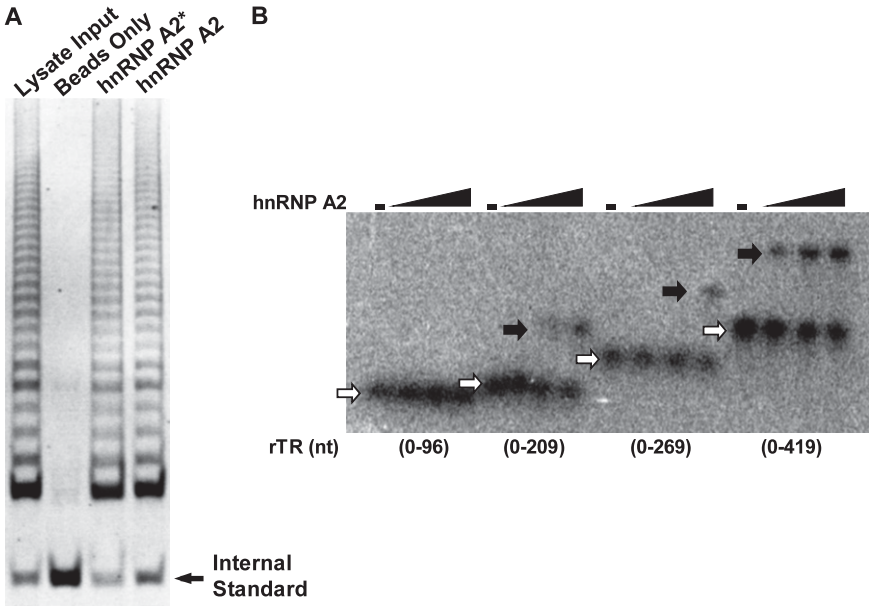

Fig. S4. (*A*) TRAP assay of telomerase activity pulled down from rat cell lysate using hnRNP A2* or hnRNP A2 immobilized on nickel beads. (*B*) Binding to the RNA component of rTR of hnRNP A2 at increasing concentrations (0, 0.25, 0.5, 1 μM) examined by EMSA. The solid arrow shows rTR/hnRNP A2 complex, and the open arrow shows free rTR. Assay conditions were the same as in Fig. 4 *A* and *B*, respectively.

