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The structurally conserved TELR region on shelterin protein TPP1 is essential for telomerase processivity but not recruitment

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The shelterin protein TPP1 is involved in both recruiting telomerase and stimulating telomerase processivity in human cells. Assessing the in vivo significance of the latter role of TPP1 has been difficult, because TPP1 mutations that perturb telomerase function tend to abolish both telomerase recruitment and processivity. The *Saccharomyces cerevisiae* telomerase-associated Est3 protein adopts a protein fold similar to the N-terminal region of TPP1. Interestingly, a previous structure-guided mutagenesis study of Est3 revealed a TELR surface region that regulates telomerase function via an unknown mechanism without affecting the interaction between Est3 and telomerase [T. Rao et al., *Proc. Natl. Acad. Sci. U.S.A.* 111, 214–218 (2014)]. Here, we show that mutations within the structurally conserved TELR region on human TPP1 impaired telomerase processivity while leaving telomerase recruitment unperturbed, hence uncoupling the two roles of TPP1 in regulating telomerase. Telomeres in cell lines containing homozygous TELR mutations progressively shortened to a critical length that caused cellular senescence, despite the presence of abundant telomerase in these cells. Our findings not only demonstrate that telomerase processivity can be regulated by TPP1 in a process separable from its role in recruiting telomerase, but also establish that the in vivo stimulation of telomerase processivity by TPP1 is critical for telomere length homeostasis and long-term viability of human cells.

shelterin protein TPP1 | separation-of-function | telomere extension | telomerase processivity | long-term cell viability

Human telomeric DNA consists of a long duplex region of tandem TTAGGG repeats terminated at a 3′ single-stranded overhang (1–3). The reverse transcriptase telomerase extends telomeres by using a short segment of its RNA subunit as template to add new repeats to telomeric overhangs (4). In most human cells capable of continuous division, a homeostatic state of telomere length is maintained by balancing the lengthening effect of telomerase and the shortening effect of nucleolytic degradation and the end replication problem (5–8). Inhibition of telomerase disrupts this balance, causing progressive telomere shortening and ultimately cellular senescence (7, 9–11).

A key regulator of telomerase is the TPP1 subunit of shelterin, a multi-subunit protein complex that associates with telomeres (12). Within the shelterin complex, telomeric binding proteins TRF1 and TRF2 bind with sequence specificity to the duplex telomeric repeats (13, 14), while the POT1/TPP1 heterodimer binds to the telomeric terminal overhangs (15, 16). TIN2 simultaneously interacts with TRF1, TRF2, and TPP1 (17–20), linking the double-stranded and single-stranded regions of telomeres and spreading POT1/TPP1 along the duplex telomeric tracts. TPP1 regulates two aspects of telomerase function. First, TPP1 is essential in vivo for recruiting telomerase to its site of action at telomeric termini, and second, TPP1 stimulates the in vitro processive addition of TTAGGG repeats by telomerase to a telomeric substrate in the presence of POT1 or when tethered to the telomeric DNA by TRF2 and TIN2 (21, 22). Both of these activities are mediated by a group of surface residues known as the TEL patch (TPP1 glutamate [E] and leucine [L]-rich patch) located within the N-terminal OB-fold domain of TPP1 (23–26), as mutations in the TEL patch disrupted telomerase recruitment and also abolished the stimulatory effects on enzyme processivity. A direct interaction with telomerase is critical for both activities of TPP1, as revealed by the repression of a charge-swap mutation in the TEL patch by a compensatory charge-swap mutation in the N-terminal domain (TEN-domain) of the human telomerase catalytic subunit (TERT), while either mutation on its own impaired telomerase recruitment and processivity (27). TPP1-regulated telomerase function is essential for the continued proliferation of human cells, since homozygous TEL patch mutations in human induced pluripotent stem cells (iPS cells) caused progressive telomere shortening and ultimately cellular senescence (28).

However, left unresolved by the above analysis was whether the inability to maintain telomeres in response to a homozygous defect in the TEL patch of TPP1 is due to a recruitment defect, a processivity defect, or both. We sought to address this by asking if there was an additional surface on TPP1 that regulated only one of these two telomerase functions. To do so, we turned our attention to the *Saccharomyces cerevisiae* Est3 protein, which interacts transiently with yeast telomerase late in the cell cycle (29). It adopts a protein fold that is strikingly similar to the N-terminal OB-fold domain of TPP1 (Fig. 1*A*) even though their primary sequences are considerably different (30). A structure-guided mutagenesis of the entire Est3 surface identified two clusters of residues that are each essential for telomerase function in vivo (30). One

Significance

Telomerase directs the synthesis of new telomeric repeats at chromosome ends, enabling cells to overcome the end replication problem and to continue to divide. The shelterin protein TPP1 interacts with telomerase, promoting both telomerase recruitment and processivity (the addition of multiple telomeric repeats after a single substrate binding event). Here, we identify separation-of-function mutants of TPP1 that eliminate telomerase processivity but leave the telomerase recruitment function intact. When introduced into human cells in a homozygous manner, these mutations can induce critical telomere shortening and cellular senescence. Our observations therefore demonstrate that TPP1 stimulation of telomerase processivity is a key regulatory step in vivo for telomerase-mediated extension of telomeres and continued cell proliferation.

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The authors declare no competing interest.

