TPP1 OB-Fold Domain Controls Telomere Maintenance by Recruiting Telomerase to Chromosome Ends

Franklin L. Zhong,[1,2] Luis F.Z. Batista,[1] Adam Freund,[1,2] Matthew F. Pech,[1,2] Andrew S. Venteicher,[1,3] and Steven E. Artandi[1,2,4,*]

[1]Department of Medicine
[2]Cancer Biology Program
[3]Biophysics Program
[4]Department of Biochemistry
Stanford University School of Medicine, Stanford, CA 94305, USA
*Correspondence: sartandi@stanford.edu

SUMMARY

Telomere synthesis in cancer cells and stem cells involves trafficking of telomerase to Cajal bodies, and telomerase is thought to be recruited to telomeres through interactions with telomere-binding proteins. Here, we show that the OB-fold domain of the telomere-binding protein TPP1 recruits telomerase to telomeres through an association with the telomerase reverse transcriptase TERT. When tethered away from telomeres and other telomere-binding proteins, the TPP1 OB-fold domain is sufficient to recruit telomerase to a heterologous chromatin locus. Expression of a minimal TPP1 OB-fold inhibits telomere maintenance by blocking access of telomerase to its cognate binding site at telomeres. We identify amino acids required for the TPP1-telomerase interaction, including specific loop residues within the TPP1 OB-fold domain and individual residues within TERT, some of which are mutated in a subset of pulmonary fibrosis patients. These data define a potential interface for telomerase-TPP1 interaction required for telomere maintenance and implicate defective telomerase recruitment in telomerase-related disease.

INTRODUCTION

The addition of telomere repeats to chromosome ends by the enzyme telomerase is essential to counter the incomplete replication of telomeres that occurs with cell division in stem cells and in cancer cells (Cech, 2004; Palm and de Lange, 2008; Artandi and DePinho, 2010; O'Sullivan and Karlseder, 2010). Disruption of this process by mutations in telomerase components causes stem cell dysfunction and results in a number of diseases in humans, including dyskeratosis conge-nita, aplastic anemia, pulmonary fibrosis, and multiple types of cancer (Savage and Alter, 2008; Calado and Young, 2009). Human telomerase consists of a minimal catalytic core, includ-ing the reverse transcriptase subunit TERT and the telomerase RNA component TERC, which are assembled into a mature enzyme along with additional holoenzyme proteins (Collins, 2008). To elongate telomeres, telomerase is thought to be recruited to chromosome ends through interactions with telo-mere-binding proteins, but the precise mechanisms of telome-rase recruitment remain incompletely understood.

Telomerase undergoes a highly orchestrated process of assembly and trafficking within the nucleus of human cells. TERC encodes the template for the reverse transcription reac-tion in telomere addition but also serves as the central scaffold for assembly of the telomerase ribonucleoprotein (RNP) (Cech, 2004; Zappulla and Cech, 2006; Egan and Collins, 2012). A newly transcribed TERC RNA molecule is bound and stabilized by the dyskerin core complex, which includes dyskerin, NHP2, and NOP10 (Darzacq et al., 2006). Loading of TERT into telomerase complexes generates an enzymatically active RNP, but this complex is unable to act on telomeres without completing additional trafficking and assembly steps in vivo. In human cancer cells and embryonic stem cells, telomerase localizes within Cajal bodies, which are nuclear sites of RNP modification and assembly (Gall, 2000; Jády et al., 2004; Zhu et al., 2004; Batista et al., 2011). RNA fluorescence in situ hybridization (FISH) studies using probes specific for TERC revealed that telomerase-containing Cajal bodies associated with a subset of telomeres specifically in S phase of the cell cycle (Jády et al., 2004; Zhu et al., 2004; Tomlinson et al., 2008). Concentra-tion of telomerase within Cajal bodies depends upon an interac-tion between the CAB box motif within TERC and TCAB1, which is a WD40 repeat protein that is part of the active telomerase holoenzyme (Cristofari et al., 2007; Tycowski et al., 2009; Ven-teicher et al., 2009). TCAB1 is required for telomere maintenance and is mutated in an autosomal-recessive form of dyskeratosis congenita (Venteicher et al., 2009; Zhong et al., 2011). Loss of TCAB1 function causes mislocalization of telomerase from Cajal bodies to nucleoli, cripples the ability of telomerase to maintain telomeres, and impairs recruitment of telomerase to

chromosome ends (Venteicher et al., 2009; Batista et al., 2011; Zhong et al., 2011; Stern et al., 2012). Depletion of the Cajal body scaffold coilin also blunts the ability of telomerase RNA to associate with telomeres, suggesting that Cajal bodies may be important for recruiting telomerase to telomeres (Stern et al., 2012).

In yeast, the telomere-binding protein Cdc13p positively regulates telomerase recruitment through an interaction with the telomerase component Est1p (Pennock et al., 2001; Taggart et al., 2002; Chan et al., 2008). In human cells, telomere-binding proteins exert both positive and negative effects on telomerase function. Human telomeres consist of long tracks of double-stranded repeats ending in a single-stranded overhang. Telomeric DNA repeats are bound by the six-protein shelterin complex (Smogorzewska and de Lange, 2004; de Lange, 2005; Verdun and Karlseder, 2007; Xin et al., 2008; O'Sullivan and Karlseder, 2010). TRF1 and TRF2, factors that bind double-stranded telomere repeats, inhibit telomerase function presumably by transducing telomere length information to the chromosome terminus (Smogorzewska et al., 2000). The single-stranded overhang is bound by a subcomplex of shelterin components in which POT1 directly contacts DNA and TPP1 bridges POT1 to TIN2, which connects to the TRF1-TRF2 double-stranded DNA-binding complex. Depletion of POT1 or TPP1 or overexpression of a POT1 variant with a deletion in the DNA-binding domain (POT1ΔOB) each leads to telomere elongation by telomerase, indicating that POT1 and TPP1 prevent telomerase action at telomeres (Loayza and De Lange, 2003; Ye and de Lange, 2004; Ye et al., 2004). In contrast to these genetic findings in cultured cells, biochemical studies in vitro have established that recombinant TPP1 and POT1 enhance processivity of telomerase on oligonucleotide substrates, suggesting that TPP1 and POT1 act as positive cofactors in telomerase catalysis (Wang et al., 2007; Zaug et al., 2010). These dual functions of the TPP1-POT1 complex in regulating telomerase function remain to be resolved.

Experiments designed to address telomerase recruitment in human cells have exploited "supertelomerase" (S-T) cells, which are cancer cells in which both TERT and TERC are overexpressed to enable telomerase detection at telomeres (Cristofari and Lingner, 2006; Cristofari et al., 2007; Abreu et al., 2010). Loss-of-function studies showed that TPP1 and TIN2 were required for efficient recruitment of telomerase to telomeres in S-T cells (Abreu et al., 2010). TERT has been shown to interact with the oligonucleotide/oligosaccharide-binding (OB)-fold of TPP1 (Xin et al., 2007), and this same domain of TPP1 was implicated in recruiting telomerase to telomeres in S-T cells (Abreu et al., 2010). However, TPP1 serves to tether POT1 to telomeres; therefore, inhibition of TPP1 leads to loss of the TPP1-POT1 complex from telomeres and the induction of a DNA damage response at telomeres (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004; Kibe et al., 2010; Tejera et al., 2010; Takai et al., 2011). In addition, loss of TPP1 in vivo is accompanied by reduced levels of TIN2 (Rai et al., 2011), suggesting that TPP1 might serve a structural role in the shelterin complex. Therefore, separating the putative recruitment function of TPP1 from its end-protection function is inherently challenging.

In this study, we investigate mechanisms of telomerase recruitment to telomeres in human cancer cells. We find that the OB-fold domain of TPP1 recruits telomerase to telomeres and that this is an essential step in telomere maintenance. We identify a putative interaction surface governing this interaction and show that this binding region is mutated in a subset of patients with diseases caused by telomerase mutations.

RESULTS

Neo-Cajal Bodies Form at Telomeres in Supertelomerase Cells

To investigate telomerase recruitment, we employed a modified S-T assay that uses transient, plasmid-based expression to overcome inherent limitations in expressing TERC from a retrovirus (Figures S1A–S1C available online). Elevated expression of TERC, together with hemagglutinin (HA)-tagged TERT, resulted in uniform colocalization of telomerase with telomeres by RNA FISH and by immunostaining with an anti-HA antibody, respectively (Figures 1A and 1B). In the absence of coexpressed TERC, HA-TERT was detected by immunofluorescence in a nucleoplasmic pattern. When transfected alone in cells lacking overexpressed TERT, transient TERC was found in Cajal bodies and rarely colocalized with telomeres (Figure S1D). To investigate whether other telomerase holoenzyme components were also found at telomeres, we performed immunofluorescence for dyskerin and TCAB1. Dyskerin and TCAB1 efficiently colocalized with telomeres in S-T cells but showed minimal overlap with telomeres in cells expressing HA-TERT alone, where they accumulated in their typical nuclear compartments, nucleoli and Cajal bodies, respectively (Figures 1C and 1D). Taken together, these results indicate that telomerase foci at telomeres in S-T cells contain the entire holoenzyme.

