SUPPORTING ONLINE MATERIALS

MATERIALS AND METHODS

Dyskerin complex purification

Dyskerin with an N-terminal AH3 tag was expressed in and purified from suspension HeLa S3 cells for mass spectrometry analysis as previously described (S*1*). To "replace" endogenous dyskerin with AH3-dyskerin, HeLa S3 cells were first transduced with a retrovirus expressing AH3-dyskerin in the pMGIB vector and then subsequently transduced with the sh-dyskerin-B knockdown vector. Note that the AH3-dyskerin cDNA contains four silent mutations in the region targeted by the sh-dyskerin-B vector (see below for details). For complex purification, 15 L of HeLa-AH3-dyskerin^{+shRNA} cells were grown to a density of 5.5 x 10^5 cells/mL at >95% viability in Joklik's MEM/5% newborn calf serum/1% penicillin-streptomycin. Nuclear extracts dialyzed to 150 mM KCl were bound to rabbit IgG resin (Sigma), washed, and eluted with TEV protease. TEV eluants were precleared with mouse IgG resin and then bound again to 12CA5 anti-HA resin. After washing, both mouse IgG (negative control) and anti-HA resins were eluted with 3.5M $MgCl_2$ and precipitated using methanol-chloroform for mass spectrometry analysis. All steps were performed at 4 °C.

Mass spectrometry

Purified dyskerin complexes were fractionated by SDS-PAGE and stained with Coomassie blue. Gel slices from the entire lane were trypsin-digested and extracted peptides were then analyzed by nanoflow reversed-phase liquid chromatography (RPLC)-tandem mass spectrometry (MS/MS) as described previously (S*1*). In brief, the nanoflow RPLC column was coupled online to a linear ion-trap (LIT)-mass spectrometer (ThermoElectron, San Jose, CA) using a nanoelectrospray source with an applied electrospray potential of 1.5 kV and capillary temperature of 160 °C. The LIT-mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by five MS/MS scans, where the five most abundant molecular ions were dynamically selected for MS/MS using a normalized collisional-induced dissociation (CID) energy of 35%. Dynamic exclusion was utilized to minimize redundant MS/MS acquisition. The CID spectra were analyzed using SEQUEST against a UNIPROT derived human proteome database downloaded from the European Bioinformatics Institute. Scaffold-01_07_00 (Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

Small scale cell culture and retroviral transductions

Adherent HeLa cultures were grown in DMEM/5% newborn calf serum/1% penicillin-streptomycin (PS); 293T cultures were grown in DMEM/10% bovine growth serum/1% PS. Retroviruses were generated from 293T cells by cotransfecting plasmids encoding RSV(Gag+Pol), VSV-G, and the retroviral expression or shRNA plasmids (S*2*) by calcium phosphate precipitation. To generate "replacement" cell lines, HeLa cells were first transduced with N-terminal Flag-tagged shRNA-resistant expression constructs in the pMGIB vector, selected with blasticidin S, transduced with the corresponding LMP shRNA vector, and finally selected with puromycin. For shRNA vectors, hairpin sequences were amplified, ligated, and sequenced as previously described (S*2*). Template sequences were: NAF1 A – 5'- tgctgttgacagtgagcgctgggatggtttcaagtattatt

agtgaagccacagatgtaataatacttgaaaccatcccaatgcctactgcctcgga-3'; TCAB1 A – 5'-tgctgttgacagtgagcgaggttcctgcatcttgaccaattagtgaagccacagatgtaattggtcaagatgcaggaaccgt gcctactgcctcgga-3'; TCAB1 B – 5'-tgctgttgacagtgagcgcgccgggagaacccgattcatatagtgaa gccacagatgtatatgaatcgggttctcccggcttgcctactgcctcga-3'; TCAB1 E – 5'-tgctgttgacagtgag cgcggtgataccatctatgattactagtgaagccacagatgtagtaatcatagatggtatcaccttgcctactgcctcgg-3'. Dyskerin and pontin shRNA sequences were previously described (S*1*). To generate expression cDNAs for NAF1, dyskerin, and TCAB1 that were not targeted by the shRNA vectors, the following silent point mutations were introduced (mutations are shown in red): (1) sh-NAF1-A targets 5'-gggatggtttcaagtatta-3', which was changed to 5'-ggcatggtaagctcgatca-3'; (2) sh-dyskerin-B targets the sequence 5'- gctgaagaatttcttatca-3', which was changed to 5'-gctgaagagttcttaatca-3'; and (3) shTCAB1-A targets the sequence 5'-gttcctgcatcttgaccaa-3', which was changed to 5'- gaagttgtattctaacaaa-3'.

