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Telomerase Recruitment Requires both TCAB1 and Cajal Bodies Independently

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The ability of most cancer cells to grow indefinitely relies on the enzyme telomerase and its recruitment to telomeres. In human cells, recruitment depends on the Cajal body RNA chaperone TCAB1 binding to the RNA subunit of telomerase (hTR) and is also thought to rely on an N-terminal domain of the catalytic subunit, hTERT. We demonstrate that coilin, an essential structural component of Cajal bodies, is required for endogenous telomerase recruitment to telomeres but that overexpression of telomerase can compensate for Cajal body absence. In contrast, recruitment of telomerase was sensitive to levels of TCAB1, and this was not rescued by overexpression of telomerase. Thus, although Cajal bodies are important for recruitment, TCAB1 has an additional role in this process that is independent of these structures. TCAB1 itself localizes to telomeres in a telomerase-dependent but Cajal body-independent manner. We identify a point mutation in hTERT that largely abolishes recruitment yet does not affect association of telomerase with TCAB1, suggesting that this region mediates recruitment by an independent mechanism. Our results demonstrate that telomerase has multiple independent requirements for recruitment to telomeres and that the function of TCAB1 is to directly transport telomerase to telomeres.

Telomeres are protein-nucleic acid structures at the ends of linear chromosomes, which protect the DNA termini from degradation and inappropriate processing as damaged DNA. Despite this protective role, telomere shortening still occurs in most normal human somatic cells during DNA replication due to inherent limitations in the replication machinery, and this shortening is the basis of cellular senescence (5, 14, 28). Approximately 85 to 90% of human cancers counteract this shortening and avoid senescence by activating the ribonucleoprotein telomerase to extend telomeres (13, 30). Active telomerase consists of three core components essential for activity (7): hTERT, the reverse transcriptase catalytic subunit (26); hTR, the RNA subunit, used as a cognate template for reverse transcription of telomeric DNA (12); and the protein dyskerin, which is essential for hTR stability (23). The extension of telomeres by telomerase is preceded by a complex series of events involving enzyme biogenesis, transport from sites of enzyme assembly, and trafficking of telomerase in the nucleus at the appropriate phase in the cell cycle. The factors involved in these steps and how these stages are integrated are not fully understood.

Regions of hTERT have been identified that are essential for the enzyme to extend the life span of untransformed cells but which are dispensable for enzyme function *ex vivo* (2, 4). These regions, which separate the *in vivo* functionality of the enzyme from *ex vivo* telomerase activity, were termed DAT for "dissociates the activities of telomerase." A potential explanation for this observation is a failure of the enzyme to be transported or recruited to telomeres. Fusion of the single-stranded telomeric DNA binding protein POT1 to N-terminal DAT mutants rescued telomere elongation, and this depended on POT1 telomere localization, suggesting that the N-DAT domain of hTERT is important for telomerase recruitment to the telomere (3). This observation predicts a protein-protein interaction for the DAT domain. Recent data have indicated that TPP1, which bridges POT1 and the proteins which bind the double-stranded portion of telomeres, mediates a key final step in the recruitment process via its OB fold (1). TPP1 was also shown to enhance telomerase processivity through an interaction with the DAT domain (37), suggesting that telomerase recruitment involves a protein-protein interaction between TPP1 and DAT.

Cajal bodies (CB) are dynamic subnuclear structures in stem cells and cancer cells comprised of several obligate subunit proteins, including coilin and TCAB1; depletion of these proteins leads to dispersal of factors which typically accumulate in these structures (10, 21). CB appear to function in diverse pathways, including snRNA modification and assembly, maturation of spliceosomal RNPs, and histone gene transcription (27). Knockdown of the subunit coilin revealed a role in proliferation in HeLa cells (6, 18). Depletion studies of coilin have also demonstrated a role for this protein in viability, fertility, and fecundity (36). CB were found to accumulate telomerase and transiently associate with telomeres (16, 39), although subsequent studies of CB in mice show no connection with telomerase recruitment (31). It remains unknown for human cells whether coilin and CB are required for telomerase recruitment to telomeres.

The accumulation of telomerase in CB is mediated by the protein WDR79 (34, 35). Human telomerase RNA (hTR) contains a 4-nucleotide (nt) sequence, known as the CAB box, that is a feature of small Cajal body RNAs (scaRNAs) and directs this localization (15). Interestingly, shRNA against WDR79 reduced hTR localization to telomeres and over time resulted in telomere shortening, demonstrating a role for WDR79 in the transport of telomerase to telomeres. Consequently this protein was renamed "telomere Cajal body protein 1" (TCAB1) (35). The precise way in which TCAB1 positively regulates telomerase recruitment to telo-

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meres is unclear, as is the role of CB in this process. During S phase, a proportion of CB colocalize with telomeres or telomerase (16, 19). When injected into *Xenopus* oocytes, *Xenopus* telomerase RNA (xTR) accumulates in CB, although disruption of its CAB box largely eliminates this accumulation without hindering telomerase assembly (19). By comparison, mouse TR does not accumulate in CB (31). The CAB box in mTR is identical to that in hTR, yet exogenous hTR expressed in these same mouse cells does accumulate in CB, demonstrating the functionality of this trafficking pathway. Since both mTR and hTR were competent to localize to telomeres in the mouse cells, this raises the possibilities that CB are not essential for recruitment of human telomerase to telomeres, that the presence of TCAB1 itself is the determinant for recruitment, and that the CB localization is incidental.

Here we study the importance of CB association with telomerase for recruitment and the role of TCAB1 and the hTERT DAT domain in this process. Our results indicate that TCAB1 remains associated with DAT mutants, demonstrating that two independent determinants of telomerase recruitment, the DAT domain and the CAB box, are simultaneously required for recruitment. We also demonstrate that TCAB1 is itself present at the telomere as part of the recruitment process. Finally, we provide evidence that intact CB are essential for endogenous telomerase recruitment. When telomerase is overexpressed, TCAB1 is required for telomerase recruitment, although CB are dispensable, indicating a direct role for TCAB1 in this process.

MATERIALS AND METHODS

Cell culture, transfections, and cell cycle synchronization. HEK 293T cells (from T. Adams, CSIRO, Melbourne, Australia; verified to be mycoplasma free) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum at 37°C in a humidified incubator with 5% CO_2. Transfections of expression constructs were performed using FuGene (Roche). Four small interfering RNAs (siRNAs) against coilin (#1, Qiagen Flexitube, catalogue no. SI00350343 [5′-AACG ACUGCCUCAGAGUUAAA-3′]; #2, SI04169718 [5′-CCGAGUCGUCA CAGAUCUCAU-3′]; #3, SI04311146 [5′-CUGCGUAUCUUGUCUCAA UAA-3′]; #4, SI04330830 [5′-CAGCAAUUAAAUGACGUGGUA-3′]) and Invitrogen Stealth siRNA duplexes against TCAB1 and DKC and one control (Stealth siRNA TCAB1 duplex #2 [5′-GUGGAAGGUGAUACCA UCUAUGAUU-3′]; DKC duplex #2 [5′-AACACCUGGAAGCAUAAUC UUGGCC-3′]; duplex control [5′-CACGUUGCUUCCCGAAACAAGU UAU-3′]) were tested for their ability to reduce expression of coilin, TCAB1, or DKC. Qiagen Allstars negative-control siRNA was used for coilin experiments. For immunofluorescence (IF) experiments, cells were seeded in 60-mm dishes (Becton Dickinson) and the following day transfected with 90 pmol of siRNA using 5.5 μl RNAimax (Life Technologies) in 3.5 ml of medium. For siRNA chromatin immunoprecipitation (ChIP) experiments, the day prior to transfection with siRNA, cells were seeded in 150-mm plates (Corning) to be 20% confluent the following day. Cells were transfected in 20 ml of medium with 600 pmol of TCAB1 or 300 pmol of coilin siRNA in Optimem containing 30 μl RNAimax and transfected with telomerase expression constructs the following day. After 48 h, the cells were harvested with ice-cold phosphate-buffered saline (PBS). Expression constructs for hTR, hTERT, or hTERT/dyskerin combined were constructed in pApex parental vector (17). G414C and the NAAIRS mutations at amino acid positions 122 to 127 and 176 to 181 and the hTERT R132D mutant were constructed with the Stratagene Quikchange II XL site-directed mutagenesis kit according to the manufacturer's instructions using wild-type (WT) hTR and hTERT, respectively, as the templates; the hTERT mutant with changes at position 170 to 175 (hereinafter called the +170 mutant in line with previous nomenclature [2]) has been previously described (17). G_1/S-phase cell cycle synchronization was achieved by incubating cells with 2 mM thymidine (Sigma) for 16 h, releasing for 9.5 h, then incubating with 2 μg/ml aphidicolin (Sigma) for 16 h, followed by release for 4 h. Progress through the cell cycle was assessed by DNA content at selected time points by permeabilizing cells on ice for 60 min with 3% (vol/vol) Triton X-100 in the presence of 500 μg of RNase and 25 μg of propidium iodide in a total volume of 250 μl and then analying on a FACSDiva fluorescence-activated cell sorter (Becton Dickinson). Generally, for 293T cells, mid-S phase was achieved after 4 h of release from aphidicolin.