The striking localization of telomerase holoenzyme components to telomeric foci in S-T cells suggested the possibility that Cajal bodies were forming de novo at telomeres. To test this idea, we stained for telomeric DNA and for coilin, an established marker of Cajal bodies. Remarkably, we found colocalization of coilin at most telomeres in S-T cells, whereas in control cells, coilin was detected in classical Cajal bodies that typically did not colocalize with telomeres (Figure 1E). The number of total Cajal bodies in S-T cells (17.5 ± 5.5 per nucleus, n = 75) far exceeded that of cells expressing HA-TERT alone (3.0 ± 1.5 per nucleus, n = 150, $p < 0.001$ by Fisher's exact test) (Figure 1E), suggesting that telomerase overexpression results in "neo-Cajal bodies" at telomeres and that these new foci may contain other Cajal body components. To test this hypothesis, we stained S-T cells for other well-characterized Cajal body components, including the small Cajal-body-specific RNA (scaRNA) U85, fibrillarin, and SMN, which do not participate in telomerase function. In control cells expressing HA-TERT alone, scaRNA U85 was detected exclusively in three to five strong nuclear foci by RNA FISH, which is consistent with its Cajal body localization (Figure 1F, top); fibrillarin was found in both Cajal bodies and the nucleolus, which is consistent with its role in modification of splicing RNAs and ribosomal RNA (rRNAs) (Figure 1G, top); and SMN protein was detected primarily in 3–10 foci per nucleus, which is consistent with its localization in both nuclear gems and
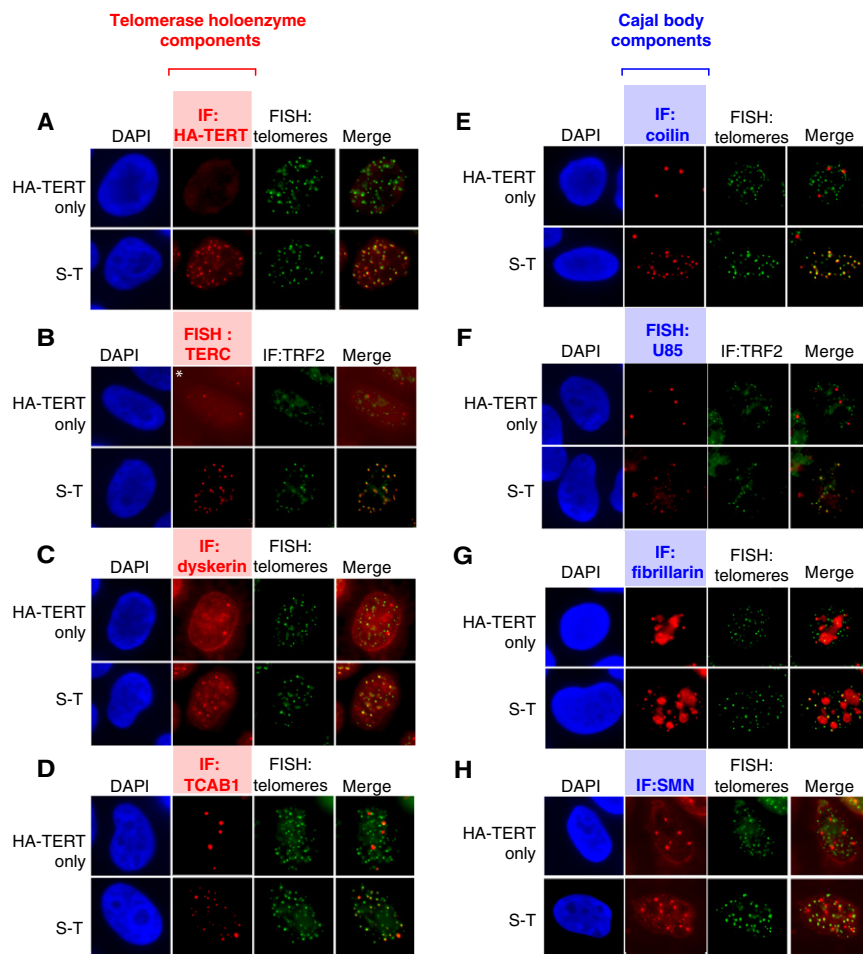
Figure 1. Telomerase Holoenzyme Components at Telomeres and Formation of Neo-Cajal Bodies in S-T Cells

HeLa cells transduced with an HA-TERT retrovirus were transfected with a TERC expression plasmid (S-T, supertelomerase) or empty vector (HA-TERT only).

(A–D) Immunofluorescence or FISH for telomerase components and telomeres using (A) anti-HA antibody (red), DNA FISH for telomeres (green); (B) RNA FISH for TERC (red), anti-TRF2 antibody (green), asterisk indicates longer exposure; (C) anti-dyskerin antibody (red), DNA FISH for telomeres (green); and (D) anti-TCAB1 antibodies (red), DNA FISH for telomeres (green).

(E–H) Immunofluorescence or FISH for Cajal body components and telomeres using (E) anti-coilin antibody (red), DNA FISH for telomeres (green); (F) RNA FISH for U85 (red), anti-TRF2 antibody (green); (G) anti-fibrillarin antibody (red), DNA FISH for telomeres (green); (H) anti-SMN antibodies (red), DNA FISH for telomeres (green).

See also Figure S1.

which is consistent with previous studies showing a requirement for TCAB1 in localization of endogenous TERC to telomeres (Figure S2A) (Venteicher et al., 2009; Stern et al., 2012). To determine whether Cajal bodies themselves are required for telomerase recruitment, we treated cells with coilin siRNA, which efficiently depleted coilin protein by western blot (Figure 2C) and resulted in loss of coilin-positive Cajal bodies in both HeLa cells and S-T cells (Figures 2D and 2E). Coilin depletion eliminated HA-TERT foci at telomeres in S-T cells (Figure 2D) without affecting HA-TERT protein levels or telomerase activity (Figures 2C, S2B, and S2C). These data show that coilin, which serves as a scaffold for assembly of Cajal bodies, is required for formation of telomerase foci at telomeres in S-T cells.