Immunoprecipitations, western blots, and northern blots

Cells were lysed in NP40 buffer (25 mM HEPES-KOH, 150 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.5% NP40, 5 mM 2ME, pH 7.5 supplemented with protease inhibitors) for 10 min on ice. Extracts clarified by centrifugation for 16,000 g for 10 min were quantified by Bradford assay. Flag-tagged proteins were immunoprecipitated/immunodepleted with 10-15 uL M2 anti-Flag resin (Sigma), and endogenous proteins were immunoprecipitated/depleted with 1 ug affinity-purified antibody per 1 mg extract supplemented with 10 uL protein A resin (Sigma) for 1-2 hr at 4 °C. Resins were then washed 5 times for 10 min each with 1 mL NP40 buffer. Where indicated, RNase A was included during the immunoprecipitation at 0.1 mg/mL. For immunoprecipitation-TRAP assays, resins were preblocked in 5% nonfat dry milk at least 30 min prior to use.

For western blots, samples were boiled in Laemmli sample buffer, and fractionated by SDS-PAGE. Gels were transferred to nitrocellulose filters (Whatman S&S), blocked in 2.5% nonfat dry milk in TBST for 30 min, and incubated in primary antibody overnight at 4 °C. Primary antibodies were used at the following: Flag M2 – 100 ng/mL; TCAB1 – 20 ng/mL; NAF1 – 20 ng/mL; pontin – 10 ng/mL; TERT – 300 ng/mL; dyskerin (serum; (S*3*)) – 1:30,000; α-tubulin (DM1A; Sigma) – 1:150,000. Anti-TERT and anti-pontin antibodies were validated previously (S*1*). The next day, filters were washed 3 times for 10 min each in TBST, incubated for 1 hr in 1:10,000 peroxidase-conjugated secondary antibody (Jackson Immunoresearch), washed again 5 times for 5 min each in TBST and developed with ECL+ (Amersham).

For northern blots, equal portions for each immunoprecipitation (into which a recovery control RNA was spiked to control for differential recovery in subsequent steps) was extracted with phenol:chloroform:isoamyl alcohol and precipitated with glycogen carrier. The recovery control RNA is a fragment of TERC (bases 1-170) generated by *in vitro* transcription using T7 RNA polymerase. The template for *in vitro* transcription was a PCR product using T7 forward (5'-gtaatacgactcactatagggc-3') and hTR-170 reverse (5'-aacgaattcgtttgctctagaatgaacgg-3') primers and the pCMV-hTR500 plasmid (a kind gift from K. Collins). RNA pellets were boiled in formamide loading buffer, loaded onto 5% polyacrylamide-8 M urea gels, transferred to Hybond N+ (Amersham), and hybridized with ^{32}P-α-dCTP labeled full-length TERC, U3, or U1 probe in Ultrahyb (Ambion) as previously described (S*1*). For all other RNAs, ^{32}P-γ-ATP end-labeled antisense DNA oligonucleotides were used as northern probes. For total RNA analysis, 2 ug of Trizol (Invitrogen) extracted RNA was used.

Polyclonal antibody production

ORFs for full-length NAF1 and TCAB1 were cloned into pMAL-c2 and pGEX6P-3 vectors to make recombinant MBP and GST tagged versions, respectively. All fusions were expressed in BL21(DE3) bacteria, induced for 4 hr with 0.3 mM IPTG at 32 °C, and produced soluble proteins. MBP fusions, purified using standard amylose affinity chromatography (NEB) and competitive maltose elution, were injected into rabbits for polyclonal antibody production (Covance). All antibodies were affinity-purified. For this step, the corresponding GST fusions were expressed and crosslinked to glutathione resin (Amersham) using disuccinimidyl suberate (DSS; Pierce). Serum was passed over the glutathione resin, washed, and eluted using 0.1 M glycine, pH 2.5.

TRAP assays

TRAP assays used PAGE-purified oligonucleotides and were used based on the original description (S*4*), but instead used ^{32}P-γ-ATP labeled TS primer and incorporated an internal control template and reverse primer (template: 5'-aatccgtcgagcagagttaaaaggccgagaagcgat-3'; rev: 5'-atcgcttctcggcctttt-3').

Immunofluorescence

HeLa cells grown on coverslips were fixed with 4% paraformadehyde in PBS, permeabilized with PBS/0.2% Triton-X100, and blocked in PBS/1% BSA. Primary antibodies (dyskerin (RU8 serum; gift from U. T. Meier) – 1:1000, TCAB1 – 20 ng/mL, p80-coilin (Abcam) – 20 ng/mL, HA (3F10; Roche) – 20 ng/mL) were incubated for 1 hr in PBS/1% BSA. Coverslips were washed 5 times with PBS for 2 min each. Secondary antibodies (at 1:1,000 dilutions and pre-adsorbed to prevent species crossreactivity; Jackson Immunoresearch) were incubated for 1 hr in PBS/1% BSA. Coverslips were again washed 5 times with PBS for 2 min each and mounted using Vectashield + DAPI (Vector labs).