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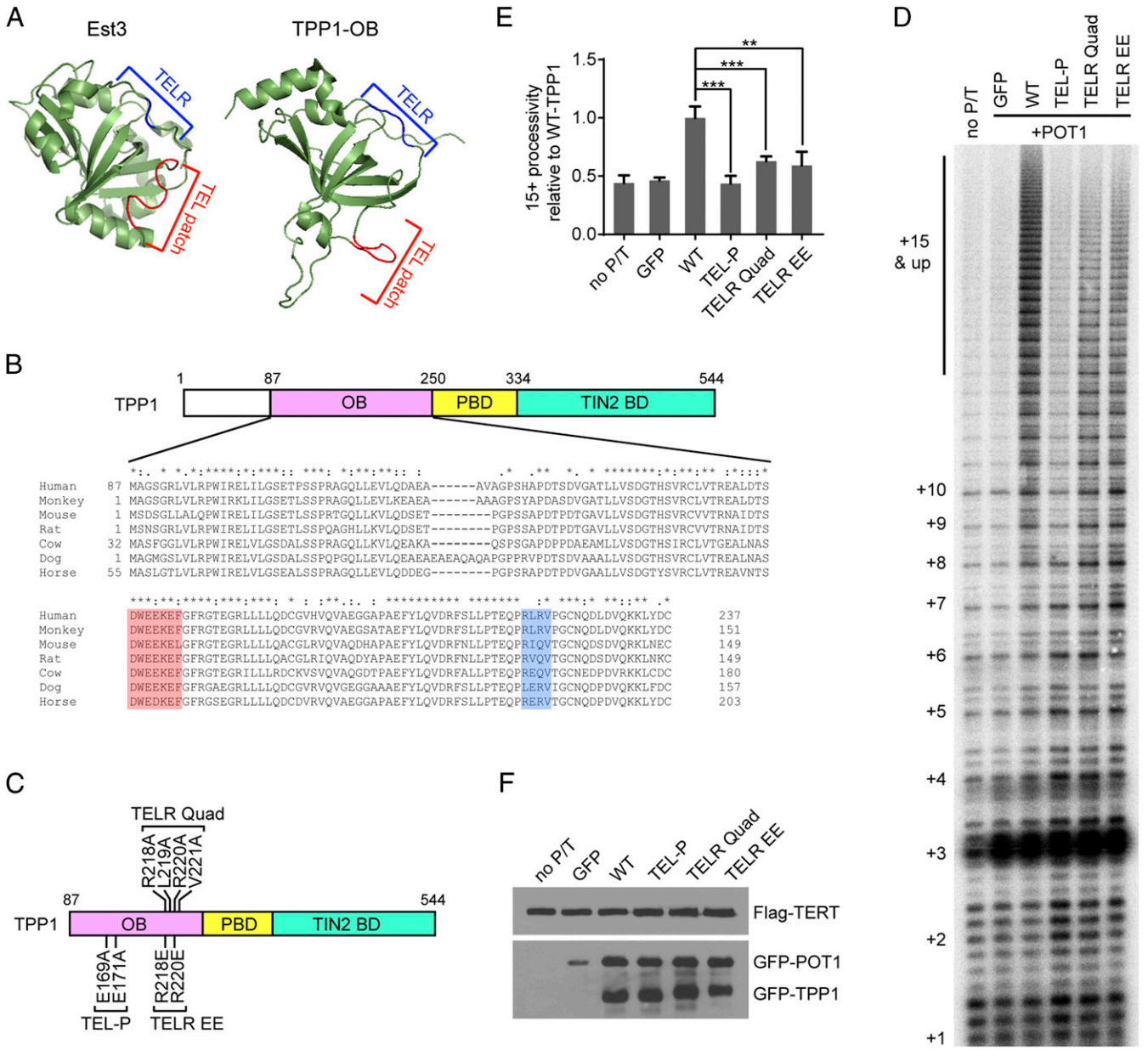

Fig. 1. Mutations in the TELR region impaired TPP1 stimulation of telomerase processivity. (*A*) The TEL patch and TELR regions within Est3 and TPP1 OB-fold domain. (*B*) Sequence alignment of the indicated mammalian TPP1 OB-fold domains. The TEL patch acidic loop is highlighted in red and the TELR basic loop in blue. (*C*) Schematic of the TPP1 mutant constructs used in this study. (*D*) Direct telomerase activity assay using extracts from 293T cells cotransfected with expression constructs for telomerase RNA, Flag-TERT, GFP-POT1, and the indicated GFP-TPP1 alleles. "no P/T" denotes transfection without POT1 and TPP1. The number of telomeric repeats added to the oligonucleotide primer are marked along the left. (*E*) Quantification of telomerase processivity relative to that obtained with wild-type TPP1. Bars represent mean values of three independent experiments and SDs. *P* values (***$P < 0.001$ and **$P < 0.01$) were calculated by two-tailed Student's *t* tests. (*F*) Immunoblots performed with extracts from parallel transfection of *D* to examine Flag-TERT, GFP-POT1 and GFP-TPP1 expression levels.

cluster largely overlaps with the TEL patch on TPP1 and mediates the interaction between Est3 and telomerase, arguing for a striking level of functional and structural conservation between the Est3 and TPP1 proteins. In addition, this mutagenesis identified a second cluster of residues on the surface of Est3, named the TELR region, that also regulates telomerase function through a separate mechanism that was not determined (30).

Here, we identified mutations in the structurally conserved TELR region on TPP1 that impair telomerase processivity without affecting recruitment of telomerase to chromosome termini. Human cell lines containing homozygous TELR mutations underwent progressive telomere shortening that led to cellular senescence, despite the presence of abundant telomerase in these cells. Our observations show that a second structural element of TPP1, in addition to the TEL patch, can control telomerase activity. Furthermore, these results establish that the in vivo stimulation of telomerase processivity by TPP1 is critical for telomere length homeostasis and long-term cell viability.