Antibodies. For immunofluorescence, immunoblots, immunoprecipitation, and ChIP experiments we used anticoilin (SC-32860) and Nopp140 (SC-28672) from Santa Cruz Biotechnology, anticoilin (ab11822) and antifibrillarin (ab4566) from Abcam, and anti-TCAB1 (NB100-68252) antibody from Novus Biologicals . We generated sheep polyclonal antibodies against hTERT as previously described (8). Sheep polyclonal antibodies against dyskerin were generated in a similar fashion (at IMVS Veterinary Services, Adelaide, Australia) against the synthetic dyskerin peptide H-CHGKPTDSTPATWKQEY-NH_2 (~10 mg, ≥90% purity; synthesized by Auspep Pty. Ltd.) conjugated to diphtheria toxoid. This dyskerin epitope corresponds to amino acids 401 to 416 (except for the N-terminal cysteine used for conjugation to DPT). Affinity purification of antibodies was performed as previously described (8). For immunoblots, secondary antibodies used were Santa Cruz goat anti-mouse antibody–horseradish peroxidase (HRP) and Dako goat anti-rabbit antibody–HRP. For secondary antibodies in immunofluorescence experiments, we used donkey anti-rabbit and donkey anti-mouse antibodies conjugated to Alexa Fluor 647, 594, and 350 from Invitrogen.

Immunoblotting and cell lysis. Cells were lysed in ice-cold buffer A (20 mM HEPES-KOH buffer [pH 7.9], 2 mM $MgCl_2$, 300 mM KCl, 10% [vol/vol] glycerol, 0.1% [vol/vol] Triton X-100, 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1× complete protease inhibitors [CPI; Roche]), rotated at 4°C for 1 h, and clarified by centrifugation at 16,000 × *g* for 10 min at 4°C. For immunoblots, 10 μl of lysate was added to 10 μl of water and 5 μl of Laemmli sample buffer (250 mM Tris-Cl [pH 6.8], 10% [wt/vol] SDS, 0.01% bromophenol blue, 50% [vol/vol] glycerol, 1.8 M β-mercaptoethanol), heated at 70°C for 10 min, loaded onto NuPage 4 to 12% Bis-Tris precast gels (Invitrogen), and electrophoresed in MOPS (morpholinepropanesulfonic acid) running buffer at 200 V for 35 min with the addition of NuPage antioxidant (Invitrogen). Protein was transferred to Hybond ECL (GE Healthcare Life Sciences) according to the manufacturer's NuPage protocol, prehybridized with 2% skim milk in phosphate-buffered saline (MP Biomedicals) and 0.1% Tween 20 (PBST) for 1 h at 4°C with rotation before incubating with primary antibody (1:5,000 dilution) in PBST overnight at 4°C. The membranes were washed according to the ECL Plus detection kit protocol (GE Healthcare Life Sciences). Membranes were then incubated with secondary antibody (1:10,000 dilution) conjugated to HRP in PBST for 1 h and washed as before. Detection of HRP was performed as indicated in the ECL Plus protocol, and membranes were visualized on a FujiFilm LAS 4000 imager.

DTAs. Direct telomerase assays (DTAs) were performed with oligonucleotide substrate in solution phase. Telomerase was immunoprecipitated from 1-ml cell lysates from 5×10^6 cells (8) using 20 μg anti-hTERT or antidyskerin or 0.5 μg anti-TCAB1, collected in microspin columns (GE Healthcare Life Sciences), and washed with 5 ml buffer A. After removing traces of buffer with brief centrifugation, the column was plugged and 50 μl of assay solution (8) was added supplemented with 4 μM 5′-biotin-CTAGACCTGTCATTCA$(TTAGGG)_3$-3′ and 100 μCi [α-^{32}P]dGTP (Perkin Elmer). Substrate oligonucleotides were purchased from Sigma-Genosys in desalted lyophilized form, gel purified on 20% acrylamide-8 M urea gels, and resuspended in 10 mM Tris-Cl, pH 7.5, before use. The columns containing the reaction mixture were incubated for 2 h (overexpressed telomerase) or 6 h (endogenous telomerase) at 37°C followed by incubation with 40 μl of Neutravidin Plus Ultralink resin (50% [vol/vol]; Thermo Scientific) for 60 min at room temperature

to capture biotinylated oligonucleotides, with mixing every 15 min. Immobilized oligonucleotides were washed with 5 ml 1 M NaCl and 5 ml TE (10 mM Tris-Cl, pH 8.5, 1 mM EDTA), followed by incubation with 0.5 mM free biotin in formamide loading buffer (90% deionized formamide, 10% Tris-borate-EDTA [TBE], 0.1% bromophenol blue, 0.1% xylene cyanol) and heated to 90°C for 10 min. The eluted oligonucleotides were collected by centrifugation and run on a 10% acrylamide-7 M urea sequencing gel in TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA) for 2 h at 85 W; the gel was dried for 1 h at 80°C, and the radioactive signal from the telomerase activity detected on a phosphorscreen.

Immunofluorescence and FISH against hTR and telomeres. Cells were harvested with ice-cold PBS and kept on ice while counted, and 20,000 cells were cytospun onto Menzel-Glaser Superfrost Plus slides in a Shandon Cytospin 4 at 800 rpm for 5 min. Cells were fixed in 4% (vol/vol) formaldehyde in PBS for 10 min followed by incubation in 0.5% IGEPAL-CA-630 in PBS for 10 min, followed by rinsing in Milli-Q H_2O two times, then further permeabilized and dehydrated in 1:1 acetone-methanol for 10 min. Slides were air dried and immediately overlaid with phosphate-buffered gelatin (PBG: 1% cold water fish gelatin [Sigma], 0.5% bovine serum albumin [BSA; fraction V; Roche] in PBS) for 1 h, followed by antibody diluted 1:500 in PBG. Cells were incubated with antibody at 4°C overnight, washed with PBG three times for 5 min each, and overlaid with secondary antibody (1:1,000 dilution) in PBG for 30 min at room temperature (RT), followed by washing in PBG three times. Slides were then refixed in 10% (vol/vol) formaldehyde in PBS, washed as before, and then subjected to ethanol dehydration with 70% (2 min), 90% (2 min), and 100% ethanol (2 min) followed by air drying prior to overlaying with 30 μl of fluorescent *in situ* hybridization (FISH) buffer (Singer lab: 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNase-free BSA, 1 μg/μl *Escherichia coli* tRNA [Roche], 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide) containing 5 ng each of five Alexa Fluor 488-labeled anti-hTR oligonucleotides (Sigma) and 5 ng of Texas Red-labeled telomere probe (Panagene, South Korea). The probe dilution was denatured at 90°C for 2 min and then cooled on ice prior to adding to the FISH hybridization solution. The DNA oligonucleotide probes against hTR were hTR 1 (5′-AF488-GCTGACATTTTTTGTTTG CTCTAGAATGAACGGAAGGCGGCAGGCCGAGGCTT-3′), hTR 2 (5′-AF488-TCCAGGCGGGGTTCGGGGGTGGGCAGGCGACCCGCC GCAGGTCCCCGGGAGGGGCGAA-3′), hTR 3 (5′-AF488-CGCGGCT GACAGAGCCCAACTCTTCGCGGTGGCAGTGGTGCCTCCGGAGA AGCCCCGG-3′), hTR 4 (5′-AF488-CTCCGTTCCTCTTCCTGCGGCC TGAAAGGCCTGAACCTCGCCCTCGCCCCCGAGAG-3′), and hTR 5 (5′-AF488-ATGTGTGAGCCGAGTCCTGGGTGCACGTCCCACAGCT CAGGGAATCGCGCCGCGCGC-3′), and the DNA oligonucleotide telomere probe was 5′-TxRed-CCCTAACCCTAACCCTAACCCTAACCCT AACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAA-3′. Slides were covered with coverslips and heated to 80°C on a Hybaid Omnislide *in situ* PCR machine for 3 min before being allowed to hybridize in a humidified chamber overnight at 37°C. The slides were then washed 2 times for 30 min at 37°C in 50% (vol/vol) formamide, 2× SSC (pH 7), 0.1% (wt/vol) SDS, followed by three 5-min washes at 42°C in 4× SSC, 0.1% Tween 20. Following staining, slides were air dried for 20 min in the dark and overlaid with Prolong Gold (Invitrogen) and a coverslip and cured for a minimum of 6 h prior to microscopic analysis. Staining was visualized at RT on a Zeiss Axio Imager M1 microscope, with a Plan-Apochromat 63× oil objective (numerical aperture, 1.4), and an AxioCam MR digital camera (Carl Zeiss). Exposure times between treatments were consistent. Image brightness and contrast were adjusted in Axiovision and Adobe Photoshop for presentation.