TERC fluorescence in situ hybridization

A cocktail of three fluorescently-labeled antisense probes recognizing distinct regions of TERC were used to detect endogenous TERC in HeLa cells as previously described (S*5, S6*). For analysis of TERC/telomere associations, S-phase cells were enriched by double thymidine arrest followed by a four hr release as described (S*6*). The combined FISH/IF analysis was performed as described (S*6, S7*), and anti-p80-coilin and anti-TRF2 antibodies were used to mark Cajal bodies and telomeres, respectively.

Telomere length analysis

HTC75 cell cultures were grown in DMEM/10% bovine calf serum/1% PS. After transduction with TCAB1 shRNA retroviruses or an empty shRNA vector control, each cell line was maintained in puromycin selection (2 ug/mL). After approximately 30 population doublings, each cell line was re-transduced with its original shRNA vector (or empty vector control) to maintain knockdown. Isolated genomic DNA was digested with RsaI and HinfI and fractionated as described previously (S*8*). Membranes were prepared by Southern transfer and hybridized to a radioactively end-labeled $(ttaggg)_4$ oligonucleotide probe as described previously (S*9*).

SUPPLEMENTAL FIGURES

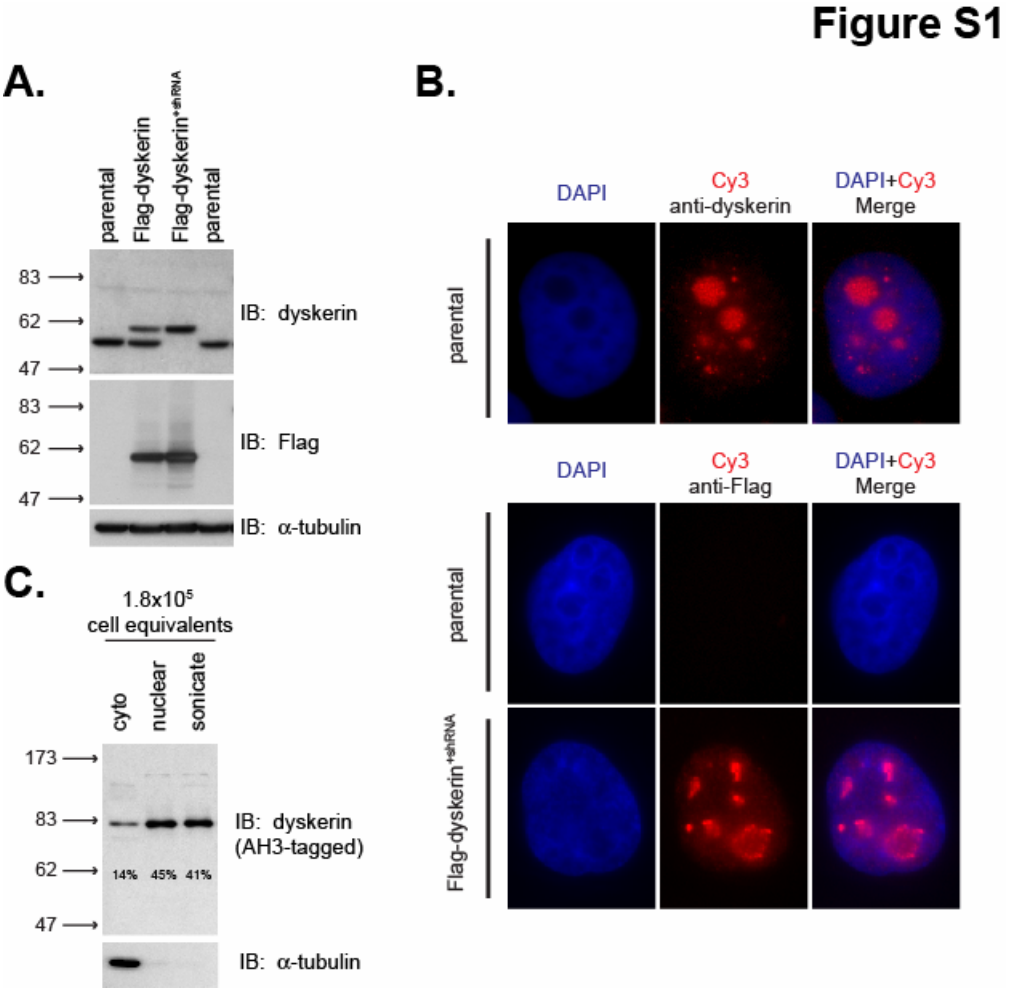
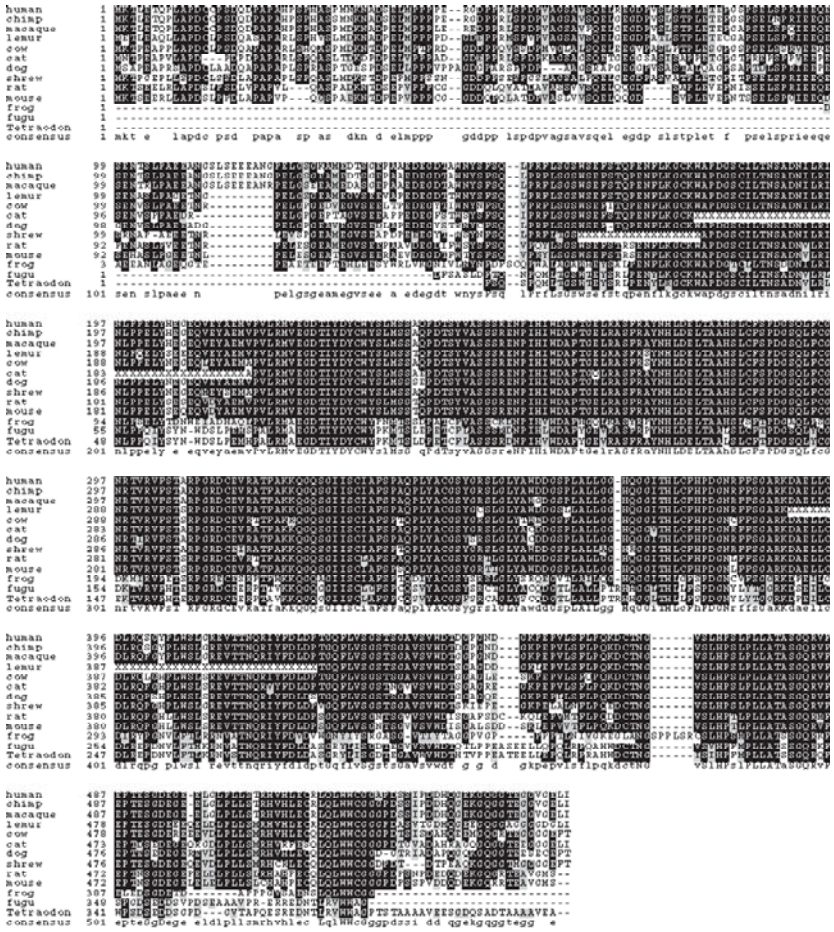