Results

Mutations in the TPP1 TELR Region Impair Telomerase Processivity. The TELR region maps to a loop connecting the β5-strand and

the αC-helix of the TPP1 OB-fold (Fig. 1 *A* and *B*). We made two mutants in this region (Fig. 1*C*): the TELR Quad mutant, in which four residues were mutated to alanines (R218A/L219A/R220A/V221A), and the TELR EE mutant, in which two arginines were mutated to the oppositely charged glutamic acids (R218E/R220E). As a control, we also made a TEL-P mutant, which contained the E169A/E171A double mutations in the TEL patch region. This mutant had been reported to disrupt TPP1's interaction with telomerase and abolish all telomerase-associated functions of TPP1 (24). We ectopically overexpressed these mutants and examined their effects on shelterin complex assembly and telomerase processivity.

Neither of the two TELR mutants caused any detectable disruption of the shelterin complex. Immunostaining of cells transfected with plasmids for GFP-tagged TPP1 using an anti-GFP antibody, followed by fluorescence in situ hybridization (FISH) using a telomeric repeat probe, showed that the TELR mutations did not affect the telomeric localization of TPP1 (*SI Appendix*, Fig. S1*A*). Immunoprecipitation performed using extracts of cells cotransfected with plasmids for Flag-tagged TPP1 and GFP-tagged TIN2 or POT1 showed that the TELR mutations did not impair the interactions between TPP1 and its shelterin partners (*SI Appendix*, Fig. S1 *B* and *C*).

To determine the impact of the TELR mutations on telomerase processivity, we performed the direct telomerase activity assay using extracts of cells cotransfected with plasmids for the telomerase core subunits (TR and Flag-tagged TERT) together with plasmids for GFP-tagged TPP1 and POT1 (Fig. 1*D*). As anticipated, introduction of the TEL-P mutation completely abrogated the ability of TPP1 to stimulate the processive extension of an oligonucleotide substrate by telomerase. We observed that the TELR Quad and the TELR EE mutations also significantly reduced this ability of TPP1 (Fig. 1 *D* and *E*). Western blotting analysis of the transfected cells confirmed that the respective TERT, POT1,