To understand the determinants at telomeres that control formation of telomerase foci, we used RNA interference to deplete TIN2 or TPP1, each of which had been implicated in telomerase recruitment (Abreu et al., 2010). Treatment of S-T cells with siRNAs against TIN2 or TPP1 efficiently depleted each protein (Figure 2F), which compromised telomere end protection, resulting in 53BP1-positive DNA damage foci at telomeres (Takai et al., 2003) (Figure S3). Loss of TIN2, but not TPP1, led to reduced TRF2 protein at telomeres, which is consistent with the role of TIN2 as a core shelterin component (Figure 2I) (de Lange, 2005; Takai et al., 2011). In agreement with previous work, depletion of either TIN2 or TPP1 resulted in a loss of HA-TERT foci colocalizing with telomeres (Figures 2G and 2H) (Abreu et al., 2010). Interestingly, instead of colocalizing with telomeres, HA-TERT was detected in a small number of bright foci that also stained positive for coilin in cells treated with siRNA against either TIN2 or TPP1 (2.5 ± 0.2 and 7.0 ± 0.4
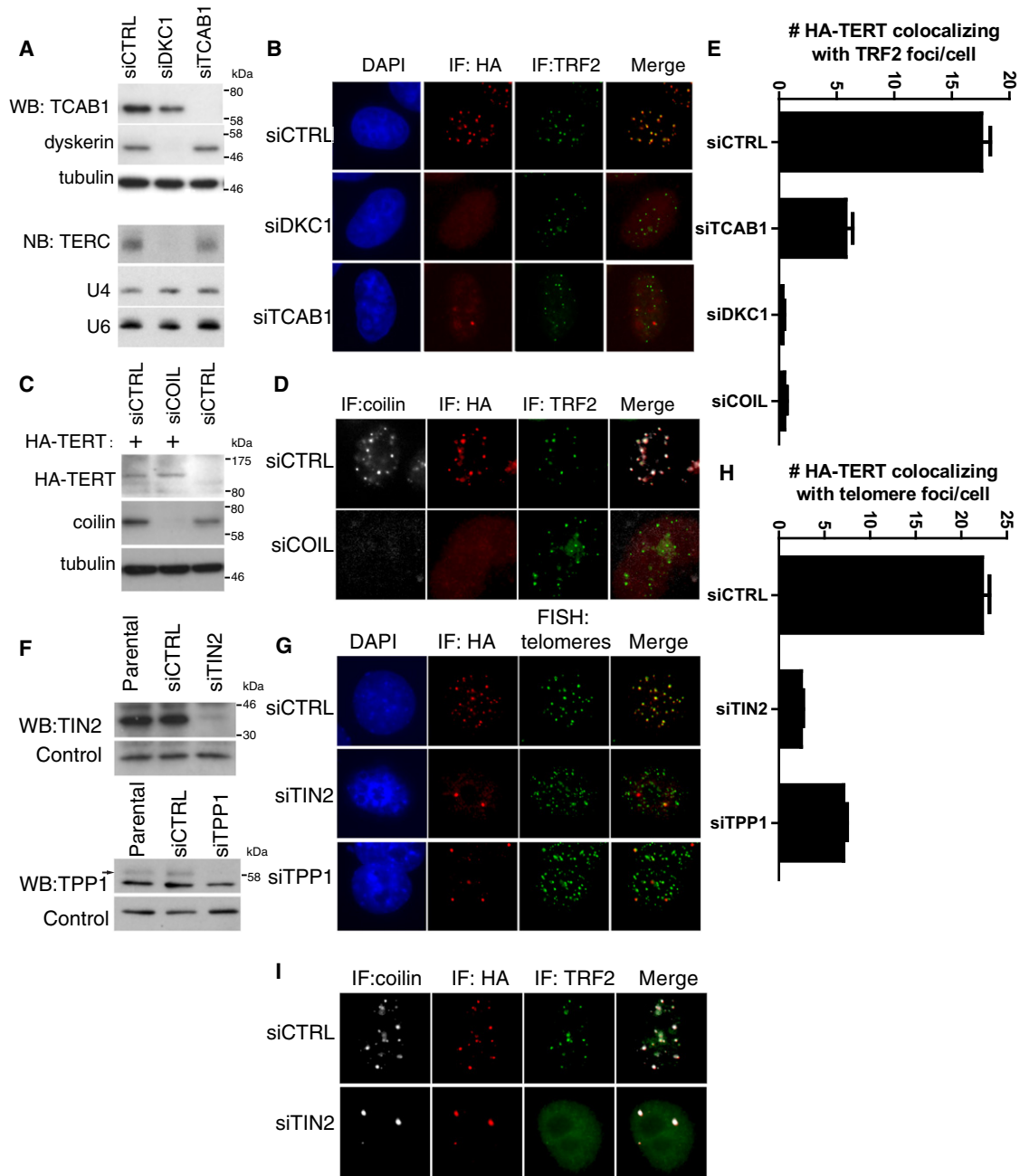
Cajal bodies (Figure 1H, top). In contrast, scaRNA U85, fibrillarin, and SMN were each readily detected at telomeres in S-T cells (Figures 1F–1H, bottom), indicating that the foci at telomeres in S-T cells resemble bona fide Cajal bodies and that overexpression of telomerase forms neo-Cajal bodies at telomeres.

Depletion of TIN2 or TPP1 Stalls Telomerase Recruitment in Conventional Cajal Bodies

To understand the requirements for formation of telomerase foci at telomeres, we depleted proteins implicated in telomerase recruitment to telomeres with short interfering RNAs (siRNAs). Dyskerin depletion led to a loss of TERC (Figure 2A) and eliminated TERT foci at telomeres in S-T cells, which is consistent with a requirement for TERC in telomerase recruitment to telomeres (Figure 2B, middle). Depletion of TCAB1 efficiently diminished the number of telomerase foci at telomeres and caused HA-TERT to mislocalize to nucleoli (Figures 2A and 2B, bottom). Some HA-TERT foci persisted at telomeres in siTCAB1-treated cells (Figure 2G; 17.6 ± 0.8 in control siRNA-treated cells versus 5.8 ± 0.6 in siTCAB1-treated cells, p < 0.0001 by Fisher's exact test), which is likely due to incomplete depletion of the protein (data not shown). These results indicate that TCAB1 is needed for efficient recruitment to telomeres,

Figure 2. Depletion of TIN2 or TPP1 Stalls Telomerase Recruitment in Conventional Cajal Bodies

(A) Western blot for TCAB1 and dyskerin in S-T HeLa cells treated with siRNAs indicated (top). Northern blot for TERC (bottom). U4 and U6 RNAs, loading controls.

(B) S-T HeLa cells were transfected with siRNAs indicated, followed by immunofluorescence using anti-HA antibody (red) and anti-TRF2 antibody (green).

(C) Western blot for HA-TERT and coilin in S-T cells treated with siRNAs indicated. Tubulin, loading control.

(D) S-T HeLa cells were transfected with coilin siRNAs, followed by immunofluorescence using antibodies against coilin (white), HA-TERT (red), and TRF2 (green).

(E) Quantification of (B) and (D), >100 nuclei were scored for number of HA-TERT foci at telomeres. Error bars represent SEM; $p < 0.005$ by Student's t test.

(F) Western blot for TIN2 and TPP1 in S-T HeLa cells treated with indicated siRNAs. Nonspecific bands, loading controls.

(G) S-T HeLa cells were transfected with TIN2 or TPP1 siRNAs, followed by immunofluorescence using antibodies against HA-TERT (red) and DNA-FISH with a telomere probe (green).

(H) Quantification of (G), >100 nuclei were scored for the number of HA-TERT foci at telomeres. Error bars represent SEM; $p < 0.005$ by Student's t test.

(I) S-T HeLa cells were transfected with TIN2 siRNAs, followed by immunofluorescence using antibodies against coilin (white), HA-TERT (red), and TRF2 (green).

See also Figures S2 and S3.

A

HA-mCherryl-lacI tag   TPP1 variants
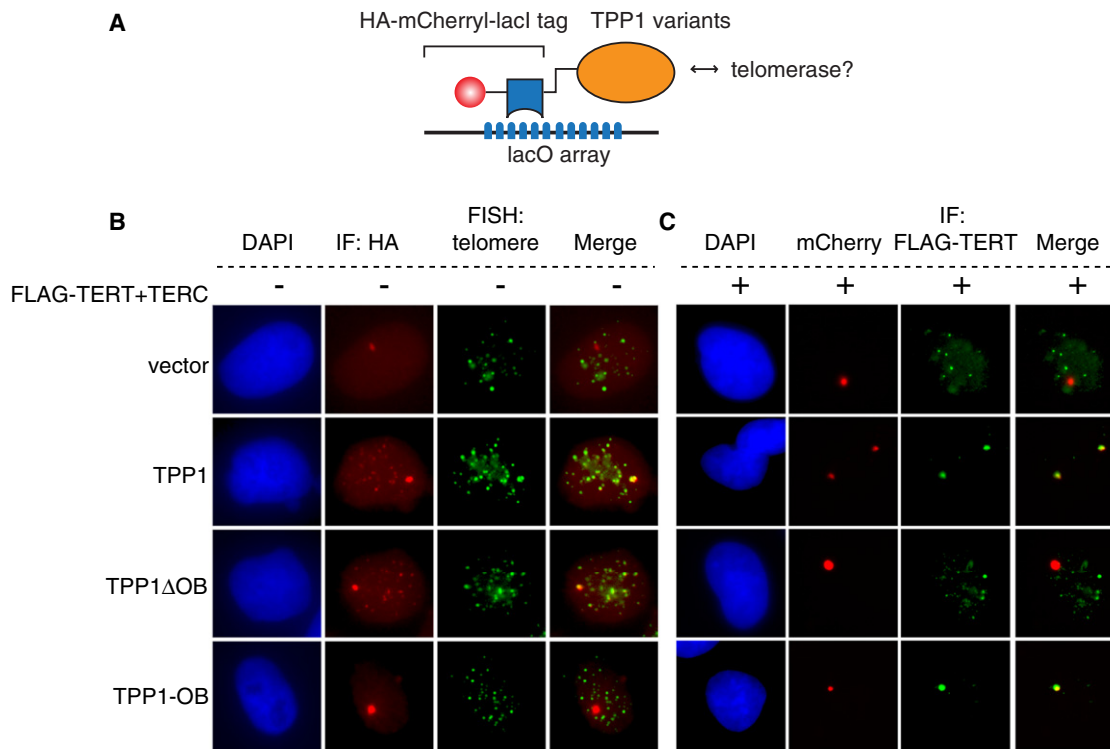
↔ telomerase?

lacO array

Figure 3. A Tethered TPP1 OB-Fold Domain Recruits Telomerase to a Nontelomeric Locus
(A) Tethering system uses TPP1 variants fused with an HA-mCherry-lacI tag transfected in cells containing an integrated lacO array.
(B) U2OS2-6-3 cells transfected with HA-mCherry-lacI-tagged TPP1 variants were stained for HA (red), followed by telomere-FISH (green).
(C) U2OS2-6-3 cells transfected with FLAG-TERT, TERC, and indicated HA-mCherry-lacI-tagged TPP1 variants were analyzed for TPP1 by mCherry epifluorescence (red) and for TERT by immunofluorescence with anti-FLAG (green).

per nucleus for siTIN2 and siTPP1, respectively, versus 22.4 ± 0.8 in control-treated cells) (Figures 2G–2I). The number and morphology of these foci were indistinguishable from Cajal bodies in control HeLa cells (data not shown), indicating that loss of TIN2 or TPP1 arrests telomerase in Cajal bodies.