Fig. S1. Substitution of endogenous dyskerin for epitope-tagged dyskerin. (**A**) N-terminal Flag-tagged dyskerin expressed from a retrovirus (lanes 2 and 3, higher molecular weight dyskerin band) under-accumulates relative to endogenous dyskerin (lower molecular weight dyskerin band) in HeLa cells. Subsequent transduction of Flag-dyskerin expressing cells with shRNA targeting endogenous dyskerin results in up-regulation of Flag-dyskerin protein to levels indistinguishable from endogenous dyskerin levels (referred to as "Flag-dyskerin^{+shRNA}"; compare intensity of Flag-dyskerin to endogenous dyskerin in lanes 3 and 4, respectively). Note that the Flag-dyskerin coding sequence contains silent mutations such that it will not be targeted by the shRNA vector. (**B**) Subcellular localization of Flag-dyskerin^{+shRNA} revealed by immunofluorescence with anti-Flag antibodies (bottom row), is indistinguishable from the localization of endogenous dyskerin in HeLa cells (top row). Anti-Flag antibody staining in parental HeLa cells was used as a negative control. (**C**) Biochemical fractionation strategy in HeLa cells expressing AH3-dyskerin^{+shRNA}. Consistent with immunofluorescence patterns shown in Fig. S1B, cytosolic extracts contain only 14% of soluble AH3-dyskerin, while nuclear AH3-dyskerin protein pools liberated using salt extraction and subsequent sonication contain over 80% of soluble AH3-dyskerin. The cytoplasm-enriched protein α-tubulin was monitored to check the quality of the fractionation.

Figure S2

A.



B.

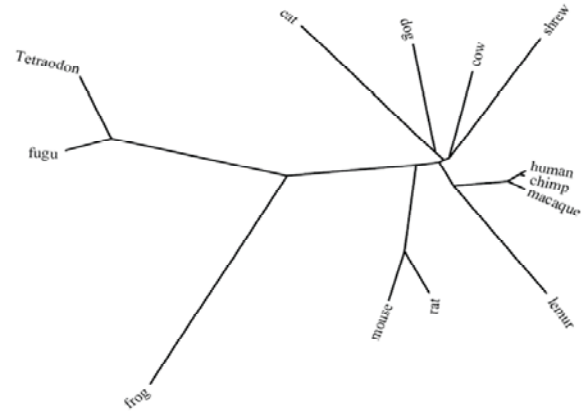


Fig. S2. TCAB1 is conserved in vertebrates. (**A**) Multiple sequence alignment of TCAB1 from sequenced genomes of vertebrate species in the Ensembl database, as of October 2008: chimp (*Pan troglodytes*), macaque (*Macaca mulatta*), lemur (*Microcebus murinus*), cow (*Bos taurus*), cat (*Felis catus*), dog (*Canis familiaris*), shrew (*Sorex araneus*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), frog (*Xenopus tropicalis*), fugu (*Takifugu rubripes*), Tetraodon (*Tetraodon nigroviridis*). Positions with highly conserved identity are shaded in black, and positions with high similarity are shaded in gray. (**B**) Phylogenetic tree of species aligned in (A).