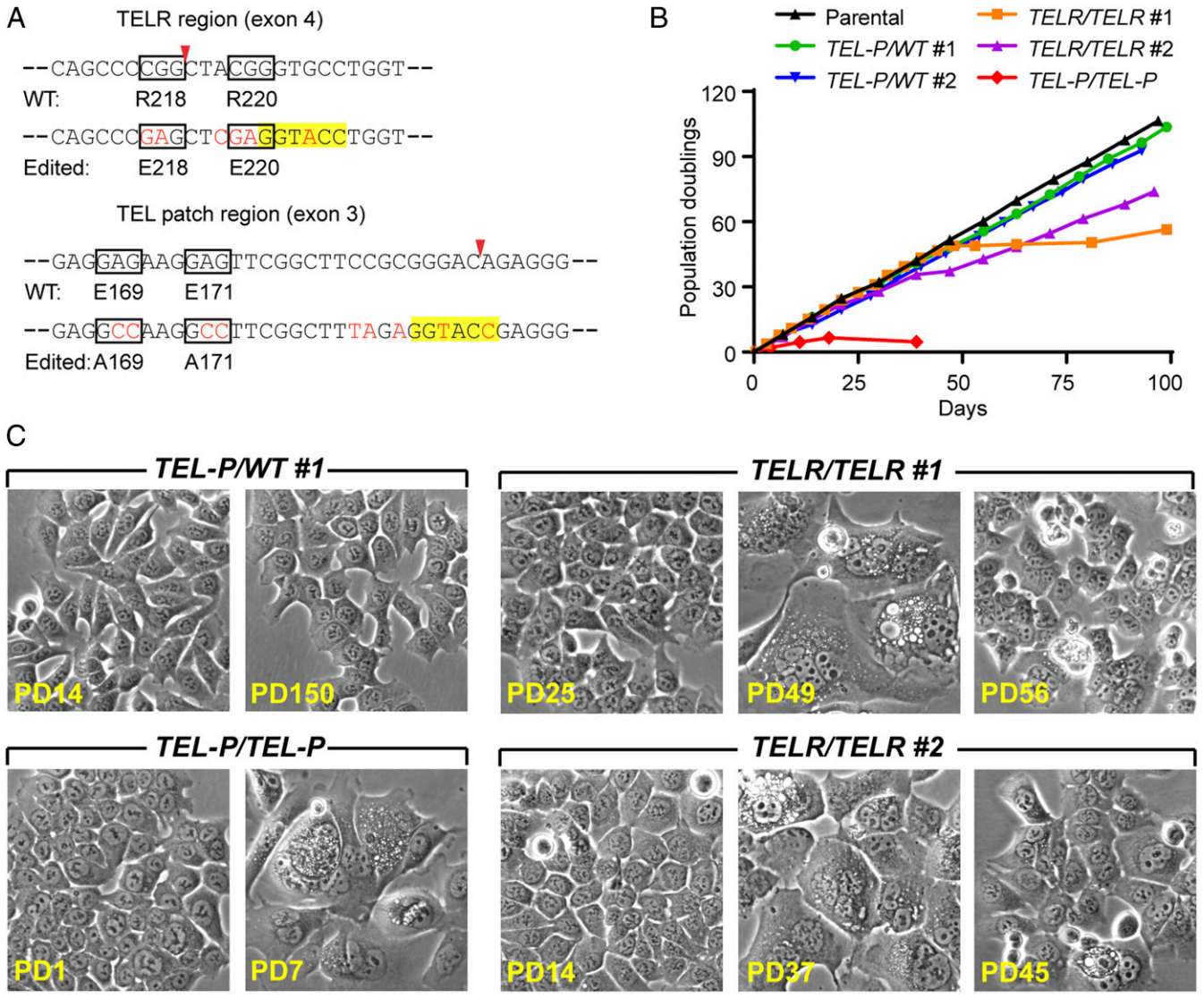

Fig. 2. The TPP1 TELR region is essential for continued proliferation of HCT116 cells. (*A*) The mutagenic ssODN sequence for the TELR or TEL patch region (with the mutated nucleotides in red text) is shown below the relevant wild-type sequence of the TPP1 gene. The mutated codons were encircled with boxes, and the Cas9 cut sites were marked with arrowheads. Silent mutations were introduced into the edited sequence to prevent recutting and to create a KpnI site (highlighted) for colony screening. (*B*) Growth curves of HCT116 cell lines containing homozygous TELR R218E/R220E mutations versus those containing heterozygous or homozygous TEL patch E169A/E171A mutations. (*C*) Phase-contrast images of the edited cell lines at the indicated population doublings (PDs). Images of pre-, peri-, or postsenescence *TELR/TELR* homozygote cells were shown.

and TPP1 proteins were expressed at similar levels across all transfections (Fig. 1*F*).

The TPP1 TELR Region Is Essential for Telomere Length Maintenance and Continued Cell Proliferation. To assess the in vivo significance of the TPP1 TELR region, we used CRISPR/Cas9-mediated genome editing to generate knock-in HCT116 cells containing TELR R218E/R220E mutations (Fig. 2*A*). HCT116 is a human colon cancer cell line that has wild-type shelterin components and, is telomerase-positive and karyotypically stable (31). To edit the TELR region, we transiently transfected HCT116 cells with plasmid constructs for the Cas9 protein and a TELR targeting guide RNA together with a single-stranded oligonucleotide (ssODN) template carrying the TELR R218E/R220E mutations (*SI Appendix*, Fig. S2 *A* and *B*). We introduced translational silent mutations into the ssODN to prevent recutting of the edited sequence and to create a KpnI restriction site to facilitate screening of candidate knock-in clones (Fig. 2*A* and *SI Appendix*, Fig. S2*B*). The TEL patch region was edited via a similar strategy (*SI Appendix*, Fig. S2 *C* and *D*) (32) to generate HCT116 cell lines containing the E169A/E171A mutations (Fig. 2*A*) as negative controls for telomerase-associated functions of TPP1. After the initial transfection, single colonies were picked, expanded, and screened by the KpnI digestion assay. Cell clones that showed KpnI sensitivity in the targeted genomic region were subjected to Sanger DNA sequencing to identify those that incorporated the desired mutations. For the TPP1 TELR R218E/R220E mutations, we isolated two independent homozygote clones (*TELR/TELR* clone #1 and #2); for the TEL patch E169A/E171A mutations, one homozygote clone (*TEL-P/TEL-P*) and two heterozygote clones (*TEL-P/WT* clone #1 and #2) were obtained (*SI Appendix*, Fig. S3 *A* and *B*). TPP1 protein levels in all edited clones were comparable to the parental HCT116 cells (*SI Appendix*, Fig. S3*C*), suggesting that these mutations do not significantly change TPP1 protein stability.

During continuous passaging, the HCT116 parental cells proliferated at a steady rate while maintaining consistent cell morphology (Fig. 2 *B* and *C*). The two clones of the *TELR/TELR* homozygote cells initially proliferated at a rate indistinguishable from the parental cells but later entered a senescence state during which the cells became multinucleated, flattened, greatly enlarged, and vacuolated (Fig. 2 *B* and *C*). We observed progressive telomere shortening in the *TELR/TELR* homozygote cells compared with the stable telomere length in parental HCT116 cells (Fig. 3 *A* and *B*). The onset of cellular senescence in each *TELR/TELR* clone coincided with the shortening of telomeres to a minimum mean length of ~2 kb (Figs. 2*B* and 3*A*), suggesting that this senescence state was induced by critical telomere shortening. Interestingly, both *TELR/TELR* homozygote clones contained a small number of cells that eventually emerged from senescence (the growth stall period was ~30 d for clone #1 and ~6 d for clone #2) (Fig. 2*B*). Telomere lengths remained relatively short in the post-senescence *TELR/TELR* cells (Fig. 3 *A* and *B*).

For the TEL patch E169A/E171A mutations, the two clones of the *TEL-P/WT* heterozygote cells proliferated at a rate indistinguishable from the parental cells and maintained consistent cell morphology during continuous passaging (Fig. 2 *B* and *C*). Telomeres in each *TEL-P/WT* heterozygote clone underwent some initial shortening before stabilizing at a new length (Fig. 3 *A* and *B*). The new homeostatic state was apparently established before telomeres reached critical length since cell proliferation was not negatively impacted (Fig. 2*B*). The *TEL-P/TEL-P* homozygote cells, in contrast, went through just a few divisions before entering senescence (Fig. 2*B*). This was not surprising since their telomeres were already quite short when we obtained the clone (Fig. 3*A*). Morphology of the *TEL-P/TEL-P* senescent cells resembled that of the *TELR/TELR* clones (Fig. 2*C*), except that we did not observe cells emerging from senescence even after >100 d of culturing.