A Tethered TPP1 OB-Fold Domain Recruits Telomerase to a Nontelomeric Chromatin Locus

Studying telomerase recruitment by using loss-of-function approaches is limited by the following: (1) the interdependence of many shelterin components for optimal accumulation (Rai et al., 2011; Takai et al., 2011) and (2) the fact that perturbing shelterin proteins induces a DNA damage response at telomeres, which could in turn affect recruitment of telomerase. To test potential interactions between telomerase and candidate interacting partners outside the context of both the shelterin complex and telomeric DNA, we employed a tethering strategy that allows the expression of a lacI fusion protein "bait" to be visualized as a strong single nuclear focus at a multimerized lacO array stably integrated into a single genomic locus in U2OS2-6-3 cells (Janicki et al., 2004) (Figure 3A). We used this approach to study potential interactions between telomerase and TPP1 at a heterologous chromatin site and in isolation from the effects of DNA damage responses at uncapped telomeres. Whereas HA-lacI-mCherry localized only in a single lacO array focus in the nucleus, the HA-lacI-mCherry-TPP1

fusion protein localized both to the lacO array and to telomeres, indicating that the TPP1 fusion protein retains the ability to be incorporated into the shelterin complex at telomeres (Figure 3B, first and second row). In addition, telomere signals were readily detected within the HA-lacI-mCherry-TPP1 focus at the lacO array by using a telomere FISH probe, indicating that the immobilized TPP1 fusion protein recruits telomeres to the lacO focus. In U2OS2-6-3 cells cotransfected with the HA-lacI-mCherry tag, Flag-TERT and TERC, HA-lacI-mCherry remained in a single lacO array focus and did not interfere with the ability of telomerase to localize to telomeres (Figure 3C, first row; data not shown). In contrast, in cells expressing HA-lacI-mCherry-TPP1, FLAG-TERT localization to telomeres was diminished, and instead, FLAG-TERT was detected, together with HA-lacI-mCherry-TPP1, in the lacO array focus (Figure 3C, second row). In this setting, HA-lacI-mCherry-lacI-TPP1 acted as a sink to preferentially recruit telomerase to the lacO array, effectively competing for telomerase-binding sites at telomeres.

To determine whether the OB-fold of TPP1, previously implicated in binding TERT (Xin et al., 2007; Abreu et al., 2010), mediated recruitment of telomerase to the lacO array, we constructed a fusion protein lacking the OB-fold (HA-lacI-mCherry-TPP1ΔOB) and a minimal fusion protein comprising only the OB-fold of TPP1 (amino acids 87–250) (HA-lacI-mCherry-TPP1OB). HA-lacI-mCherry-TPP1ΔOB localized to the lacO array and to telomeres (Figure 3B, third row) but was

unable to recruit telomerase to the lacO array and could no longer compete telomerase away from telomeres (Figure 3C, third row). Conversely, HA-lacI-mCherry-TPP1OB, which localized only to the lacO array (Figure 3B, bottom row), effectively recruited FLAG-TERT to the lacO array and blocked telomerase binding to telomeres (Figure 3C, bottom row). Importantly, telomeres were not detected at the lacO focus in cells expressing HA-lacI-mCherry-TPP1OB (Figure 3B, bottom row), indicating that HA-lacI-mCherry-TPP1OB likely recruited telomerase in the absence of other shelterin components. Taken together, these results show that the OB-fold domain of TPP1, when isolated from telomeric DNA and other shelterin components, is necessary and sufficient to recruit telomerase to a heterologous chromatin locus.

Specific Loop Residues within the TPP1 OB-Fold Mediate Recruitment of Telomerase to Telomeres

To further understand the interaction between telomerase and the TPP1 OB-fold domain, we tested whether overexpressed TPP1 OB-fold could effectively compete with endogenous TPP1 for telomerase binding. Because TPP1-OB lacks the ability to be incorporated into the shelterin complex at telomeres, we reasoned that an isolated and untethered TPP1-OB would sequester telomerase away from telomeres in a dominant-negative manner. To investigate this hypothesis, we developed a "competitive sequestration" assay. Specifically, mCherry-tagged TPP1 OB-fold was cotransfected along with green fluorescent protein (GFP)-TERT and TERC in HeLa cells (Figures 4A–4C). Similar to S-T cells described above, GFP-TERT localized to telomeres in the presence of TERC (Figure 4C, top row). In contrast to the mCherry vector, expression of mCherry-TPP1 OB (mCherry-OB) abolished localization of GFP-TERT to telomeres and caused GFP-TERT to be sequestered within conventional Cajal bodies (Figure 4C, middle row), which are results reminiscent of TPP1 depletion in S-T HeLa cells (Figure 2G). Unexpectedly, mCherry-OB itself strongly accumulated within Cajal bodies together with GFP-TERT, indicating that a telomerase-mCherry-OB complex was sequestered in Cajal bodies. Localization of mCherry-OB in Cajal bodies was not observed without coexpressed TERT and TERC. Taken together, we conclude that overexpressed TPP1 OB-fold acts as a competitive inhibitor of telomerase recruitment, presumably by blocking an interacting surface on telomerase that is engaged by endogenous TPP1 during normal telomerase action at telomeres. These results further support the necessity and sufficiency of TPP1-OB in recruiting telomerase from Cajal bodies to telomeres.

The structure of the TPP1 OB-fold domain is closely related to the structure of certain OB-folds in telomere-associated or telomerase-associated proteins from other species (Wang et al., 2007; Xin et al., 2007). In *Saccharomyces cerevisiae* and *Candida albicans*, Est3 is a telomerase-associated cofactor whose OB-fold shows structural similarity to TPP1 OB-fold when modeled using structure prediction algorithms. Sequences within Est3 that are responsible for binding yeast telomerase have been identified using functional and biochemical assays (Lee et al., 2008; Yu et al., 2008). Using structure-guided mutagenesis, we sought to identify specific amino acids in the

TPP1 OB-fold domain required for association with human telomerase. We chose residues that were solvent exposed based on the TPP1 OB-fold crystal structure, including those that were conserved in mammals, present in loop regions connecting β strands, and near the analogous Est3-yeast telomerase association site (Figure S4). Using the competitive sequestration assay described above, we tested each TPP1-OB variant for its ability to inhibit telomerase localization to telomeres and to sequester telomerase within Cajal bodies (Figure S5).

Many mutations in TPP1-OB had no effect on the efficiency of mCherry-OB in blocking localization of GFP-TERT to telomeres (Figures 4D and S5), indicating that these residues are dispensable for TPP1-OB association with TERT. These included double mutants R159A;E160A and D163A;T164A, both of which reside in a short α helix (helix αβ) (Figures 4D and S5A–S5D). Mutation of a conserved serine in loop L_{A1} (S111A) similarly had no effect on the activity of mCherry-OB (Figures 4D and S5A–S5D). In marked contrast, a double charge swap mutation—D166R;E168R, hereafter referred to as OB-RR—in conserved residues in loop L_{34} completely eliminated the activity of mCherry-OB (Figures 4A and 4C). mCherry-OB-RR was expressed at similar levels compared to wild-type mCherry-OB but failed to inhibit localization of GFP-TERT to telomeres and as a result was not detected in Cajal bodies (Figures 4B and 4C, bottom row). Deconvolution of this double mutant revealed that E168R was more severely impaired in its ability to sequester telomerase away from telomeres than D166R (Figures 4C and S5A–S5D), suggesting that the inactivity of the OB-RR mutant is largely due to mutation of E168. Introduction of K170A at a nearby residue in the same loop caused a modest reduction in the activity of mCherry-OB in this assay (Figures 4A, 4D, and S5A–S5D). Introduction of the RR mutations into full-length TPP1 revealed that both wild-type mCherry-TPP1 and mCherry-TPP1-RR localized to telomeres in HeLa cells. Although wild-type mCherry-TPP1 did not interfere with the localization of GFP-TERT at telomeres, mCherry-TPP1-RR effectively blocked the ability of GFP-TERT to localize to telomeres, resulting in localization of GFP-TERT to Cajal bodies (Figures 4E and 4F). In this case, mCherry-TPP1-RR inhibited telomerase recruitment to telomeres, presumably by competing away endogenous TPP1 from the shelterin complex and replacing it with a mutant defective in the ability to associate with telomerase. Taken together, these data show that residues D166, E168, and K170 are required for telomerase association and may define a critical surface required for interaction between TPP1 and telomerase.