Chromatin immunoprecipitation. Except for fixation, all steps were carried out on ice or at 4°C. Two days after transfection with expression constructs encoding telomerase core components, cells were harvested at 80% to 90% confluence with ice-cold PBS and kept on ice while counted, and the nuclei from 5×10^6 cells were isolated by incubating cells for 10 min in 1 ml of ice-cold ChIP lysis buffer (5 mM HEPES [pH 8.0], 85 mM KCl, 0.4% IGEPAL-CA-630, 1 mM PMSF, and 1× CPI). Nuclei were pelleted at 5,000 × *g* for 15 s at 4°C, the supernatant was removed, and the nuclei were resuspended in 1 ml ChIP lysis buffer lacking IGEPAL-CA-630 but containing 1% formaldehyde at room temperature. Nuclei were incubated for 10 min at room temperature and mixed once during the incubation. The fixed nuclei were then incubated with 75 μl of 1.5 M glycine for 5 min at room temperature, mixing once, to quench cross-linking. Nuclei were collected by centrifugation at 5,000 × *g* for 15 s at 4°C, and the supernatant was removed. To the nuclei, 50 μl of nuclei lysis buffer was then added (50 mM Tris-Cl [pH 8.1], 1 mM EDTA, 1% [wt/vol] SDS, 1 mM PMSF, and 1× CPI) and incubated on ice for 10 min followed by the addition of 750 μl of buffer A (see above) without DTT. Samples were then sonicated on a mixture of ice and ethanol using a Branson 450 sonifier probe tip until the size of chromatin fragments ranged from 200 to 2,000 bp, based on agarose gel electrophoresis of proteinase K-treated and purified DNA. The solubilized chromatin was then clarified by centrifugation at 16,000 × *g* for 10 min at 4°C and the supernatant was stored at −80°C. For the immunoprecipitation step, the amount of chromatin was equalized between samples. This was achieved by incubation of a 50-μl aliquot at 45°C for 3 h with 100 μg proteinase K (Roche) in 100 μl of elution buffer (50 mM $NaHCO_3$, 1% [wt/vol] SDS) followed by purification using a Qiagen PCR purification kit and spectrophotometric determination of DNA concentration. Antibody (10 μg for hTERT and dyskerin, 1 μg for TCAB1) was added to 225 μl ChIP buffer (20 mM HEPES-KOH buffer [pH 8], 150 mM KCl, 1.0% [vol/vol] Triton X-100, 1.5 mM EDTA, 1× CPI, and 1 mM PMSF), which was then mixed with the remaining chromatin. After 1 h of rotation at 4°C, 40 μl of nuclease-free BSA-blocked protein G beads (8) was added and samples were rotated at 4°C overnight. The next day, beads were collected by centrifugation for 30 s at 12,000 × *g* and supernatant was removed, and then the beads were washed 4 times with 1 ml of RT buffer A, followed by 1 ml TE (10 mM Tris-Cl, pH 8, 1 mM EDTA). For each wash, the beads were collected by microcentrifugation for 30 s at 12,000 × *g*. Immunoprecipitated DNA was eluted from the protein G beads by digestion with 100 μg of proteinase K in 100 μl of elution buffer and incubation at 45°C for 3 h and purified using a Qiagen PCR purification kit, eluting in 140 μl TE. The purified DNA (70 μl) was added to 490 μl of 0.46 M NaOH, heated to 95°C for 5 min, and cooled on ice before dot blotting on Hybond XL (GE Healthcare Life Sciences) according to the manufacturer's protocol. Membranes were air dried for 5 min and then cross-linked with 240 mJ/cm^2 using a Stratalinker at 254 nm followed by prehybridization in Church and Gilbert hybridization buffer (1% [wt/vol] BSA fraction V grade, 1 mM EDTA, 500 mM $NaHPO_4$, pH 7.2, 7% [wt/vol] SDS) for 30 min at 55°C and then hybridized overnight with a DNA oligonucleotide probe (5′-CCCTAACCCTAACCCTAA-3′) end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Membranes were washed 3 times for 10 min in 0.1× SSC-0.1% SDS and exposed to phosphorscreens, followed by imaging and quantification of signal using the array analysis function on the ImageQuant program (GE Healthcare Life Sciences), using unused wells for background subtraction. The amount of telomeric DNA immunoprecipitated was normalized for each sample based on the amount of input telomeric DNA determined by signal intensity from standard curves for each sample. To control for nonspecific ChIP, purified ChIP samples were also probed for Alu sequence (gift from T. de Lange, Rockefeller University, New York, NY) labeled using Ambion's Decaprime II kit. For both ChIP and IF/FISH experiments, data were analyzed by Student's *t* test for pairwise comparisons or single-factor analysis of variance (ANOVA) followed by Tukey-Kramer for multiple comparisons.

RESULTS

Endogenous telomerase recruitment requires coilin. CB are dynamic, mobile structures that accumulate telomerase (15, 39) as a result of hTR binding to TCAB1 (35). In S phase, a proportion of CB colocalize with telomeres (1, 16). Given the mobility of both CB (29) and telomeres (24) and the observation that these struc-