Mutations in the TELR Region Do Not Affect Telomerase Recruitment. We next examined how telomerase recruitment was impacted in the HCT116 knock-in cells. To facilitate the detection of telomerase, we transiently transfected the respective knock-in clones with plasmids encoding telomerase RNA and the catalytic subunit (TR and TERT). We then performed immunofluorescence staining against shelterin proteins TRF1 and TRF2 to detect telomeres, followed by FISH against telomerase RNA to detect telomerase. As anticipated, telomerase was recruited to ~85% of telomeres in parental cells, while the homozygous TEL patch mutations nearly completely abrogated telomerase recruitment (Fig. 4 *A* and *B*). The homozygous TELR mutations, in contrast, did not show any negative impact on telomerase recruitment; telomerase was recruited to ~85% and ~89% of telomeres in the respective *TELR/TELR* clones (Fig. 4 *A* and *B*). This observation, in combination with the results obtained from the direct telomerase activity assay (Fig. 1 *D* and *E*), indicated that mutations in the TELR region impaired telomerase processivity while leaving telomerase recruitment unperturbed. We note that in the respective *TEL-P/WT* heterozygote clones, telomerase was only recruited to ~32% and ~41% of telomeres (Fig. 4 *A* and *B*). The twofold reduction of telomerase recruitment caused by the heterozygous TEL patch E169A/E171A mutations provides an explanation for why these two clones reset telomere length homeostasis at a shorter length (Fig. 3 *A* and *B*).

One potential concern with the above experiment is that the TELR mutations might have a partial defect in telomerase recruitment that was masked in cells that overexpressed telomerase. To address this, we also assessed the recruitment of endogenous telomerase to telomeres in the edited cell lines. To do so, we carried out immunofluorescence staining for shelterin proteins TRF1 and TRF2, as well as the Cajal body component coilin, followed by FISH for endogenous telomerase RNA (Fig. 4*C* and *SI Appendix*, Fig. S4). The localization of TR at Cajal bodies was included in this assessment because TR processing and maturation occurs in these nuclear compartments. Since recent studies of telomerase using super-resolution live imaging analysis suggest that telomerase recruitment and/or extension occurs outside of Cajal bodies (33, 34), we quantitated TR-telomere associations that lacked coilin. As expected, this category of TR-telomere foci was significantly reduced by the homozygous TEL patch mutations in TPP1; in contrast, the homozygous TELR mutant cell lines were indistinguishable from the parental cell line (Fig. 4*D*). A similar result was observed even when all TR-telomere associations were assessed (both with and without coilin), which were once again significantly reduced by the homozygous TEL patch mutations, but not by the TELR mutations in TPP1 (Fig. 4*E*). Finally, because of the low sensitivity of this assay (TR-telomere foci, either with or without coilin association, were not detected in ~40% of cells), we quantitated the percentage of cells that displayed more than one colocalization event between TR and telomeres that lacked coilin. A total of ~13% of the parental HCT116 cells and 13 to 14% of homozygous TELR mutant cells exhibited more than one TR-telomere foci (excluding coilin) per nucleus, whereas this category was reduced to ~1% in homozygous TEL patch cells (Fig. 4*F*). These results demonstrate that mutations in the TELR region of TPP1 do not affect recruitment of endogenous telomerase to telomeres.

Ectopic Expression of the TPP1 TELR Mutants in Cultured Human Cells Causes Telomere Shortening without Affecting Telomerase Recruitment. We also examined how ectopically expressing TPP1 TELR mutants (Fig. 1*C*) affected telomere length maintenance and telomerase recruitment. We infected HCT116 cells with lentivirus expressing Flag-tagged TPP1 (Fig. 5*A*) and collected stably infected cells for bulk telomere length analysis. Overexpression of wild-type TPP1 led to robust telomere lengthening. The TELR Quad and TELR EE mutants, in contrast, each induced progressive telomere shortening