Telomerase-TPP1 OB-Fold Association Is Essential for Telomere Maintenance

To test the functional significance of the telomerase-TPP1 OB interaction in telomere length maintenance, we tested whether isolated TPP1 OB-fold domain could prevent telomerase from elongating telomeres. We used retroviral transduction to express wild-type TPP1 OB-fold domain, TPP1-OB-RR, or GFP as a negative control in HTC75 cells, a telomerase-positive fibrosarcoma cell line widely used to study telomere maintenance (Smogorzewska et al., 2000). After selection, each culture was
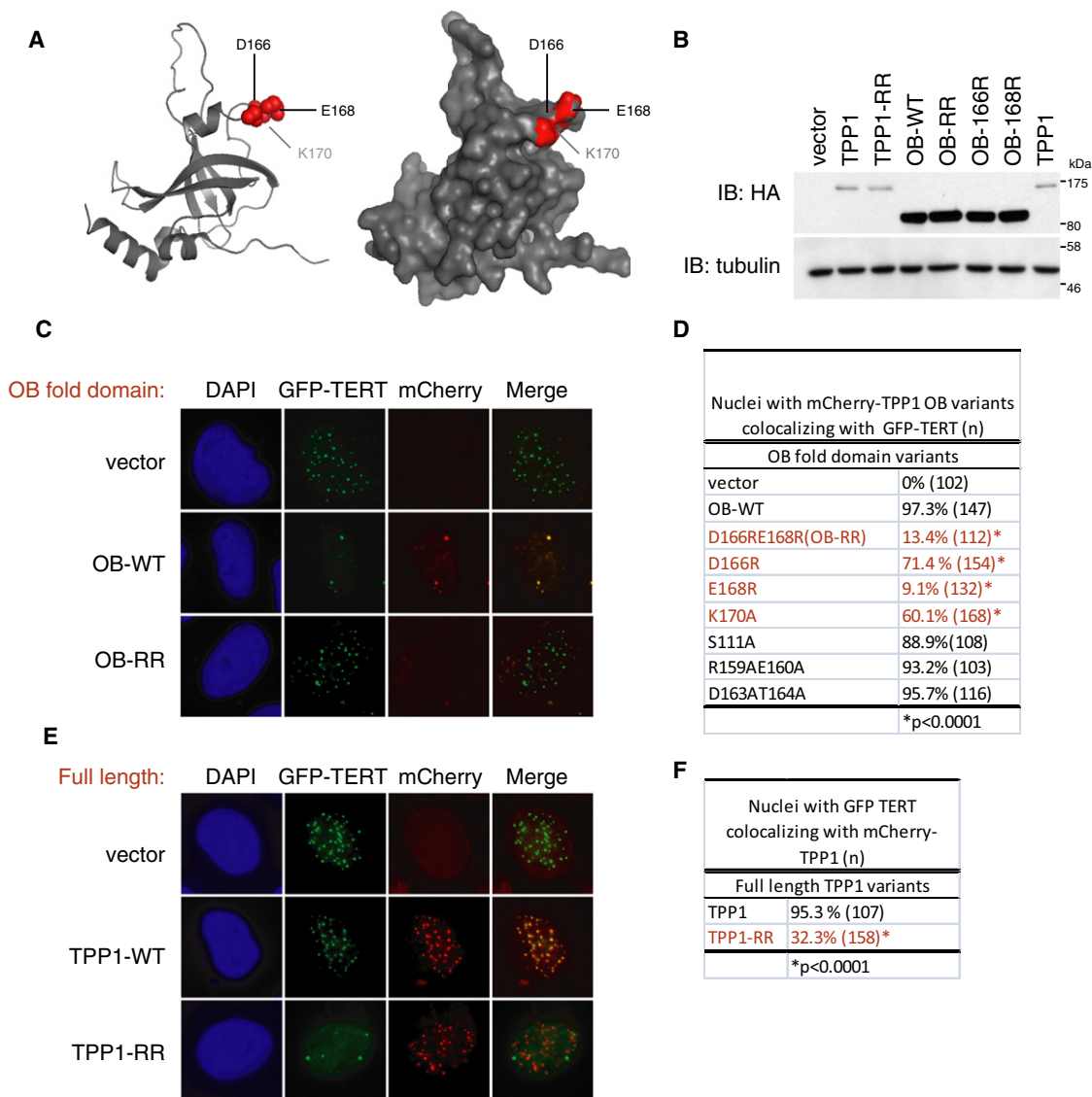
Figure 4. Loop Residues in TPP1-OB Are Required for Telomerase Recruitment

(A) Structural representation of TPP1-OB domain (PDB 2i46). Residues required for telomerase interaction shown in red.

(B) Western blot for TPP1 variants transfected in HeLa cells, assayed by using anti-HA antibody.

(C) Competitive sequestration assay in HeLa cells transfected with GFP-TERT and TERC together with mCherry-tagged TPP1-OB, TPP1-OB-D166RE168R (OB-RR), or empty vector. Epifluorescence for GFP-TERT (green) and mCherry-OB (red).

(D) Quantification of colocalization between mCherry-TPP1-OB mutants and GFP-TERT from assay in (C). More than 100 nuclei scored. p value, Fisher's exact test.

(E) Competitive sequestration assay in HeLa cells transfected with GFP-TERT, TERC, and mCherry-tagged full-length TPP1 or TPP1-D166RE168R (TPP1-RR). Epifluorescence for GFP-TERT (green) and mCherry-OB (red).

(F) Quantification of (E). More than 100 nuclei scored. p value, Fisher's exact test.

See also Figures S4 and S5.

transduced either with an empty vector or with a retrovirus expressing Myc-POT1(ΔOB), which is a POT1 variant that lacks the N-terminal OB-fold domain. Myc-POT1(ΔOB) causes rapid telomere elongation by telomerase, presumably by relieving inherent negative regulation at the chromosome terminus (Loayza and De Lange, 2003). As expected, telomeres significantly elongated in cells expressing GFP and Myc-Pot1(ΔOB) through successive population doublings (Figure 5A, lane 1

versus 4 and 7 versus 10). In comparison, telomere elongation by Myc-Pot1(ΔOB) was abrogated by prior expression of wild-type TPP1-OB (Figure 5A, lane 2 versus 5 and 8 versus 11). This inhibitory effect of TPP1-OB was dependent on its association with telomerase because expression of TPP1-OB-RR exerted no inhibitory effect on telomere elongation by telomerase in Myc-Pot1(ΔOB) cells. Furthermore, telomeres in cells expressing wild-type TPP1-OB (without Myc-POT1(ΔOB)) showed
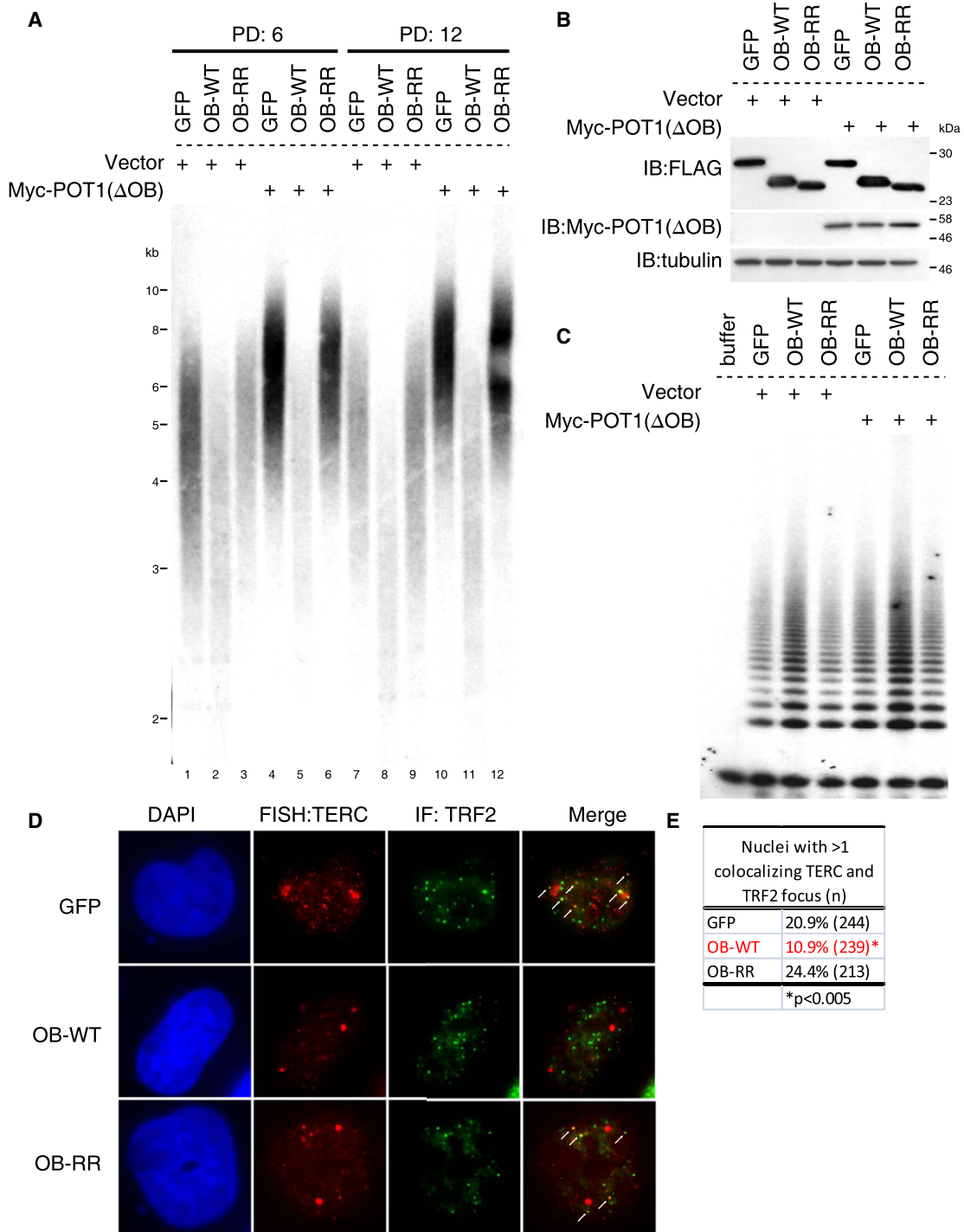
Figure 5. TPP1-OB Inhibits Telomere Length Maintenance by Telomerase and Blocks Endogenous Telomerase Recruitment