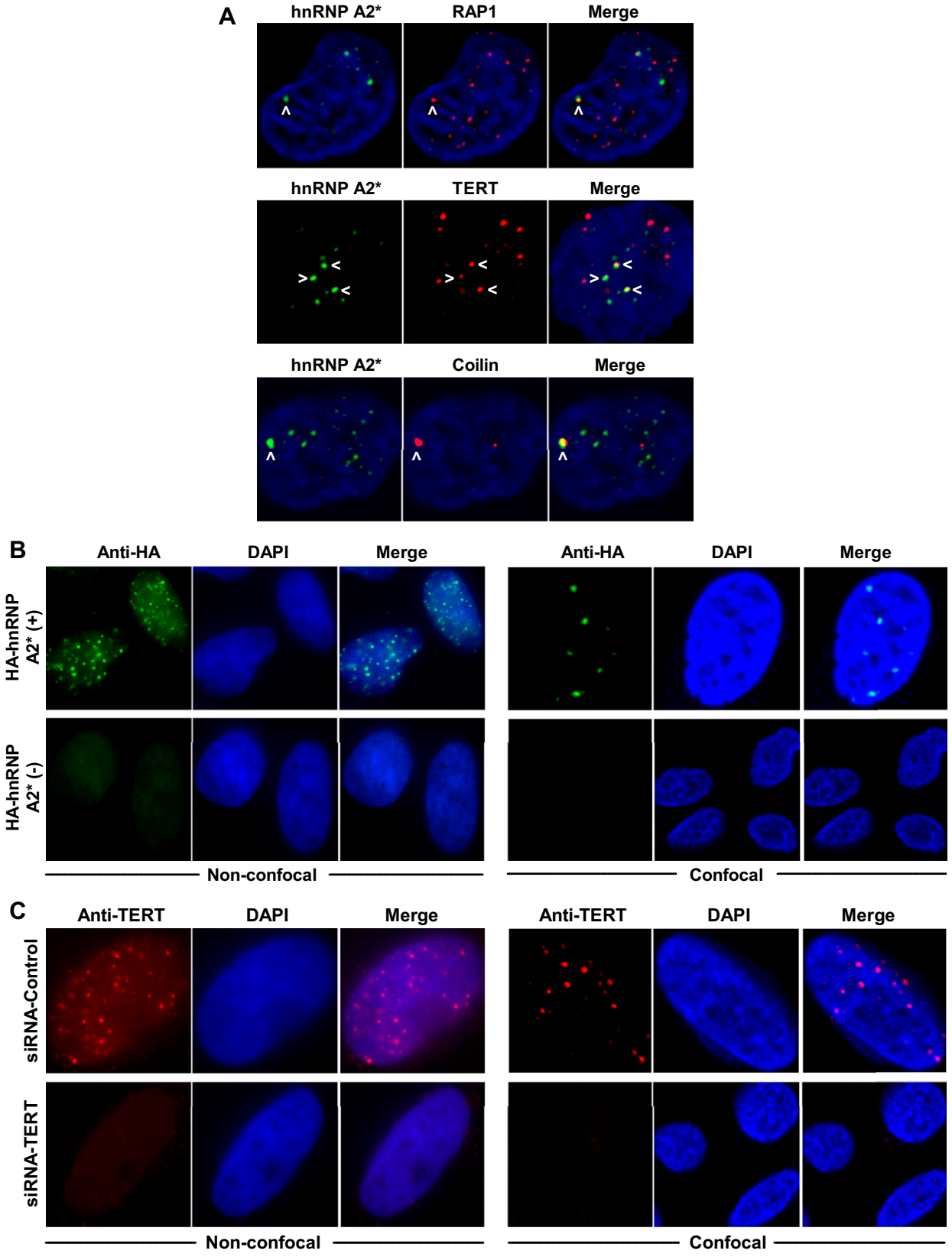

Fig. S5. (*A*) Colocalization of hnRNP A2* with RAP1, TERT, and Coilin in cultured rat cells detected by immunofluorescence. RAP1 and Coilin are markers for telomere and Cajal body, respectively. (*B*) Immunofluorescent staining of cultured HA-hnRNP A2*–expressing and HA-hnRNP A2*–negative rat cells using anti-HA antibody. (*C*) Immunofluorescence detection of rat TERT is suppressed by siRNA against TERT. Representative images were obtained from three independent experiments. The same exposure time was used to take all pictures. *B* and *C* include pictures of cells visualized with both a regular fluorescent microscope (to avoid false-negative results) and with a confocal microscope.

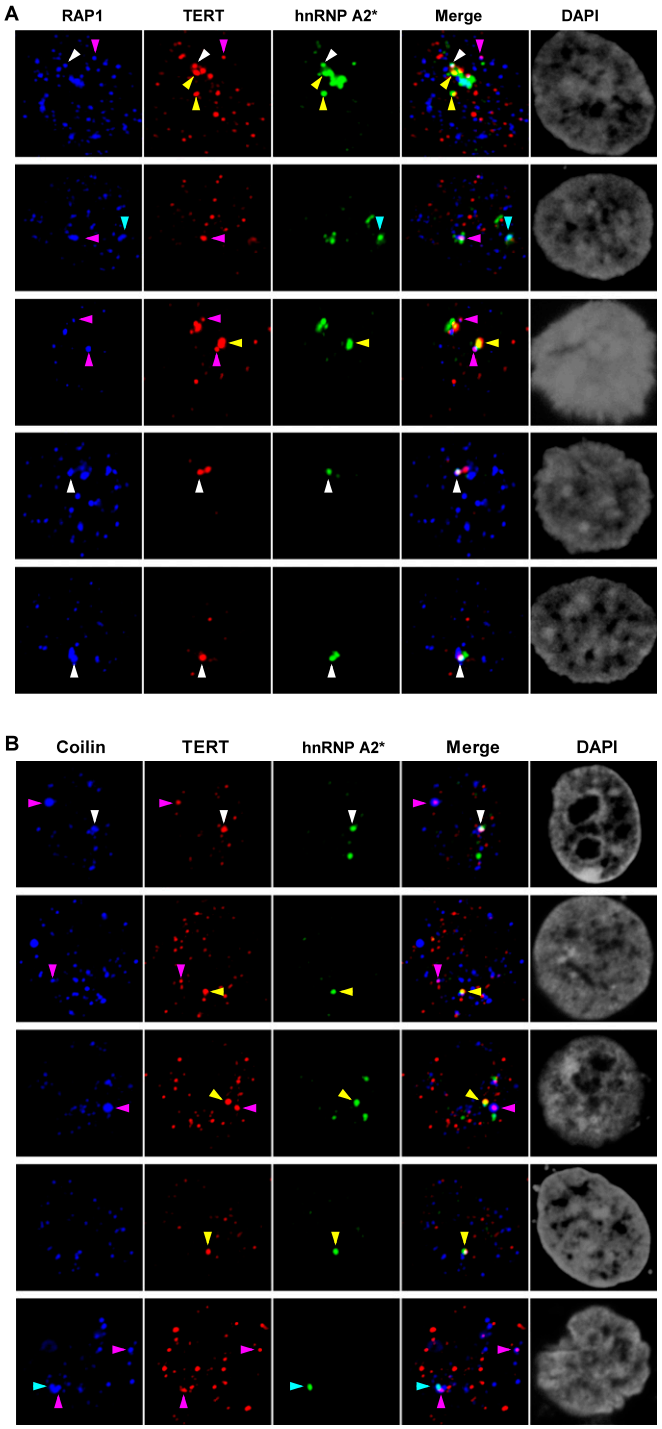

Fig. S6. (*A*) Representative colocalizations observed among RAP1, TERT, and hnRNP A2* by three-color immunofluorescence in cultured rat cells. RAP1 is a marker for telomeres. (*B*) Representative colocalizations observed among Coilin, TERT, and hnRNP A2* by three-color immunofluorescence in cultured rat cells. Coilin is a marker for Cajal bodies.