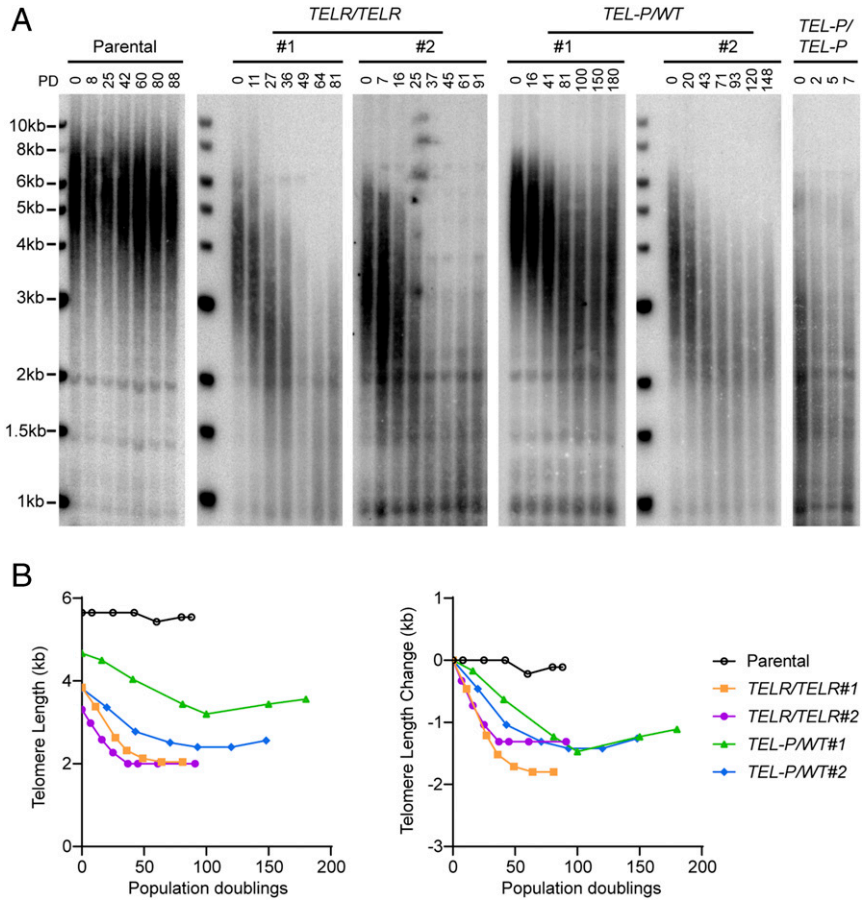

Fig. 3. Homozygous mutations in the TPP1 TELR region caused critical telomere shortening in HCT116 cells. (*A*) Telomere restriction fragment (TRF) analysis of the edited cell lines as compared with the parental HCT116 cells over continuous passaging. (*B*, *Left*) Mean telomere lengths in *A* were determined by the ImageQuant software and plotted against PDs. (*Right*) Quantification of changes in mean telomere length plotted against PDs.

(Fig. 5 *B* and *C*). Similar telomere shortening was also observed in cells overexpressing the TEL patch E169A/E171A mutant (Fig. 5 *B* and *C*). Unlike the *TEL-P/TEL-P* and *TELR/TELR* homozygotes, in HCT116 cells overexpressing the TPP1 TEL-P and TELR mutants, telomeres stabilized at a mean length slightly above 3 kb and continued to proliferate at a constant rate, most likely due to the presence of wild-type TPP1 expressed from its endogenous locus.

To examine how ectopic expression of the TELR mutants affected telomerase recruitment, we transiently transfected HeLa1.2.11 cells (a HeLa subclone with long telomeres frequently used for immunofluorescence study of telomeric proteins) with plasmids encoding telomerase core subunits (TR and TERT) and GFP-tagged TPP1 variants. We then performed immunofluorescence staining against GFP-TPP1, followed by FISH against telomerase RNA. As all the TPP1 variants had been shown to localize normally to telomeres (*SI Appendix*, Fig. S1*A*), telomerase recruitment was assessed by quantifying the colocalization between telomerase RNA and the GFP-TPP1 foci. Our data showed that neither the TELR Quad nor the TELR EE mutant induced any adverse effect on telomerase recruitment: Telomerase was detectable at ∼90% of the GFP-TPP1 foci in cells expressing these mutants, comparable to the level in cells expressing wild-type TPP1 (*SI Appendix*, Fig. S5 *A* and *B*). Overexpression of the TEL-P mutant, as expected, sharply decreased telomerase recruitment to ∼10% of the GFP-TPP1 foci (*SI Appendix*, Fig. S5 *A* and *B*). Results of the ectopic expression studies further corroborated our findings from the homozygous *TELR/TELR* mutant cell lines: The TELR region, although not required for telomerase recruitment, is essential for telomerase to extend telomeres.

We also examined the telomere length effect of a TPP1 L104A mutant. L104A was previously reported to significantly decrease TPP1 stimulation of telomerase processivity, but not telomerase recruitment (24, 28, 35), similar to the TELR mutations. We found that overexpression of TPP1 L104A in HCT116 cells led to steady telomere extension, although the extension rate was less dramatic than that induced by the wild-type TPP1 (Fig. 5 *B* and *C*). Our observation is in agreement with another overexpression study showing TPP1 L104A caused telomere lengthening in HeLa cells (35). We speculate that, compared with the TELR mutants, the L104A mutant may still retain a partial degree of telomerase function in vivo. Indeed, in human iPS cells harboring a deleterious homozygous TEL patch mutation, expression of the TPP1 L104A mutant from an engineered AAVS1 genomic locus was found to prevent critical telomere shortening and rescue cell viability (28).

Ectopic Expression of Telomerase Rescues *TELR/TELR* but not *TEL-P/TEL-P* Homozygotes from Cellular Senescence Triggered by Critical Telomere Shortening. We next investigated whether telomere maintenance and cell viability in the *TELR/TELR* and *TEL-P/TEL-P* homozygotes could be rescued by ectopically expressing wild-type TPP1 or telomerase. We infected respective presenescence cells with lentivirus expressing wild-type TPP1 or the core components of telomerase (TERT + TR). Stably infected cells were then pooled, continuously passaged, and collected for cell counts and bulk telomere length analysis. Overexpression of wild-type TPP1 led