(A) Telomere lengths by Southern blot in HTC75 cells transduced first with retroviruses expressing FLAG-GFP, FLAG-OB-WT, or FLAG-OB-RR, followed by transduction with either empty vector or Myc-POT1(ΔOB). Genomic DNA harvested at population doubling (PD) 6 and 12 for Southern.

(B) Western blots for expression of OB variants and Myc-POT1(ΔOB) in cells used in (A).

(C) TRAP assays for telomerase activity in cells used in (A).

(D) RNA FISH for endogenous TERC colocalization (red) with TRF2 (green) by immunofluorescence using anti-TRF2 antibody in HeLa cells transduced with retroviruses expressing GFP, TPP1-OB, or TPP1-OB-RR. Arrows, TERC foci colocalizing with telomeres.

(E) Quantification of TERC and TRF2 colocalization in (D). More than 200 nuclei scored. p value, Fisher's exact test.

See also Figure S6.

rapid telomere shortening as compared to GFP-expressing cells in which telomere lengths were maintained with passage. After 12 population doublings, the mean telomere length in cells expressing TPP1-OB was 1.5 kb shorter than cells expressing GFP (Figure 5A, lane 7 versus 8). In contrast, TPP1-OB-RR showed no effect on telomere maintenance (Figure 5A, lane 1 versus 3 and 7 versus 9), despite similar expression compared to wild-type TPP1-OB protein (Figure 5B). The strong inhibitory effect of TPP1-OB was not due to a reduction in telomerase catalytic function; catalytic assays performed on extracts from these cells showed no inhibition of enzymatic activity by expression of TPP1-OB (Figure 5C). In addition, TPP1-OB did not interfere with cell growth (Figures S6A and S6B) or telomere protection, as there was no increase in DNA damage foci at telomeres (Figures S6C and S6D) when the telomere lengths were assayed. These findings demonstrate that TPP1 OB-fold inhibits telomere length maintenance by telomerase both at the basal level and in the context of rapid telomere elongation induced by POT1(ΔOB).

To understand how expression of TPP1-OB variants affected recruitment of endogenous telomerase to telomeres, we performed RNA FISH for endogenous TERC in HeLa cells stably transduced with TPP1-OB or TPP1-RR. The frequency of cells harboring TERC foci that overlapped with TRF2 was significantly diminished by overexpression of TPP1-OB, but not by TPP1-OB-RR (Figures 5D, 5E, and S6E). These findings corroborate our observations from our competitive sequestration assay (Figure 4) at the endogenous level and provide functional evidence that association between the OB-fold domain of TPP1 and telomerase is essential for telomerase to be efficiently recruited to telomeres and to synthesize telomere repeats.

IPF Mutations in TERT Block Recruitment and Show Diminished Association with TPP1 OB-Fold

Based on these findings indicating a functionally important association between TPP1 and telomerase, we hypothesized that specific amino acid residues within TERT are required for its recruitment to telomeres. To address this question, we carried out domain-mapping studies on TERT by generating a panel of TERT deletion mutants (Figure S7A). HA-tagged TERT mutants and TERC were coexpressed in HeLa cells and assayed for their localization by triple immunofluorescence staining for HA-TERT, TRF2, and coilin. We found three distinct classes of localization patterns for these deletion mutants (Figure S7A). Whereas wild-type TERT showed robust colocalization with TRF2, the N-terminal truncations of TERT were detected in a nucleoplasmic pattern (Figure 6B; ΔTEN). C-terminal deletions within TERT abrogated telomere localization but instead showed strong accumulation in coilin-positive Cajal bodies (Figure 6B; ΔCTE). Based on these results, we conclude that Cajal body localization and telomere association are each governed by distinct structural domains of TERT. The N-terminus of TERT, including the TEN and TRBD domains, is essential for Cajal body localization, whereas the CTE is required for recruitment to telomeres.

Upon establishing the roles of the TEN and CTE domains in telomerase trafficking, we examined a panel of disease-associated or engineered TERT point mutants in the TEN and CTE domains (Figure 6A). Four mutants—G100V, V144M, E1117X, and F1127NAA (C-DAT)—were found to be defective in localizing to telomeres when coexpressed with TERC (Figures 6C and 6D) and strongly accumulated in Cajal bodies. This pattern was reminiscent of the relocalization of TERT into Cajal bodies upon depletion of TPP1 or upon expression of the OB-fold of TPP1 (Figures 2 and 4). To determine whether these mutants failed to be recruited to telomeres because of a defect in association with TPP1, we coexpressed each mutant with mCherry-TPP1-OB. Three of the four mutants—G100V, V144M, and E1117X—were significantly impaired in capturing mCherry-TPP1-OB into Cajal bodies, which is consistent with a defect in association between these mutant TERT proteins and the OB-fold of TPP1. The C-DAT mutant retained the ability to colocalize within mCherry-OB in Cajal bodies, although mCherry fluorescence intensity was reduced in Cajal bodies as compared to wild-type TERT, which is evidence for weaker association with mCherry-OB (Figures 6E and 6F). These data indicate that the mutations in the TEN and CTE domains of TERT block association with TPP1 OB-fold, and this defect explains their inability to be recruited to telomeres.

Importantly, the ability of the examined TERT variants to localize to telomeres was unrelated to their catalytic activity measured in vitro (Figure S7B). The V144M and E1117fsX mutants derive from patients with idiopathic pulmonary fibrosis (Yamaguchi et al., 2005; Armanios et al., 2007; Tsakiri et al., 2007). Although E1117fsX has diminished catalytic activity, V144M retained wild-type activity in in vitro assays (Tsakiri et al., 2007; Tsang et al., 2012). Our results suggest that defective trafficking from Cajal bodies to telomeres underlies the telomerase dysfunction in patients with the V144M mutation. G100V is an engineered mutation in the TEN domain and has been shown to be essential for the enhancement of telomerase processivity mediated by recombinant TPP1-POT1 (Zaug et al., 2010). Thus, an impaired interaction between TERT-G100V and TPP1-OB-fold may explain both the absence of processivity enhancement in this mutant and the defect in telomerase recruitment. F1127NAA (C-DAT) is an engineered mutation that "dissociates activities of telomerase" (DAT) by preserving enzymatic function while interfering with the ability of telomerase to immortalize primary human cells (Banik et al., 2002; Armbruster et al., 2003). It is worth noting that all the recruitment-disrupting mutations lie within or close to the previously characterized DAT domains in the TEN and CTE domains, raising the possibility that the DAT domains represent structural motifs directly involved in recruitment. Taken together, these data show that specific residues within TERT govern recruitment to telomeres via interaction with the TPP1-OB-fold domain and that this recruitment step is impaired in a subset of patients with pulmonary fibrosis.