Figure S3

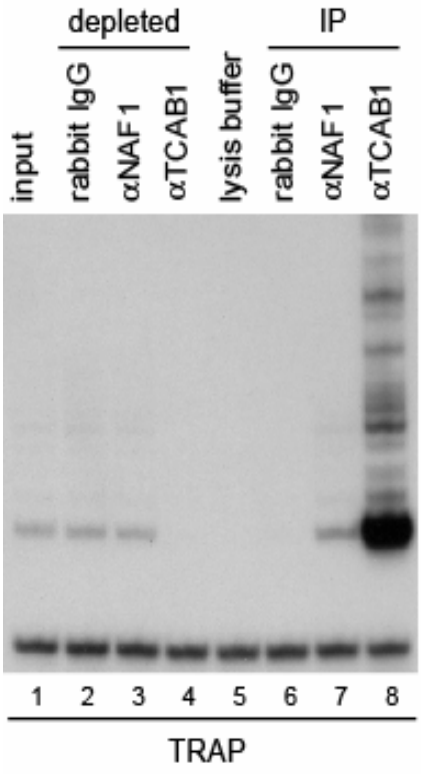


Fig. S3. Endogenous TCAB1 is associated with nearly all telomerase activity in cell extracts. Telomerase activity was assessed using TRAP assays in HeLa cell extracts before and after immunoprecipitation using rabbit IgG, anti-NAF1 antibodies, or anti-TCAB1 antibodies (lanes 1-4). Immunoprecipitations were assayed for telomerase activity in parallel (lanes 6-8). This is a lighter exposure of the TRAP assay displayed in Fig. 2B.