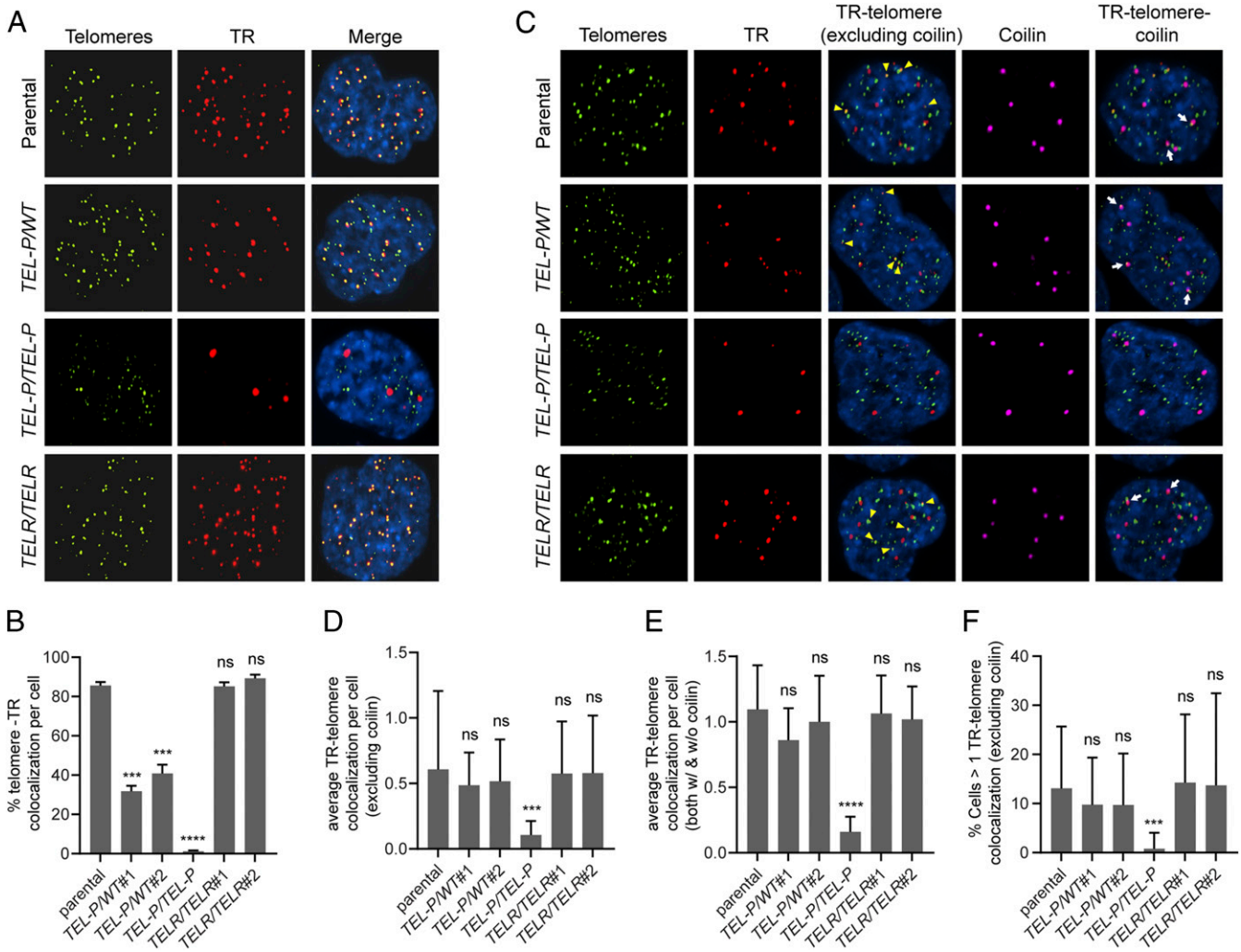

Fig. 4. TPP1 TEL patch mutations, but not TELR mutations, impaired telomerase recruitment. (*A*) Each edited cell line was cotransfected with expression constructs for telomerase (TR + TERT), and telomerase recruitment was assessed by colocalization of overexpressed telomerase RNA and telomeres. Immunostaining was performed using anti-TRF1 and anti-TRF2 antibodies to detect telomeres (green), followed by RNA FISH to detect TR (red). (*B*) Percentage of telomeres colocalizing with overexpressed TR. Bars represent mean values of ~50 nuclei from 10 fields of view and SEMs. (*C*) Telomerase recruitment was assessed by colocalization of endogenous telomerase RNA and telomeres in each edited cell line. Images are from the subpopulation of cells that displayed more than one colocalization event between TR and telomeres (excluding coilin). Additional images are shown in *SI Appendix*, Fig. S4. Arrowheads indicate TR-telomere associations that lacked coilin; arrows indicate TR-telomere-coilin associations. Asynchronous cells were stained with anti-TRF1 and TRF2 antibodies to detect telomeres (green) and anti-coilin antibody to detect Cajal bodies (purple), followed by RNA FISH to detect TR (red). (*D*) Average number of TR-telomere associations (excluding coilin) per nucleus. (*E*) Average number of all TR-telomere associations (both with and without coilin) per nucleus. (*F*) Percentage of cells containing more than 1 TR-telomere colocalization event (excluding coilin). For *D*, *E*, and *F*, bars represent mean values and SDs obtained from ~200 nuclei for each cell line. All quantifications were carried out blindly. *P* values (****$P < 0.0001$; ***$P < 0.001$; ns, not significant) were calculated by one-way ANOVA with Dunnett's multiple comparison test at 95% confidence level, comparing all with parental cells.

to almost identical levels of telomere lengthening and the bypass of senescence in *TELR/TELR* and *TEL-P/TEL-P* cells (Fig. 6 *A* and *B*), suggesting that the TELR mutant, similar to the TEL patch mutant, does not have a dominant-negative effect over wild-type TPP1's ability in promoting telomerase function. Overexpression of telomerase, in contrast, extended telomeres in the *TELR/TELR*, but not the *TEL-P/TEL-P*, cells (Fig. 6*A*). Conceivably, *TELR/TELR*, but not *TEL-P/TEL-P*, homozygotes were rescued from the critical telomere shortening-triggered senescence by telomerase overexpression (Fig. 6*B*). Telomere repeat amplification protocol (TRAP) analysis showed that overexpressing telomerase increased telomerase activity to comparable levels in *TELR/TELR* and *TEL-P/TEL-P* homozygotes (Fig. 6*C*), suggesting that the failed rescue in the latter cells cannot be attributed to a lack of core telomerase enzymatic activity.