DISCUSSION

A Stepwise Model for Telomerase Recruitment to Telomeres

Recruitment of large protein complexes to their sites of action on chromatin is a critical rate-limiting step for many biological processes, such as the formation of kinetochores at
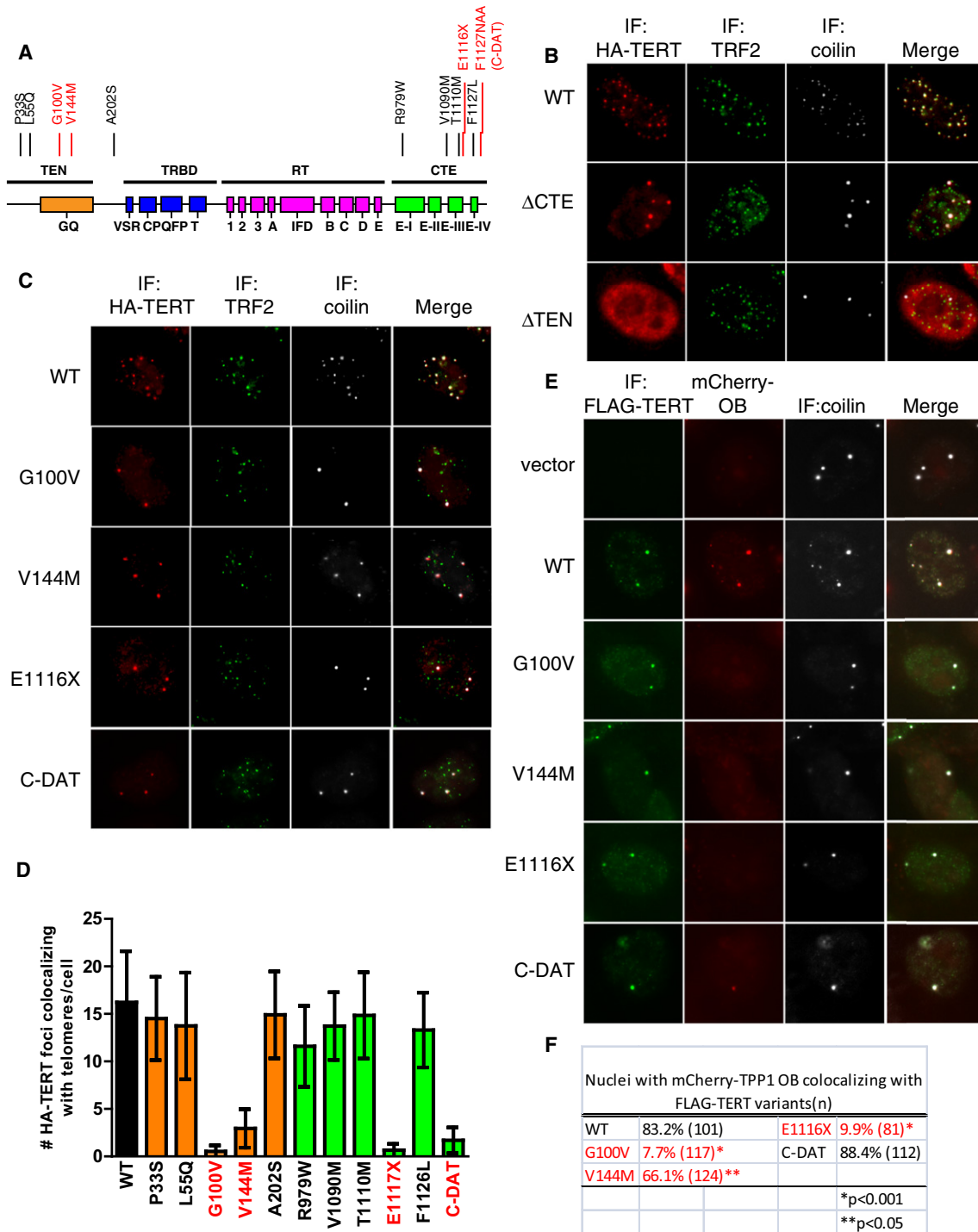
Figure 6. Subset of TERT Mutations in IPF Arrest Telomerase in Cajal Bodies due to Impaired Interaction with TPP1-OB

(A) Summary of TERT functional domains adapted from Podlevsky and Chen (2012). Point mutations in the TEN and CTE domains indicated; recruitment-defective mutants V144M, G100V, E1117X, and C-DAT highlighted in red.

(B) Localization of HA-tagged TERT deletion variants in HeLa cells also transfected with TERC. Immunofluorescence using antibodies against HA (red), TRF2 (green), and coilin (white).

(C) Immunofluorescence for HA-TERT (red), TRF2 (green), and coilin (white) in HeLa cells transfected with TERT point mutants in the TEN and CTE domains, along with TERC.

(D) Quantification TERT mutant colocalization with telomeres from (C). At least 100 nuclei scored. Error bars represent SEM. p < 0.0001 for TERT mutants in red, by two-tailed Student's t test.
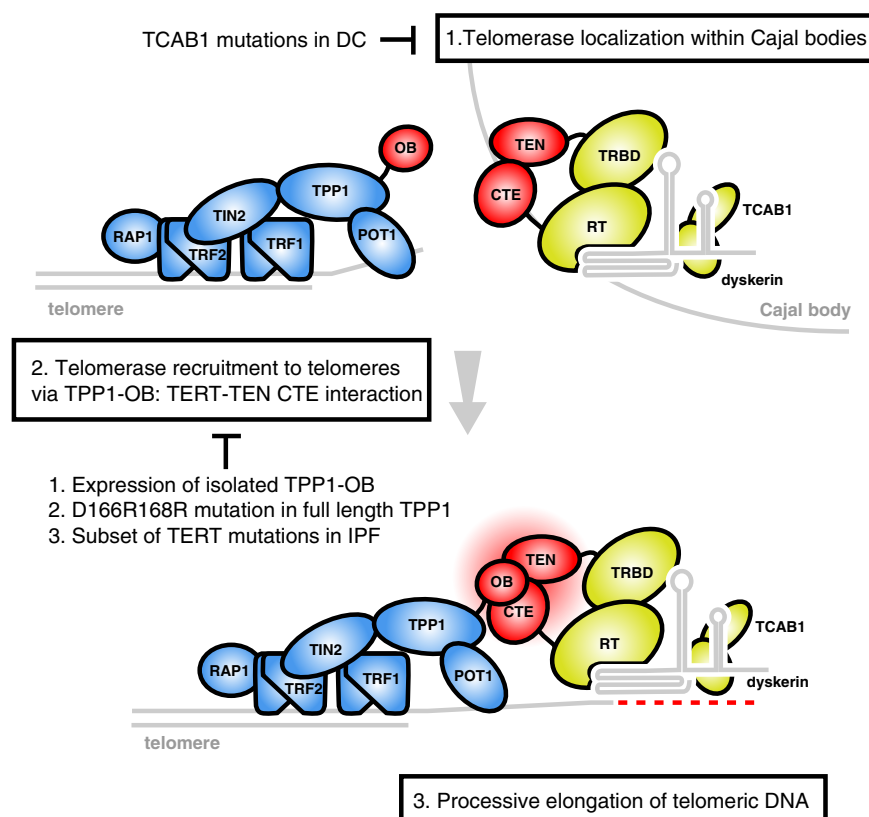
Figure 7. A Stepwise Model for Telomerase Recruitment to Telomeres

Telomerase accumulates in Cajal bodies, which is a step that is disrupted in patients with mutations in TCAB1 (1). Association between the TPP1 OB-fold and the TEN and CTE domains of TERT (colored red) mediates recruitment to telomeres, which is a step that is dependent on specific OB-fold loop residues and sequences in TERT mutated in some IPF patients (2). Once recruited to telomeres, TPP1-telomerase association may support processive elongation of telomeres (3).

protein coilin can reduce telomerase foci at telomeres (Figure 2) (Stern et al., 2012). Together, these findings establish the Cajal body as an important reservoir for telomerase, but it remains uncertain precisely what function is served by Cajal bodies and whether the bodies themselves are required for telomere synthesis by telomerase.

TPP1 OB-Fold: A Docking Platform for Telomerase Recruitment and an Enzymatic Cofactor for Processivity

In the second step (Figure 7, step 2), telomerase RNP is recruited to telomeres via

centromeres and the origin-of-replication complexes on DNA. Similarly, telomerase must be recruited to telomeric chromatin to synthesize telomere repeats, although this process has been difficult to dissect in human cells. In this study, we identified an obligatory interaction between the telomerase RNP and the OB-fold domain of the shelterin component TPP1 that recruits telomerase from a Cajal body reservoir to telomeric chromatin. Blocking the telomerase-TPP1 OB-fold interaction inhibited telomerase recruitment to telomeres and abrogated telomere synthesis by telomerase in vivo.

Our findings suggest a stepwise mechanism for recruitment of the telomerase RNP to telomeres (Figure 7). In the first step, telomerase localizes to Cajal bodies by virtue of the interaction between the TERC CAB-box sequence and the Cajal body-enriched telomerase holoenzyme component TCAB1 (Figure 7, step 1, top). The importance of the Cajal body is suggested by observations showing that mislocalization of telomerase to the nucleolus, either by TCAB1 depletion (Figure 2) or by TERC CAB-box mutations, inhibits telomerase recruitment and telomere synthesis by telomerase (Cristofari et al., 2007; Venteicher et al., 2009; Zhong et al., 2011). Enhanced loading of telomerase on telomeres in S-T cells leads to formation of neo-Cajal bodies at telomeres, and furthermore, depletion of the Cajal body

an obligatory "docking" step, whereby the TERT TEN and CTE domains interact with the OB-fold domain of TPP1 (Figure 7, step 2). The importance of this interaction is supported by a number of observations. First, removal of TPP1 from telomeres by using siRNAs against TPP1 or TIN2 abrogated the ability of overexpressed telomerase to localize to telomeres and led to strong accumulation in Cajal bodies (Figure 2) (Abreu et al., 2010). Second, tethered TPP1 OB-fold alone was able to recruit telomerase to a heterologous nontelomeric locus (Figure 3). Third, the expression of TPP1 OB-fold competitively sequestered overexpressed telomerase away from telomeres into Cajal bodies. Furthermore, the TPP1-OB domain itself was captured by telomerase in this context, localizing in Cajal bodies (Figure 4). Fourth, mutations in TPP1 OB-fold, such as OB-RR, or TERT mutations in the TEN and CTE domain (Figure 6) abrogated this interaction and inhibited telomerase recruitment, again arresting telomerase in its prerecruitment, Cajal-body-localized state.