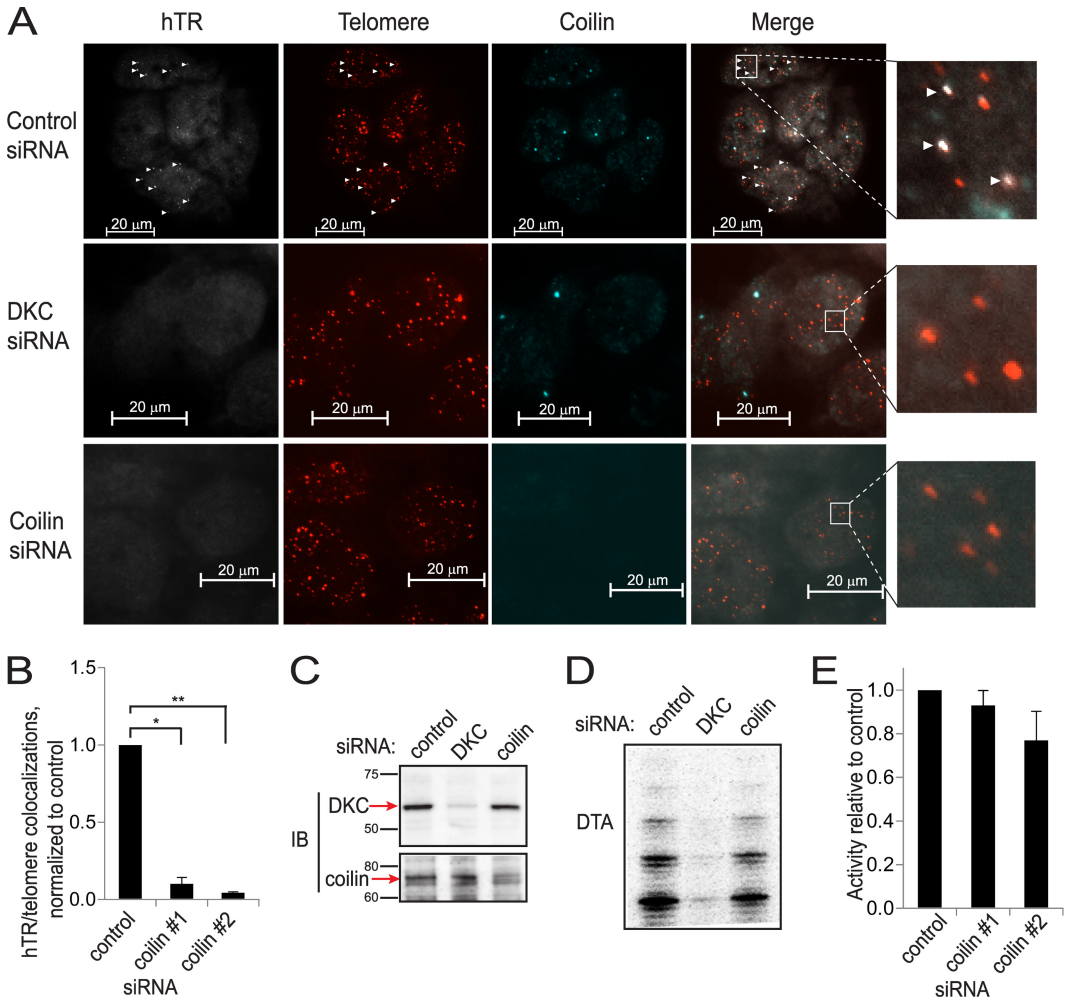

FIG 1 Recruitment of endogenous telomerase to the telomere requires coilin. (A) Representative coilin IF and FISH data for endogenous hTR and telomeres in 293T cells treated with siRNA against DKC or coilin (coilin siRNA 1). Arrowheads indicate colocalizations between hTR and telomeres. (B) Quantitation of hTR/telomere colocalizations from experiments such as that in panel A. Data are from two independent experiments for each siRNA; 60 to 140 cells counted per experiment; mean colocalizations per cell expressed normalized to control siRNA ± SD between experiments (*, $P = 0.001$; **, $P = 0.0001$; 2-tailed t test). (C) Immunoblot of DKC and coilin from cells in panel A. Size markers are in kDa. (D) Direct telomerase assay of immunopurified endogenous telomerase from cells in panel A. (E) Quantitation of telomerase activity of 293T cells after coilin siRNA ($n = 2$; mean ± SD; no significant difference; $P = 0.28$ and 0.13; 2-tailed t test).

tures colocalize during S phase when telomerase extension of telomeres takes place, we tested the hypothesis that CB mediate telomerase recruitment to telomeres. Coilin is an essential CB protein, without which CB cannot form (10). To examine the role of coilin and CB in telomerase recruitment, we used fluorescent *in situ* hybridization (FISH) for hTR and telomeres in conjunction with siRNA against coilin to disrupt CB levels in S-phase-synchronized immortal human embryonic kidney cells (293T) (see Fig. S1 in the supplemental material). When cells were treated with control siRNA, hTR accumulated at telomeres in a significant proportion of the cells (Fig. 1A). As a negative control, siRNA to DKC abolished telomerase localization to telomeres, which is consistent with the known requirement of DKC for hTR stability (22, 23). In contrast to control siRNA, coilin siRNA abolished telomerase accumulation at telomeres; in cells lacking CB, there were almost no observable hTR foci (Fig. 1A and B). Compared to controls, immunoblot analysis of cell lysates from coilin siRNA treatments indicated significantly reduced coilin levels (Fig. 1C). Silencing RNAs directed against TCAB1 and hTERT also abolished telomerase recruitment, as previously demonstrated (data not shown; 35).

CB were visualized by immunofluorescence (IF) for coilin and were readily detectable after control treatments but not after coilin siRNA treatment (Fig. 1A). To test whether the dramatic reduction in hTR localization to telomeres after coilin siRNA could be explained by reduced enzyme levels, we performed a direct telomerase assay (DTA). Coilin siRNA had a slight but statistically insignificant effect on telomerase levels (Fig. 1D and E), which is insufficient to explain the dramatic reduction in hTR accumulation at telomeres.

High cellular levels of telomerase overcome the need for coilin. It has been proposed that CB increase the efficiency of several nuclear processes by concentrating their components in one region of the nucleoplasm (25). A prediction of this model is that enhanced levels of telomerase could rescue the effect of coilin depletion on telomerase recruitment. To test this, we overex-

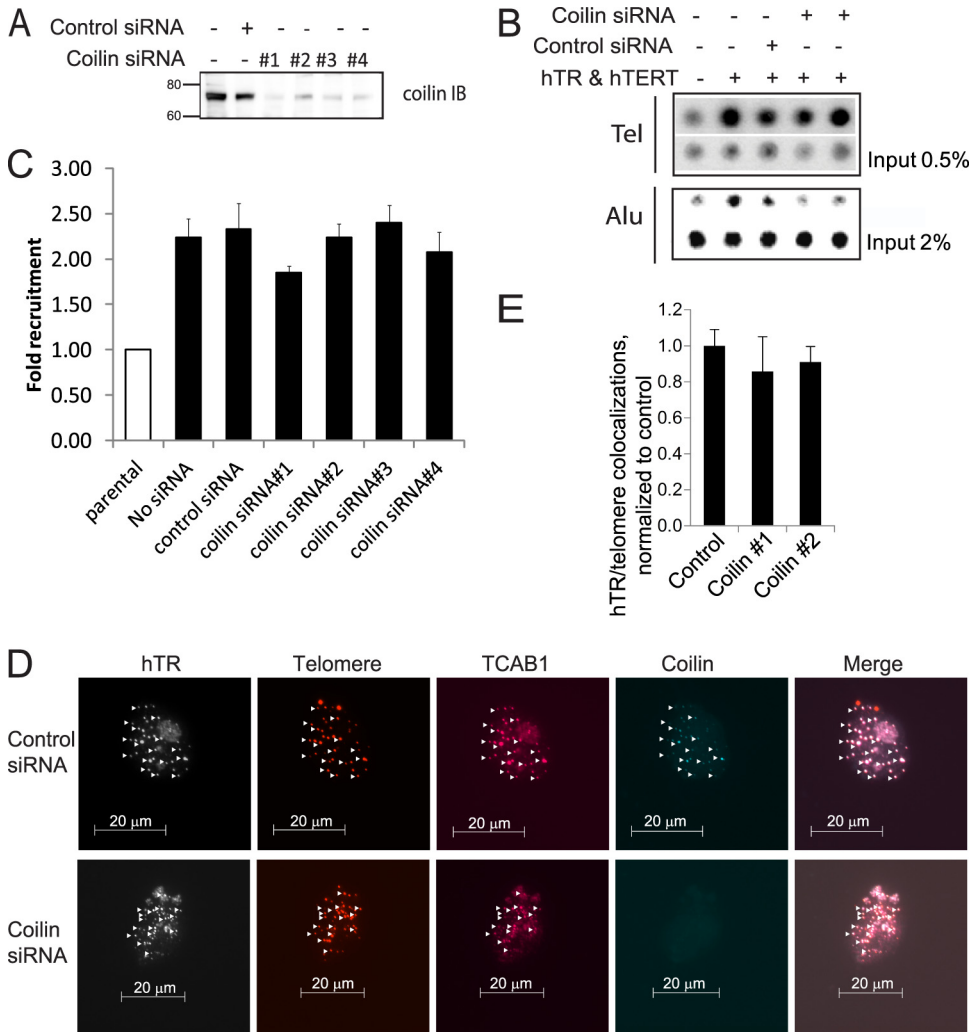

FIG 2 Overexpression of telomerase compensates for the absence of coilin. (A) Immunoblot of coilin in 293T cells overexpressing hTERT and hTR and treated with four different coilin siRNAs. (B) Representative ChIP using antibodies against hTERT on chromatin from cells treated with siRNA 1 or 2 against coilin. Immunoprecipitates were probed with oligonucleotide probes against telomeric DNA (Tel) or Alu repeats (Alu). (C) Quantitation of hTERT ChIP experiments on control cells (parental) or cells overexpressing hTERT and hTR (black bars) and treated with coilin siRNA ($n = 9$ to 11). Data are ± standard error of the mean (±SEM). No significant difference was seen between controls and coilin siRNA (single-factor ANOVA, $F_{(6,60)} = 1.45$; $P = 0.21$) (D) Representative coilin and TCAB1 IF and FISH for hTR and telomeres in cells overexpressing hTERT and hTR and treated with coilin siRNA 1. Arrowheads indicate colocalizations between hTR and telomeres. (E) Quantitation of hTR/telomere colocalizations (and TCAB1/telomere colocalizations, since hTR foci colocalize with all TCAB1 foci) from experiments such as that in panel D, counting only cells that overexpress hTR. Data are from 1 or 2 independent experiments for each siRNA; 30 to 50 cells counted per experiment; mean colocalizations per cell expressed normalized to control siRNA ± SEM between cells (coilin siRNA 1) or standard deviation (SD) between experiments (coilin siRNA 2). Analysis indicates no significant reduction in recruitment from coilin siRNA (single-factor ANOVA, $F_{(2,22)} = 0.5$; $P = 0.61$).

pressed exogenous hTERT and hTR from plasmids in 293T cells and used an anti-hTERT antibody to examine telomerase recruitment to telomeres in chromatin immunoprecipitation (ChIP) assays, following treatment with control or coilin siRNA. It is not possible to reliably detect endogenous human telomerase at the telomere using ChIP due to the enzyme's low abundance (9), but overexpression of telomerase specifically resulted in an increased recovery of telomeric DNA, as indicated by hybridization of the ChIP samples with a probe against Alu repeats (Fig. 2B). Four different siRNAs against coilin reduced coilin protein levels to between 69 and 89% in cells overexpressing telomerase (Fig. 2A), yet hTERT ChIP assays revealed little effect of coilin depletion on hTERT recruitment to telomeres (Fig. 2B and C) (single-factor ANOVA, $F(6,60) = 1.45$; $P = 0.21$).