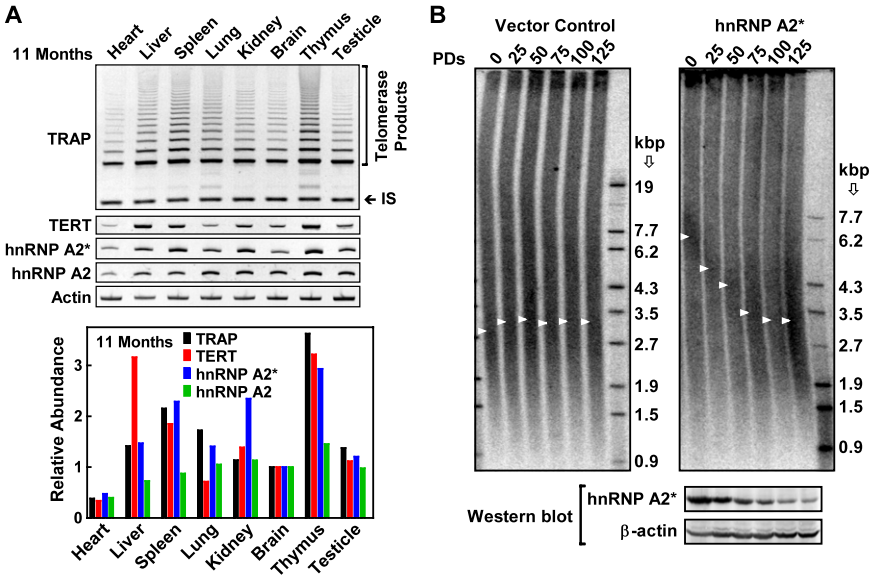

Fig. S7. (*A*) Correlation of expression of hnRNP A2* with that of hnRNP A2, TERT, and activity of telomerase in 11-mo-old rat tissues. Expression of hnRNP A2*, hnRNPA2, and TERT was analyzed by RT-PCR. Telomerase activity was assayed by TRAP. Relative abundance was obtained by normalizing band intensity to actin and then to brain. IS, internal standard. (*B*) Overexpression of hnRNP A2* increases telomere length in a concentration-dependent manner in rat cells. The density peak of each lane is indicated by a white arrowhead. HA-hnRNP A2* expression was assessed by Western blot. Other conditions were the same as in Fig. 5*B*.

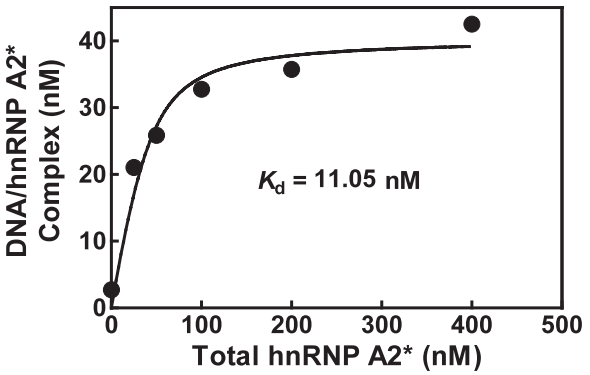

Fig. S8. Dissociation constant (K_d) of hnRNP A2* to its MBS. ^{32}P-labeled 5′-TAGGGTTAGG-3′ was incubated with increasing concentrations of hnRNP A2* and DNA/hnRNP A2* complex was separated from free DNA by EMSA.

Table S1. MALDI-TOF mass spectrometry of hnRNP A2*

m/z submitted	MH$^+$ matched	ΔDa	Start	End	Peptide sequence	Modifications
727.3752	727.4466	−0.0714	78	83	(R) VVEPKR (A)	
781.4059	781.3593	0.0466	298	305	(K) SGNFGGSR (N)	
1,087.4952	1,087.4849	0.0103	27	34	(R) NYYEQWGK (L)	
1,087.4952	1,087.4681	0.0271	35	42	(K) LTDCVVMR (D)	1PO4 1Cys-am
1,188.6464	1,188.6476	−0.0012	126	135	(K) IDTIEIITDR (Q)	
1,338.6715	1,338.7018	−0.0302	88	100	(R) EESGKPGAHVTVK (K)	
1,695.7682	1,695.7655	0.0028	142	156	(R) GFGFVTFDDHDPVDK (I)	
1,798.9305	1,798.9227	0.0077	11	26	(K) LFIGGLSFETTEESLR (N)	
1,879.9572	1,879.9666	−0.0094	102	117	(K) LFVGGIKEDTEEHHLR (D)	
2,220.0923	2,220.0712	0.0211	118	135	(R) DYFEEYGKIDTIEIITDR (Q)	