In the third step (Figure 7, step 3), telomerase engages with telomeric DNA substrates and processively synthesizes telomeric repeats. Previously, the TPP1-POT1 heterodimer has been shown to aid telomerase catalysis by enhancing its processivity on oligonucleotide substrates in vitro. A single amino acid

(E) Competitive sequestration assay in HeLa cells transfected with FLAG-TERT mutants and TERC together with mCherry-TPP1-OB. Immunofluorescence using anti-FLAG antibody (green) and anti-coilin antibody (white) together with mCherry epifluorescence to detect mCherry-TPP1-OB (red).
(F) Quantification of (E). At least 100 nuclei scored for colocalization of mCherry-OB and FLAG-TERT. p values, Fisher's exact test.
See also Figure S7.

mutation in TERT(G100V) abrogated the processivity enhancement by TPP1-POT1 (Zaug et al., 2010). We found that the same mutation severely impairs TERT-TPP1 OB interaction and telomerase recruitment. Based on these observations, we postulate that the processive elongation of telomeric DNA and telomerase recruitment rely on the same TERT TEN-CTE: TPP1-OB interaction, which persists after the docking step and throughout the entire duration of telomerase catalysis (Figure 7, bottom). In other words, TERT TEN-CTE: TPP1-OB interaction not only recruits telomerase to telomeres, but also allows telomerase to be tethered to the shelterin-bound telomeric DNA substrate, preventing premature release of the telomere substrate and/or aiding telomerase translocation on telomeric tracts (Latrick and Cech, 2010). It is possible that the residues that we have identified in the TPP1 OB-fold—D166, E168 and K170—may contribute to the actual interface at which TPP1 and telomerase interact. These residues in the L_{34} loop are solvent exposed, and form a ridge along a groove running across the TPP1 OB-fold structure. The development of small molecules targeting this region of the OB-fold could act as telomerase inhibitors to block telomere synthesis in an analogous fashion to the effects of overexpressed OB-fold that we describe here.

Dual Function of the Shelterin Complex in Telomerase Regulation

The shelterin complex has consistently been found to inhibit telomerase in its ability to lengthen telomeres. This role of the shelterin complex was supported by both loss-of-function and gain-of-function genetic experiments, which demonstrated that telomeres lengthen upon shRNA-mediated depletion of the shelterin components TIN2, POT1, and TPP1 (Loayza and De Lange, 2003; Houghtaling et al., 2004; Liu et al., 2004; Ye and de Lange, 2004; Ye et al., 2004), whereas overexpression of TRF1 and TRF2 causes telomere shortening (Smogorzewska et al., 2000). However, it has been challenging to study the function of a single shelterin protein using these approaches due to the interdependence among these proteins at telomeres (Rai et al., 2011; Takai et al., 2011). Furthermore, disrupting the stoichiometry of the shelterin complex causes a DNA damage response at telomeres, which could affect telomerase recruitment. By using a minimal TPP1 OB-fold domain to recruit TERT to a heterologous chromatin locus and by specifically interfering with telomerase-shelterin interaction through expression of the isolated OB-fold domain, we were able to separate the capping function of TPP1 from its interaction with telomerase.

Our results are consistent with a model in which the TPP1-POT1 module serves a dual function at telomeres, restricting telomerase access to the chromosome terminus through POT1 in order to prevent unscheduled telomere elongation, while recruiting telomerase to telomeres via the TPP1 OB-fold. POT1 binds single-stranded telomeric DNA with high affinity and is a potent inhibitor of telomere extension both in vivo and in vitro (Loayza and De Lange, 2003; Lei et al., 2004; Kelleher et al., 2005). Once telomerase has been recruited to telomeres, it may compete with POT1 for the terminal single-stranded overhang for productive elongation. Telomere lengthening upon the depletion of TIN2 or TPP1 may occur because of concomitant decrease in POT1 occupancy at telomeres,

favoring telomerase-telomere binding following recruitment by residual TPP1. Alternatively, additional contacts between telomerase and shelterin components could facilitate recruitment in the context of depletion of TIN2 or TPP1.

Telomerase Trafficking and Disease

Germline mutations in telomerase or in TIN2 result in very short telomeres, which in turn precipitate several disease states, including dyskeratosis congenita, aplastic anemia, cancer, liver fibrosis, and pulmonary fibrosis (Calado and Young, 2009). Certain telomerase mutations can cause disease without an apparent change in telomerase enzymatic activity. Mutations in TCAB1 cause dyskeratosis congenita by disrupting telomerase trafficking to the Cajal body while leaving telomerase enzymatic activity intact (Batista et al., 2011; Zhong et al., 2011). Our results with the TERT mutations V144M and E1116fsX from idiopathic pulmonary fibrosis (IPF) patients reveal that disease mutations can arrest telomerase trafficking in Cajal bodies, rendering telomerase unable to be recruited to telomeres. These findings expand our understanding of the contribution of telomerase trafficking defects in disease; TCAB1 mutations can force mislocalization of telomerase to nucleoli, whereas certain TERT mutations can prevent recruitment and trap telomerase in Cajal bodies. Telomerase activation confers replicative immortalization to primary human cells and is a hallmark of cancer (Hanahan and Weinberg, 2011). Our data demonstrate that telomerase-TPP1 OB interaction is rate limiting for telomere length maintenance in human cancer cells. These findings highlight a potential vulnerability in the telomerase pathway that could be exploited through the development of targeted therapeutics.

EXPERIMENTAL PROCEDURES

Small-Scale Cell Culture, cDNA and siRNA Transfections, and Retroviral Transductions

HeLa, 293T, HTC75 (a gift from T. de Lange), and U2OS2-6-3 cells (a gift from S. Janicki) were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum/1% penicillin-streptomycin. Lipofectamine 2000 (Life Technologies) was used for all cDNA transfection experiments with and without siRNAs. For transfection with siRNA alone, Dharmafect 4 (Dharmacon) was used. All siRNAs were purchased from Dharmacon as siGENOME pools. Cells were reseeded 24 hr posttransfection and were assayed 24–48 hr later. For transient overexpression, TERT and TPP1 coding sequences were cloned into pCDNA3.1 (Invitrogen) with indicated amino terminal tags. To generate cells by retroviral gene transfer, 293T cells were first transfected with RSV Gag-pol and VSV-g packaging vectors, together with retroviral plasmids. Viral supernatant was collected 24, 48, and 72 hr posttransfection and concentrated by using Retro-X concentrator (Clontech). Infected cells were selected in antibiotic-containing media up to 1 week. All TERT and TPP1 point mutants were generated by using site-directed mutagenesis (QuikChange II, Agilent). See Table S1 for primer sequences.

Immunofluorescence and In Situ Hybridization

All immunofluorescence (IF) was carried out as previously described (Zhong et al., 2011) on cells seeded on coverslips. RNA FISH was carried out by using Quasar 570 labeled oligonucleotide probes (Biosearch). See Table S2 for probe sequences. Telomere DNA FISH with PNA probes was carried out as described (Kibe et al., 2010). For combined IF and DNA/RNA FISH, IF was carried out first, and cells were refixed with 1 mM DSP in 1× PBS for 5 min. Images were subsequently acquired with a Leica wide-field fluorescence microscope. LAF AS Lite suite (Leica) and ImageJ were used for image analyses.

Telomere Repeat Amplification Protocol and Telomere Restriction Fragment Analysis

Telomere Repeat Amplification Protocol (TRAP) was carried out by using Trapeze kit according to the manufacturer's protocol (Milipore) with minor modifications. Cells were lysed in NP40 buffer (25 mM HEPES-KOH, 400 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.5% NP40, and 1 mM DTT [pH 7.5] supplemented with protease inhibitors). Each reaction was programmed with 0.5–2 μg of protein lysate. To measure telomere lengths during extended culture, HTC75 cells were grown in 6-well plates and reseeded every 3 days. Harvested cells were pelleted and digested with Proteinase K at 6 μg/ml overnight. DNA was extracted by using the standard phenol-chloroform-based method and digested overnight with Hinfl and RsaI before electrophoresis and Southern blotting with an end-labeled $(CCCTAA)_4$ oligonucleotide probe.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.07.012.

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