Figure S4

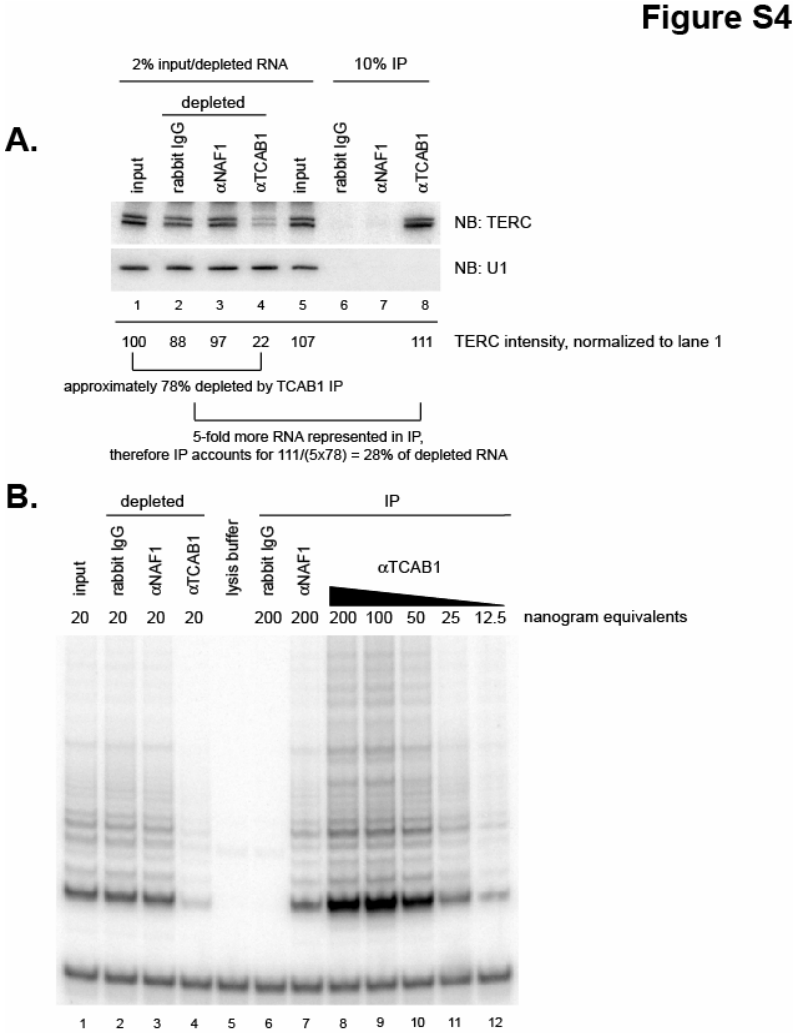


Fig. S4. Quantification of TERC and telomerase activity by endogenous TCAB1 immunoprecipitation. TERC and telomerase activity depleted by immunoprecipitation (IP) of TCAB1 from HeLa cell extracts were compared to the amount contained in the IP. (**A**) Northern blot for TERC in the TCAB1-immunodepleted extract compared with the TCAB1 IP. Two percent of input and depleted samples (lanes 1-5) were compared to ten percent of each IP (lanes 6-8). Band intensities were quantified by phosphorimager and ImageQuant software. RNAs bound to TCAB1 in the IP were recovered by elution, organic extraction, and precipitation. The precipitated and eluted TERC in the TCAB1 IP (lane 8) accounts for approximately 28% of TERC depleted from the extract (lane 4). (**B**) TRAP assay to compare telomerase activity in the TCAB1-immunodepleted extract relative to activity present in the TCAB1 IP. One mg of cell extract was immunodepleted using anti-TCAB1 antibodies. Twenty ng of extract was assayed in the input and depleted samples (lanes 1-4). For comparision, dilutions of each immunoprecipitated and immobilized complex was diluted (expressed as ng equivalents, lanes 6-8). Serial 1:1 dilutions of the TCAB1 IP on beads (lanes 9-12) were assayed in parallel. TRAP assay signals were detected using a phosphorimager. Levels of telomerase activity depleted from 20 ng of extract is comparable to the activity present in 12.5-25 ng equivalents of the TCAB1 IP, indicating that TCAB1 is quantitatively associated with active telomerase enzyme in cell extracts.