In agreement with the ChIP experiments, IF/FISH experiments also demonstrated little effect of coilin siRNA on overexpressed telomerase recruitment. Despite the absence of detectable CB (Fig. 2D), there was no significant change in the number of hTR foci at telomeres (Fig. 2D and E) (single-factor ANOVA, $F_{(2,22)} = 0.5$; $P = 0.61$) or telomerase activity (see Fig. S2 in the supplemental material).

Coilin knockdown has been reported in some cell types to lead to the formation of structures referred to as residual Cajal bodies (33), comprised of TCAB1, Nopp140, SMN, and fibrillarin (20, 33). To examine if residual Cajal bodies remained in cells overexpressing telomerase, cells were treated with siRNA against coilin and costained for TCAB1 and coilin, Nopp140, or fibrillarin (Fig. 3A to C). In cells treated with control siRNA, TCAB1 consistently

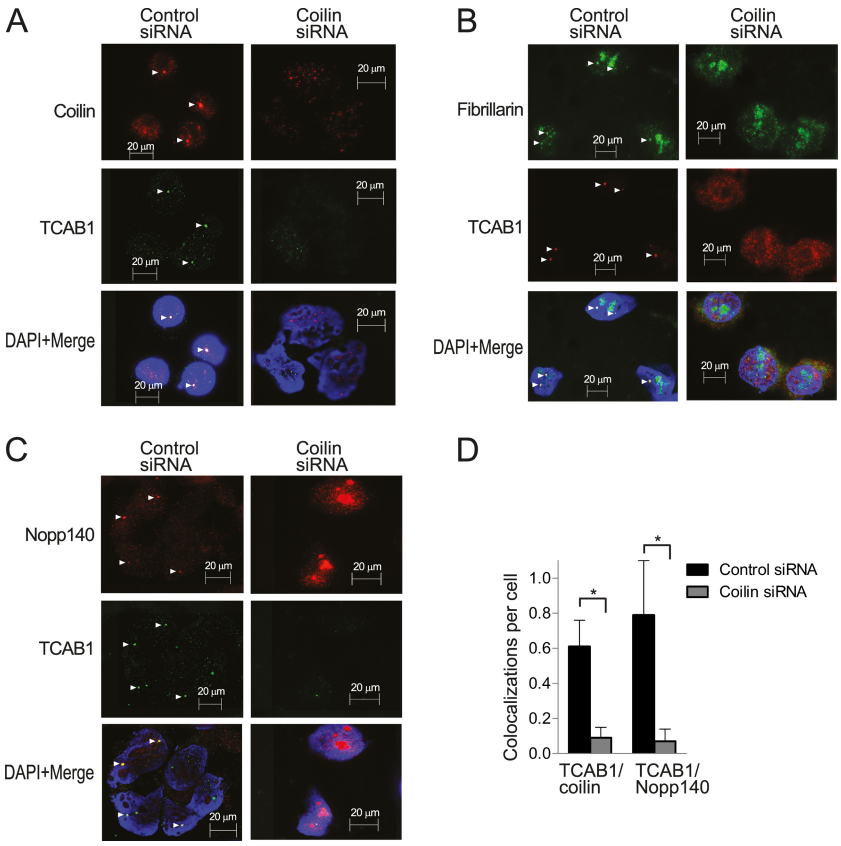

FIG 3 Residual Cajal bodies do not form in the absence of coilin. (A to C) Representative immunofluorescence using antibodies against coilin and TCAB1 (A), fibrillarin and TCAB1 (B), and Nopp140 and TCAB1 (C) in 293T cells transfected with siRNA 1 against coilin and expressing hTR and hTERT. Arrowheads indicate foci colocalizing with TCAB1. (D) Quantitation of foci containing both TCAB1 and coilin, or both TCAB1 and Nopp140, in experiments in panels A and C, counting only cells that overexpress hTR (15 to 30 cells counted per experiment; data are mean ± SEM; *, $P = 0.01$ [coilin] and 0.05 [Nopp140]; 2-tailed t test).

colocalized with the vast majority of coilin, Nopp140, and fibrillarin foci. In contrast, in coilin siRNA-treated cells overexpressing telomerase, almost no foci were seen for coilin or TCAB1 (Fig. 3A). Nopp140 and fibrillarin both appeared to accumulate in nucleoli but did not colocalize with TCAB1 (Fig. 3B to D). These data indicate that residual CB that lack coilin are unlikely to be involved in the transport of telomerase in these cells.

Overexpression of telomerase cannot compensate for the absence of TCAB1. TCAB1 is an RNA chaperone that binds to RNA molecules via a 4-nucleotide motif termed the CAB box and targets these molecules to Cajal bodies. This protein was reported to interact with telomerase via the hTR CAB box but also through a direct interaction with dyskerin (11, 34, 35). TCAB1 has been shown to be necessary for the delivery of endogenous telomerase to telomeres in HeLa cells in S phase (35). Since the overexpression of telomerase compensates for the role of Cajal bodies in this process, it seemed possible that it would also compensate for the absence of TCAB1. To test this, we carried out ChIP with an hTERT antibody in cells overexpressing telomerase in the presence of control siRNA or TCAB1 siRNA (Fig. 4A and B). TCAB1 siRNA reduced TCAB1 protein expression 84% (Fig. 4C); this had no effect on telomerase activity (see Fig. S3 in the supplemental material) yet resulted in a profound decrease in telomerase at the telomere (Fig. 4A and B) (single-factor ANOVA, $F_{(2,18)} = 6.16$; $P = 0.009$).

IF/FISH experiments on cells treated with control or TCAB1 siRNA also demonstrated a significant effect on the ability of overexpressed telomerase to recruit to telomeres (Fig. 4D and E) as a result of TCAB1 protein knockdown (84% and 85%) (Fig. 4F) (single-factor ANOVA, $F_{(2,19)} = 11.53$; $P = 0.0005$). In cells treated with TCAB1 siRNA, hTR accumulated in structures resembling nucleoli (Fig. 4D), consistent with recent observations of hTR in cells derived from patients with TCAB1 mutations that cause dyskeratosis congenita (38). In those cells in the population that have lost TCAB1 expression, there are no detectable Cajal bodies. These data agree with the observation that TCAB1 is required for the formation of CB (21).

These data indicate that high cellular levels of telomerase cannot compensate for loss of TCAB1, suggesting that this protein has a role in recruitment that goes beyond CB localization.

TCAB1 localizes to telomeres in a telomerase-dependent manner. To further define the role of TCAB1 in telomerase recruitment, we carried out TCAB1 and dyskerin ChIP to determine if these proteins remain associated with telomerase upon recruitment to the telomere. ChIP experiments were performed with antibodies against TCAB1, dyskerin, and hTERT. Expression of telomerase led to detectable levels of telomeric DNA isolated by ChIP using antibodies against all three proteins (Fig. 5A and B, blue bars). Controls using siRNA against TCAB1 demonstrated the specificity of the ChIP signal for the antibody against this pro-