Figure S5

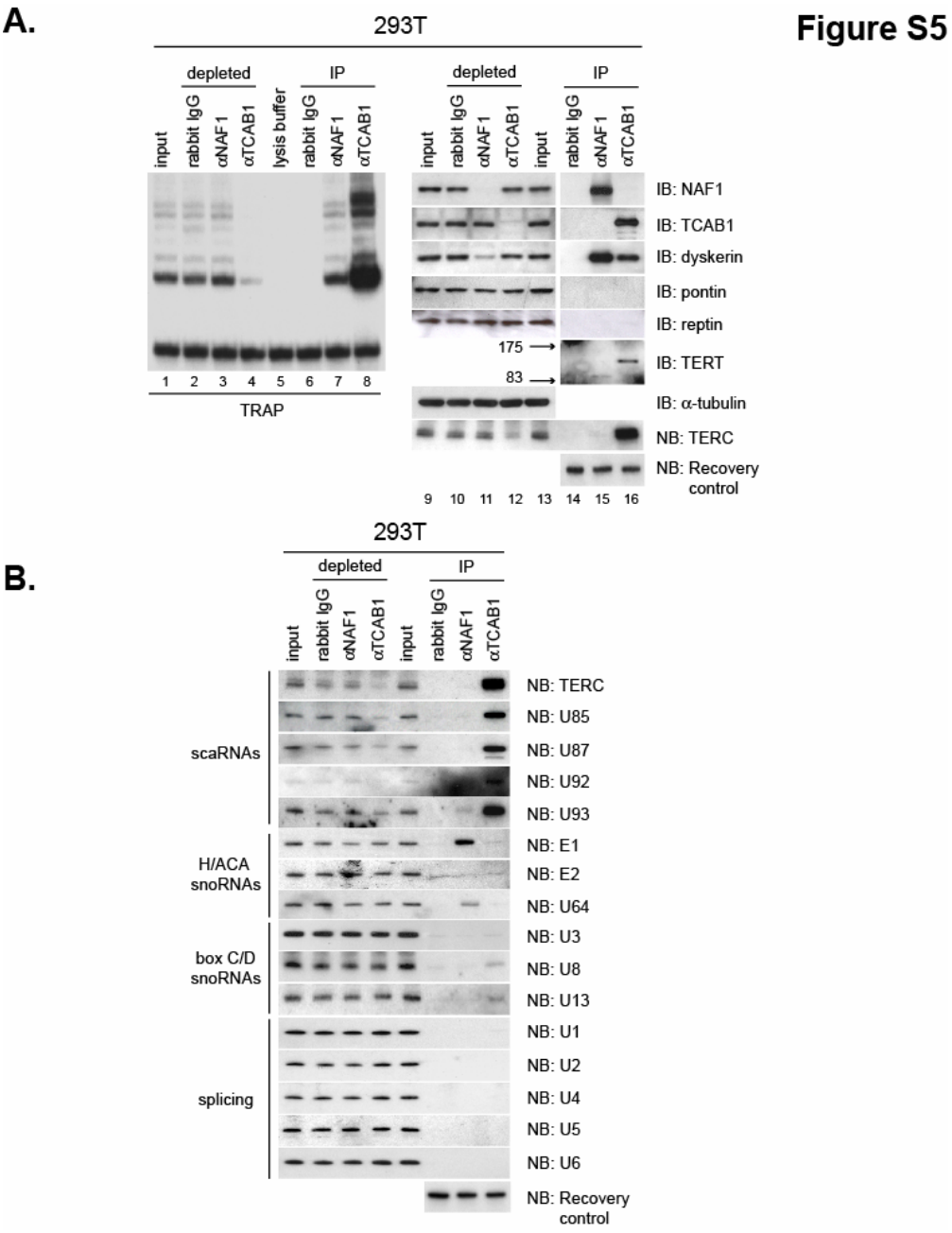


Fig. S5. TCAB1 associates with a majority of telomerase activity, TERC, and other scaRNAs in 293T cells. (**A**) Immunodepletion of endogenous TCAB1, but not NAF1, from 293T whole cell extracts co-depletes telomerase activity and TERC. Rabbit IgG was used as a negative control. Endogenous TERT is detectable in TCAB1 immunoprecipitates. Experiment was done similarly to that shown in Fig. 2B for HeLa cell extracts. (**B**) Immunodepletion of endogenous TCAB1 from 293T whole cell extracts co-depletes scaRNAs specifically, but not other classes of nuclear non-coding RNAs. Rabbit IgG and antibodies immunodepleting endogenous NAF1 was used as negative controls. Experiment was done similarly to that shown in Fig. 3D for HeLa cell extracts.

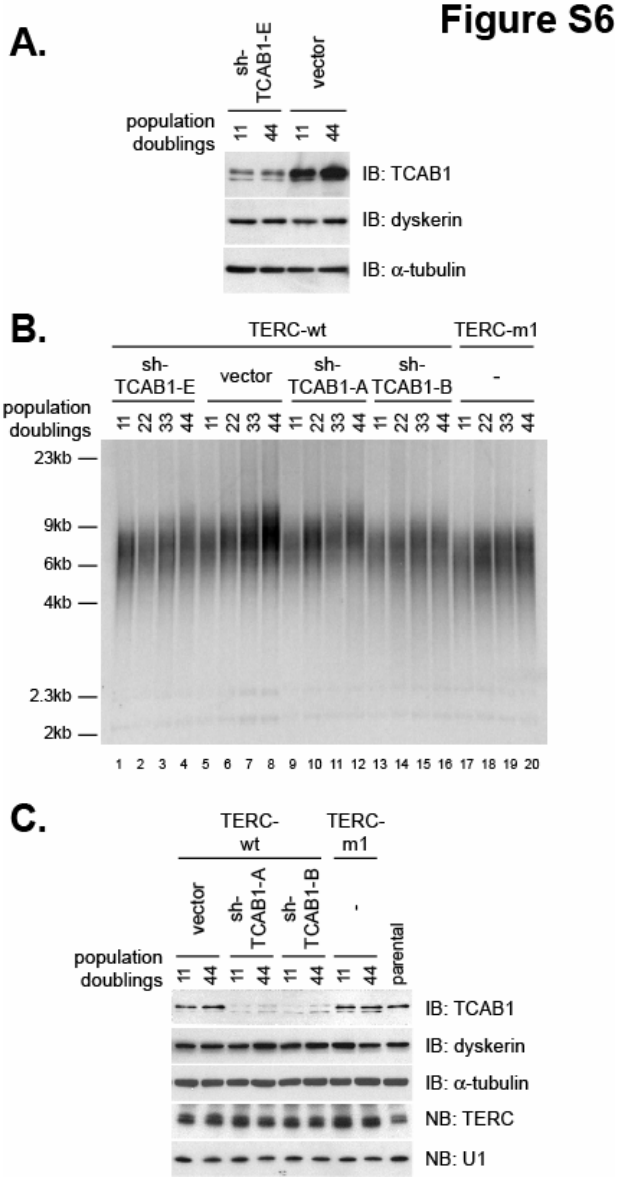


Fig. S6. Telomere hyper-elongation induced by TERC overexpression is blunted by sustained depletion of TCAB1. HTC75 fibrosarcoma cells over-expressing TERC were transduced with shRNA vectors targeting TCAB1 or an empty shRNA vector control (analyzed in Fig. 4D). (**A**) Western blot for TCAB1 in empty vector or shTCAB1-E cell lines shown in Fig. 4D. Knockdown of TCAB1 is maintained after 44 population doublings, but levels of dyskerin are unaffected. (**B**) Telomere lengths were measured by TRF Southern blot in HTC75 cells overexpressing wildtype TERC (lanes 1-16) or point mutant TERC-m1 (lanes 17-20). Cells overexpressing wildtype TERC were transduced with three independent shRNA retroviruses targeting TCAB1 or with empty vector. Cells were passaged in culture and genomic DNA was isolated at the indicated number of population doublings. (**C**) Western blot to monitor TCAB1 depletion during the course of the serial passage experiment. TCAB1 knockdown is maintained after 44 population doublings in TCAB1 shRNA-treated cells, but both levels of dyskerin protein and over-expressed TERC levels are unaffected. α-tubulin protein and U1 RNA were used as loading controls.