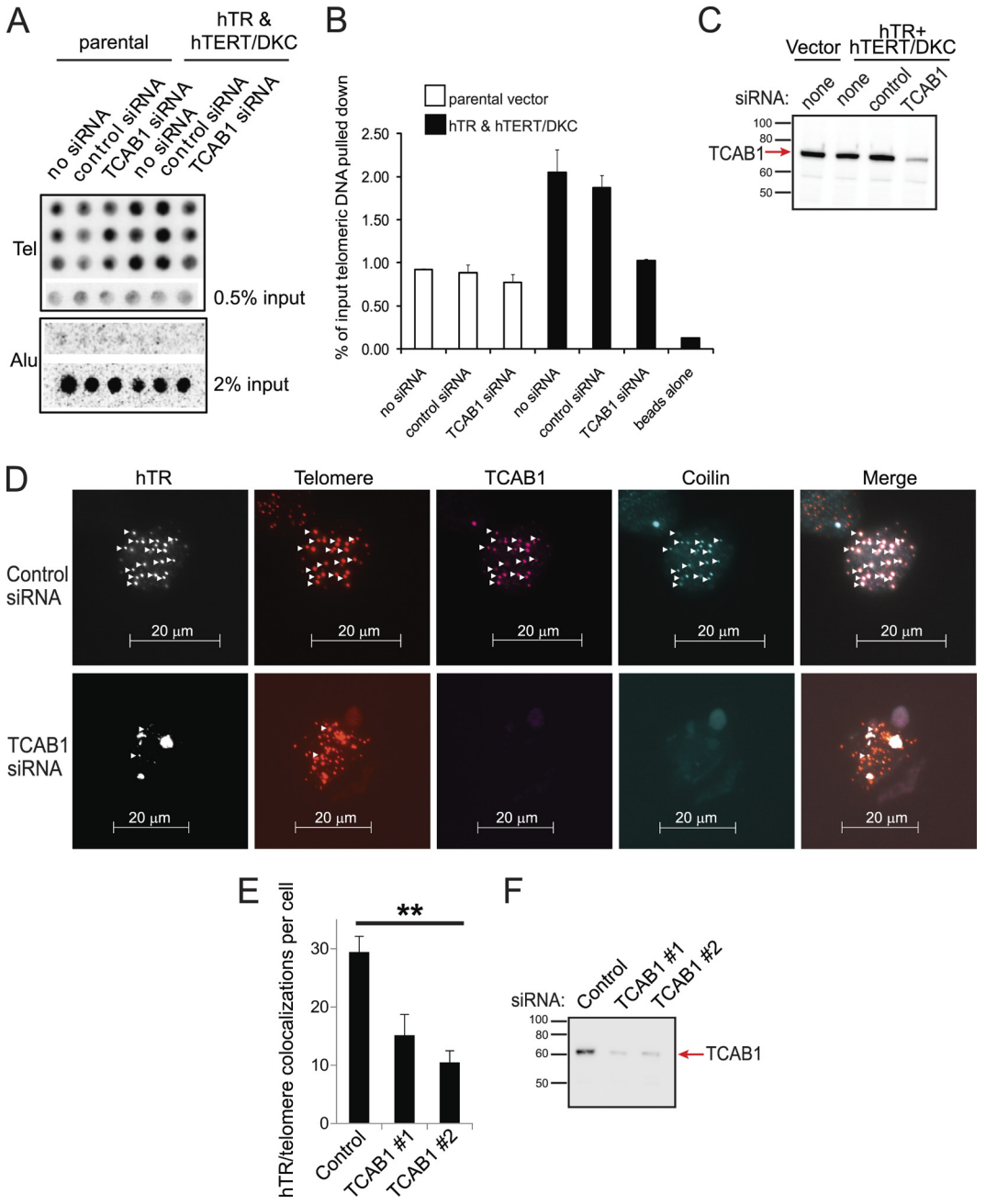

FIG 4 Overexpression of telomerase cannot compensate for the absence of TCAB1. (A and B) Representative dot blot (A) and quantitation of ChIP experiments (B) following treatment of cells with control siRNA or siRNA against TCAB1, using antibodies against hTERT on chromatin from parental vector-transfected 293T cells or cells expressing hTR, hTERT, and dyskerin. Immunoprecipitates were probed with oligonucleotide probes against telomeric DNA (Tel) or Alu repeats (Alu). Bar charts show quantitation of ChIP data ± SEM ($n = 3$). Analysis indicates a significant reduction in recruitment from TCAB1 siRNA (single-factor ANOVA, $F_{(2,18)} = 6.16$; $P = 0.009$). (C) Immunoblot of TCAB1 from cells in panel A. Size markers are in kDa. (D) Representative coilin and TCAB1 IF and FISH for hTR and telomeres in cells overexpressing hTERT and hTR and treated with TCAB1 siRNA. Arrowheads indicate colocalizations between hTR and telomeres. (E) Quantitation of hTR/telomere colocalizations from D, counting only cells that overexpress hTR (60 to 75 cells counted; mean ± SEM between cells; single-factor ANOVA, $F_{(2,19)} = 11.53$; **, $P = 0.0005$). (F) Immunoblot of TCAB1 from cells in panel D. Size markers are in kDa.

tein (see Fig. S4 in the supplemental material). These data demonstrate that TCAB1 localizes to telomeres concomitantly with telomerase.

A mutation in the CAB box of hTR (G to C at nucleotide position 414) was reported to disrupt the ability of TCAB1 to interact with hTR (11). We constructed this mutant to determine whether TCAB1 localization to the telomere is dependent on its interaction with telomerase. In contrast to wild-type hTR-expressing cells, telomerase activity was not associated with TCAB1 in cells expressing this mutation (see Fig. S5 in the supplemental material), confirming that the mutation abolishes the hTR-TCAB1 interaction in our system. Analysis of this mutant by

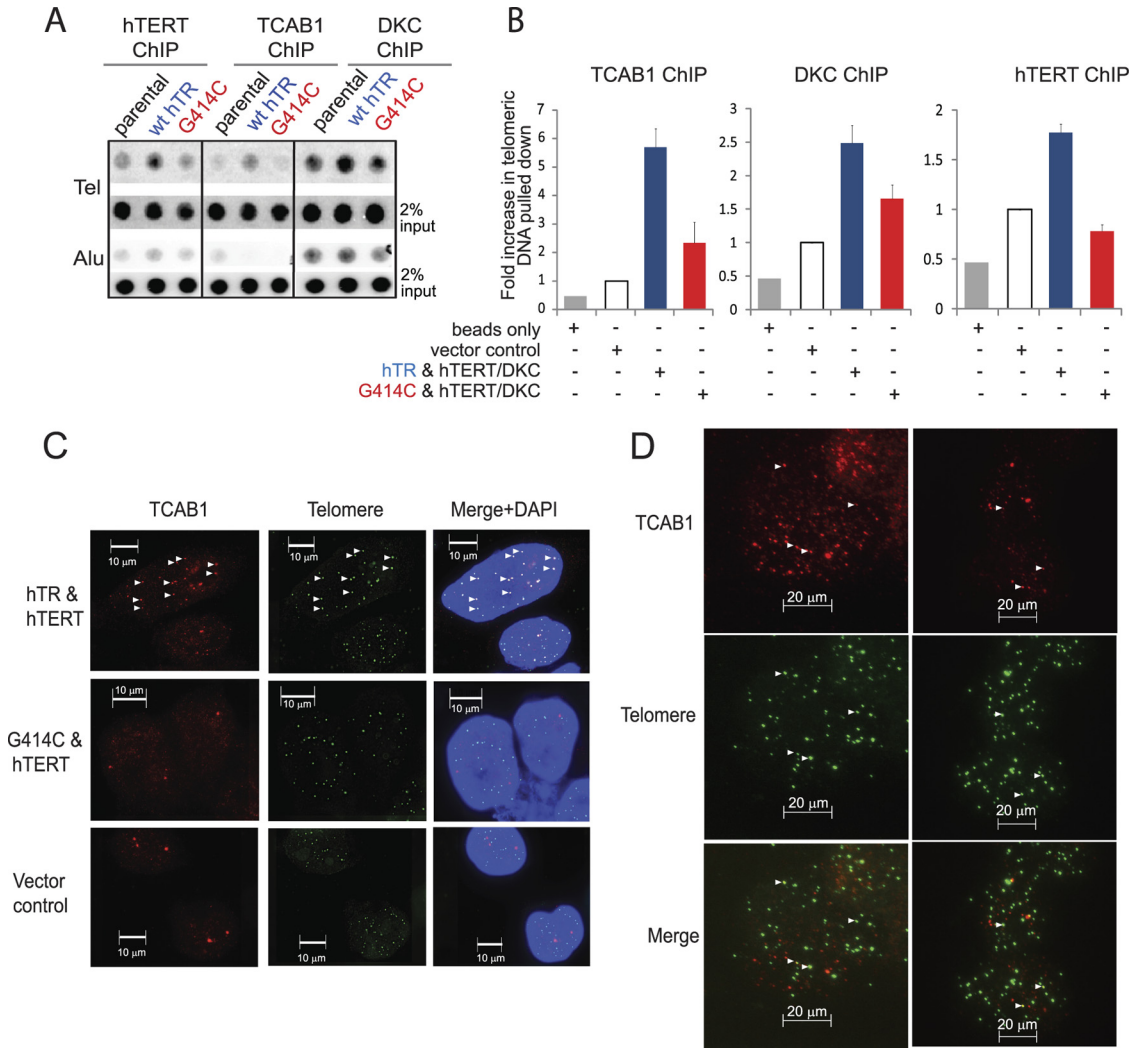

FIG 5 TCAB1 localization at the telomere is dependent on its interaction with telomerase. (A) ChIP using antibodies against TCAB1, dyskerin, or hTERT on chromatin from 293T cells transfected with parental vectors or expressing hTERT, dyskerin, and either WT hTR or CAB box mutant G414C hTR. Immunoprecipitates were probed with oligonucleotide probes against telomeric DNA (Tel) or Alu repeats (Alu). (B) Quantitation of ChIP data in panel A ±SEM (n = 4). (C) Immunofluorescence for TCAB1 together with FISH for telomeres in cells expressing hTERT and either WT hTR or CAB box mutant hTR or in parental vector-transfected cells. (D) Immunofluorescence for TCAB1 together with FISH for telomeres in 293T cells; at right and left are two representative examples. Arrowheads indicate colocalizations.