Figure S7

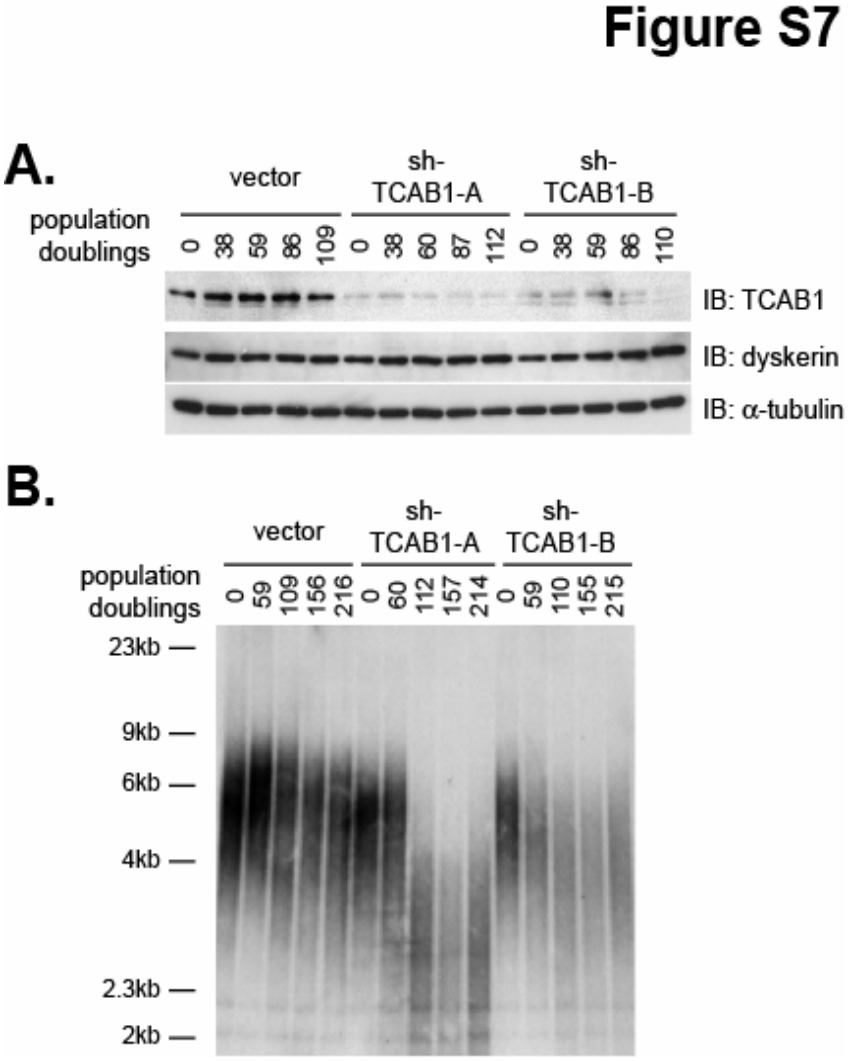


Fig. S7. TCAB1 is required for telomere synthesis by telomerase in human cancer cells. Parental HTC75 fibrosarcoma cells were transduced with two independent TCAB1 shRNAs or empty vector control (analyzed in Fig. 4E). (**A**) Western blot for TCAB1 in HTC75 cell lines transduced with TCAB1 shRNA or empty vector control. Knockdown of TCAB1 is maintained during the long-term serial passage experiment shown in Fig. 4E. Cells were counted at each passage and cumulative population doublings are indicated. (**B**) Telomere lengths were measured by TRF Southern blot in HTC75 cell lines shown in Fig. 4E through approximately 215 population doublings as indicated. Note that the samples analyzed here were taken from a wider interval of population doublings compared to Fig. 4E.

REFERENCES FOR SUPPORTING MATERIAL

S1. A. S. Venteicher, Z. Meng, P. J. Mason, T. D. Veenstra, S. E. Artandi, *Cell* **132**, 945 (Mar 21, 2008).

S2. R. A. Dickins *et al.*, *Nat Genet* **37**, 1289 (Nov, 2005).

S3. Y. Mochizuki, J. He, S. Kulkarni, M. Bessler, P. J. Mason, *Proc Natl Acad Sci U S A* **101**, 10756 (Jul 20, 2004).

S4. N. W. Kim *et al.*, *Science* **266**, 2011 (Dec 23, 1994).

S5. Y. Zhu, R. L. Tomlinson, A. A. Lukowiak, R. M. Terns, M. P. Terns, *Mol Biol Cell* **15**, 81 (Jan, 2004).

S6. R. L. Tomlinson, T. D. Ziegler, T. Supakorndej, R. M. Terns, M. P. Terns, *Mol Biol Cell* **17**, 955 (Feb, 2006).

S7. R. L. Tomlinson *et al.*, *Mol Biol Cell* (Jul 2, 2008).

S8. G. Cristofari, J. Lingner, *Embo J* **25**, 565 (Feb 8, 2006).

S9. E. J. Middleman, J. Choi, A. S. Venteicher, P. Cheung, S. E. Artandi, *Mol Cell Biol* **26**, 2146 (Mar, 2006).