TCAB1 ChIP demonstrated the requirement for an intact hTR CAB box (Fig. 5A and B, red bars) for TCAB1 recruitment to the telomere. As controls, we confirmed that the G414C mutation also abolished interaction of both hTERT (9) and DKC with the telomere (Fig. 5A and B). We confirmed that this failure was not due to differences in total levels of telomerase reconstituted in the treatments (see Fig. S3). Thus, TCAB1 is not constitutively present at the telomere; telomerase and TCAB1 are mutually dependent for their interaction with the telomere.

These data were confirmed with an independent technique, by performing IF/FISH experiments in S-phase-synchronized cells expressing wild-type or CAB box mutant hTR. We observed that TCAB1 colocalized with telomeres in wild-type hTR-expressing cells but not in cells expressing the CAB box mutant (Fig. 5C). Together, these data demonstrate that the reason for the loss of telomere recruitment of hTR mutant G414C (9) is a loss of binding between telomerase and TCAB1 and that TCAB1 has a more direct role than simply the transport of telomerase to CB. To test whether TCAB1 localized to telomeres in cells expressing endogenous levels of telomerase, we stained 293T cells for TCAB1 and telomeres. We found that in cells expressing endogenous levels of telomerase, TCAB1 was detectable at telomeres in S phase (Fig. 5D).

To further strengthen the conclusion that TCAB1 plays a role in recruitment that is independent of CB, we examined TCAB1 localization in cells overexpressing telomerase and lacking coilin (Fig. 2D). All telomeric foci contained TCAB1 as well as hTR, despite a complete lack of detectable CB (Fig. 2D and 3). These results illustrate that TCAB1 accompanies telomerase to telomeres, via a mechanism that can be independent of CB.

Recruitment via TCAB1 is independent of the DAT domain. Serial substitutions of 6 amino acids (NAAIRS) in the N and C termini of hTERT (2, 4) revealed regions in hTERT required to

extend replicative life span but dispensable for *ex vivo* activity (i.e., DAT mutants). The *in vivo* defect of these DAT mutants suggested a defect in trafficking or recruitment of the enzyme to the telomere (3). To test this hypothesis directly, we constructed one of these DAT mutants (changes at position 122 to 127, hereafter called the +122 mutant in line with previous nomenclature [2]) and, using ChIP, tested its ability to localize to telomeres. In conjunction with these experiments, we tested a mutant hTERT with a NAAIRS substitution in a nearby region of the hTERT N terminus at position 170 to 175, which we recently demonstrated to have a severe defect in catalytic activity due to a deficiency in positioning the 3′ end of the primer in the active site (17). This mutant (the +170 mutant) was originally reported to fail to immortalize normal human cells upon exogenous expression, and this was thought to arise from the fact that it exhibited no *in vitro* catalytic activity (2). However, it remains unknown whether this mutant is competent for recruitment. As a negative control, we made a NAAIRS substitution at an adjacent position (for the +176 mutant), located within an unstructured linker region of the protein; this mutant was reported to immortalize cells normally when expressed exogenously (2).

hTERT ChIP experiments demonstrated that while wild-type hTERT and the +176 mutant were recruited to telomeres, both the +122 and +170 NAAIRS mutants failed to localize to telomeres (Fig. 6A and B). To confirm this result with a less extensive mutation than the 6-amino-acid NAAIRS substitution, we also constructed a point mutation in an amino acid of the DAT domain that is located at the surface of a homology model of the hTERT TEN domain (17) (R132D). This mutant also showed a profound failure in recruitment, measured by both hTERT ChIP (Fig. 6C) and FISH for hTR (Fig. 6D and E). When expressed with this hTERT mutant, hTR localized to CB in those cells which contained these structures (Fig. 6D). In other cells, the overexpressed hTR appeared to accumulate in nucleoli (data not shown). These data indicate that the defect of the DAT mutants in immortalizing human cells likely results from a recruitment failure. The failure of the +170 mutant to localize to the telomere indicates that the region in the N terminus of TERT required for recruitment may involve more than the previously described DAT domain.

Both the DAT mutants of hTERT (Fig. 6) and siRNA to TCAB1 (Fig. 4) reduced telomerase recruitment. The loss of telomerase recruitment to the telomere with TCAB1 siRNA did not result from a decrease in telomerase activity (see Fig. S3 in the supplemental material). To determine whether the TCAB1-mediated telomerase recruitment involves an interaction with the DAT domain of hTERT, we examined the level of telomerase activity immunoprecipitated by anti-TCAB1 antibodies from cells expressing +122 hTERT, hTERT^{R132D}, and WT telomerase. The amounts of telomerase activity immunoprecipitated with TCAB1 antibodies were similar for wild-type and mutant hTERTs (Fig. 6G and H), demonstrating that TCAB1 remains associated with the telomerase complex in cells expressing DAT mutants. Furthermore, both hTR and TCAB1 colocalized with CB in cells expressing hTERT^{R132D} (Fig. 6D and F), indicating that the expression of this mutant has not perturbed the TCAB1 trafficking pathway. These data indicate that the DAT domain is unlikely to mediate telomerase recruitment via a protein-protein interaction with TCAB1. TCAB1 association with telomeres was reduced to the same extent as that of hTR in cells expressing hTERT^{R132D} (Fig. 6D and E), providing additional support for the conclusion that TCAB1 localization to the telomere depends on telomerase.

DISCUSSION

Human telomerase RNA localizes to CB (15, 16, 32, 39), suggesting that coilin may be important in telomerase trafficking, yet coilin depletion has not been examined in detail in human cancer cells. The requirement of the hTR CAB box and its binding protein TCAB1 for telomere localization of telomerase (9, 35) and the observation that CB can be colocalized with telomeres (16) further suggest a role for coilin in telomerase recruitment. Our data demonstrate that for endogenous telomerase, coilin is required for hTR to accumulate at telomeres, but that telomerase overexpression can compensate for coilin deficiency. This supports the view that a role of CB is to concentrate their components, which might normally be present at limiting levels in the nucleoplasm (25).

The RNA chaperone TCAB1 was reported to play a role in the recruitment of telomerase to CB and telomeres (34, 35). However, these studies did not ascertain whether the primary role of TCAB1 was solely in the transport of telomerase to CB or whether TCAB1 plays a more direct role in telomerase recruitment. In our study, we have used ChIP and immunofluorescence experiments to demonstrate that TCAB1 is present at the telomere in a telomerase-dependent manner. Using ChIP in conjunction with siRNA directed against TCAB1 or CAB box mutant hTR, we demonstrated that this protein is an important mediator of telomerase recruitment even in situations when CB themselves are not required. Taken together, these data suggest thatTCAB1 may directly mediate interactions with telomeric proteins essential for telomerase recruitment.

Previous reports indicated that the N-terminal DAT domain of hTERT is essential for telomerase function *in vivo* but not *ex vivo* (2, 4). Fusion of POT1 to telomerase rescued telomere elongation by N-DAT mutants and this depended on POT1 localization to the telomere, suggesting that this domain is important for recruitment (3). We used ChIP assays to demonstrate that one of these mutants, as well as a nearby mutant with a catalytic defect, was not detectable at the telomere. We further identified a point mutation in this domain which also abolishes recruitment in both ChIP experiments and FISH assays. These data provide a mechanism for the failure of N-terminal hTERT mutants to function *in vivo* and provide some insight into how this region might be targeted to interfere with telomerase function *in vivo*. Importantly, the failure of these mutants to localize to telomeres is not due to a failure to associate with the essential recruitment factor TCAB1, as the amounts of mutant and wild-type enzyme that associate with TCAB1 are similar.

Recently, it was reported that shRNA against the telomere-binding proteins TPP1 or Tin2 prevented telomerase recruitment, and while this was rescued by full-length TPP1 resistant to the shRNA, it was not rescued by TPP1 lacking the OB fold (1). These experiments strongly suggest that the OB fold of TPP1 contains an important site that mediates recruitment. We have demonstrated that mutation of the hTERT DAT domain does not compromise interaction with TCAB1. Therefore, the interaction between TPP1 and the DAT domain (37) suggests that the DAT recruitment process may instead involve TPP1.

Thus, one model for telomerase recruitment suggests that there are at least three distinct and essential requirements for recruitment to telomeres, including CB, TCAB1, and an intact

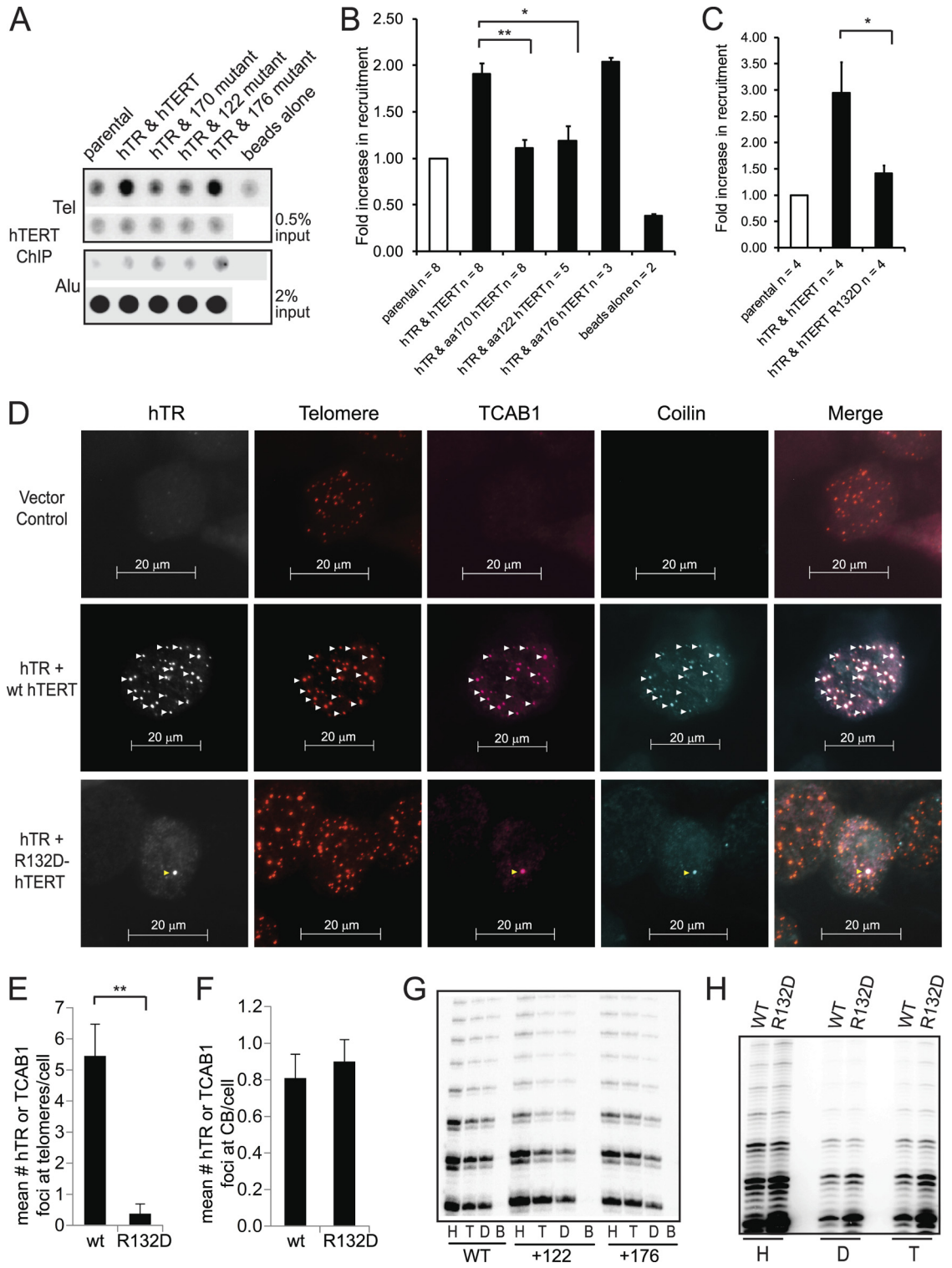

FIG 6 The TCAB1 telomerase recruitment pathway is independent of the hTERT DAT domain. (A and B) Representative dot blot (A) and quantitation of hTERT ChIP (B) from 293T cells expressing WT hTR and either WT hTERT or mutant hTERT. Data are ±SEM. (**, $P = 0.003$; *, $P = 0.04$; 2-tailed t test). (C) Quantitation of hTERT ChIP experiments from cells expressing WT hTR and either WT hTERT or hTERT^{R132D} (*, $P = 0.026$; 2-tailed t test). (D) Representative coilin and TCAB1 IF and FISH for hTR and telomeres in 293T cells overexpressing telomerase (white arrowheads indicate hTR/telomere/TCAB1/coilin colocalizations; yellow arrowheads indicate hTR and TCAB1 localization with CB). (E) Quantitation of TCAB1/telomere colocalizations (and hTR/telomere colocalizations, since TCAB1 foci colocalize with all hTR foci) from panel D, counting only cells that overexpress hTR (60 to 75 cells counted; mean ± SEM between cells; **, $P = 0.0002$; 2-tailed t test). (F) Quantitation of foci containing TCAB1, hTR, and coilin but not telomeric DNA from D, counting only cells that overexpress hTR (60 to 75 cells counted; mean ± SEM between cells; $P = 0.62$; 2-tailed t test). (G) Direct telomerase assay of immunoprecipitates from 293T cells expressing hTR and either WT hTERT or mutant hTERT, using antibodies against hTERT (lane H), TCAB1 (lane T), DKC (lane D), or beads alone (lane B) as a negative control. (H) Direct telomerase assay of immunoprecipitates from cells expressing hTR and either WT hTERT or hTERT^{R132D} using antibodies against hTERT (lane H) , DKC (lane D), and TCAB1 (lane T).

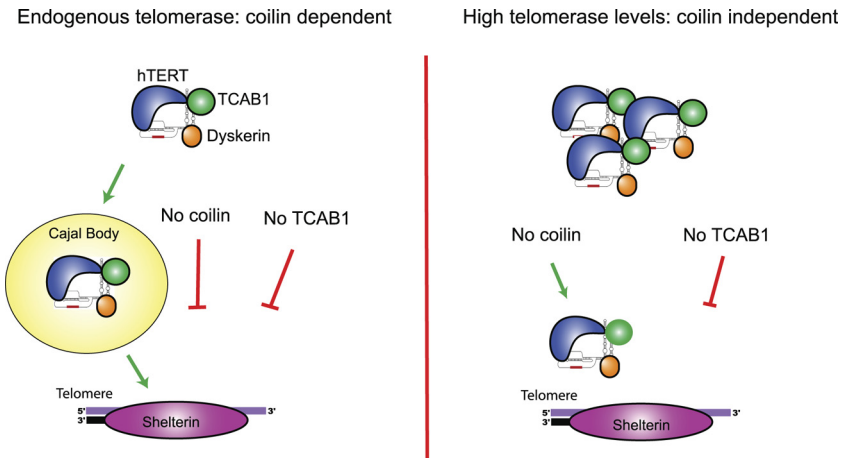

FIG 7 Multiple independent requirements for telomerase recruitment. Endogenous telomerase recruitment to telomeres depends on coilin and TCAB1, indicating a requirement for Cajal bodies. High telomerase levels circumvent the requirement for coilin but not for TCAB1, suggesting that TCAB1 may mediate a critical interaction that facilitates the ultimate steps in recruitment. The requirement for TCAB1 in telomerase recruitment is independent of the requirement for the hTERT DAT domain.

hTERT DAT domain (Fig. 7). The role of CB may be to concentrate limiting amounts of telomerase in one region of the nucleoplasm, whereas our data indicate that TCAB1 plays a direct role in transporting telomerase to the telomere.

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