Discussion

Although TPP1 has long been known to stimulate telomerase processivity in vitro, its potential in vivo importance remained unknown due to the inability to cleanly separate this role of TPP1 from that in recruiting telomerase. In this study, we show that the TPP1 TELR mutants significantly impaired telomerase processivity while leaving the telomerase recruitment function intact. Using CRISPR/Cas9-mediated genome editing technology, we generated HCT116 knock-in cell lines harboring homozygous TPP1 TELR mutations. Telomeres in the edited cells progressively shortened. When they reached a critical length, the cells entered a senescence state.

These results therefore demonstrate that TPP1-stimulated telomerase processivity is essential for telomere length maintenance and long-term cell viability. Furthermore, our data provide direct

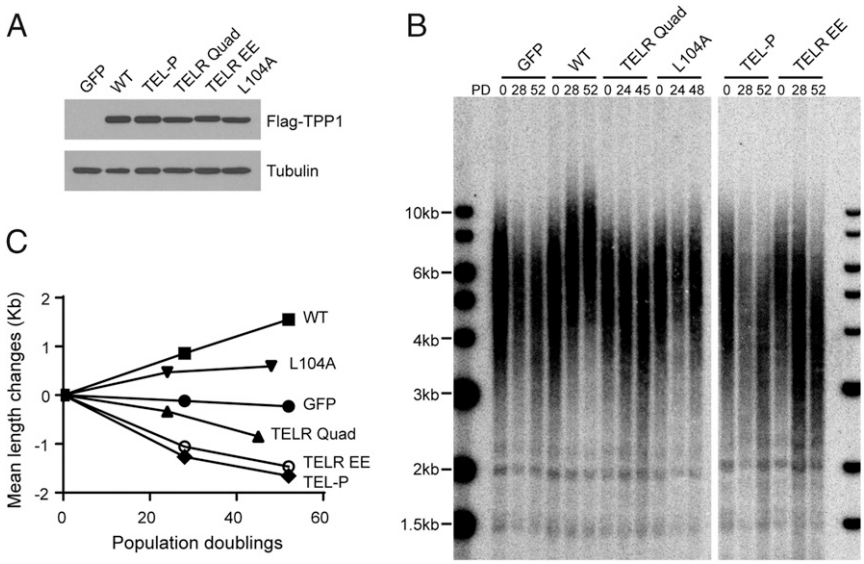

Fig. 5. Ectopic expression of the TPP1 TELR mutants in HCT116 cells led to progressive telomere shortening. (*A*) Lentiviral expression level of Flag-tagged TPP1 alleles was examined by immunoblotting using an anti-Flag antibody. Tubulin was included as the loading control. (*B*) TRF analysis of cells expressing the indicated TPP1 constructs over continuous passaging. HCT116 cells were infected with lentiviruses expressing Flag-tagged TPP1. The infected cells were pooled, continuously passaged, and collected at indicated PDs. (*C*) Mean telomere length changes were plotted against PDs.

evidence that telomerase processivity can be regulated by TPP1 in a process separable from its role in recruiting telomerase.

The exact mechanism by which the TELR region impacts telomerase processivity remains to be determined. Recent structural and single molecule studies of telomerase suggest that the TEN-RBD-RT-TRAP ring on TERT forms DNA substrate binding motifs to aid template translocation after the addition of each telomeric repeat (36–38). We note the conservation of one or two arginines within the short TELR region across mammalian TPP1 homologs (Fig. 1*B*). Whether these residues contribute to the formation of a basic DNA-binding pocket together with the TERT ring to help retain telomeric DNA substrate during template translocation will be a subject for future investigation.

In both clones of the *TELR/TELR* homozygote cells, after an extended period of stalled cell proliferation, we observed the outgrowth of a small subset of cells. Compared with the original

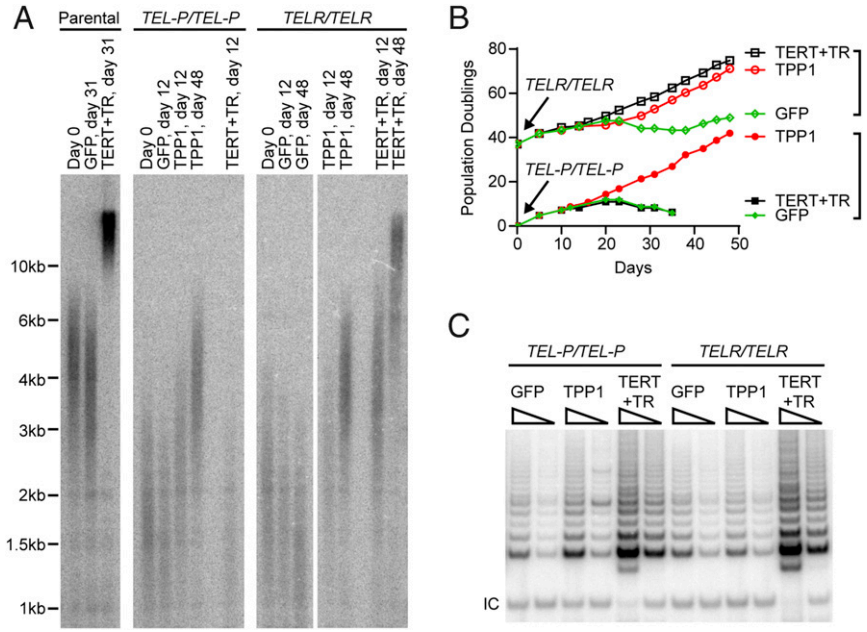

Fig. 6. Telomerase expression prevented the *TELR/TELR* homozygotes, but not the *TEL-P/TEL-P* homozygotes, from entering the critical telomere shortening–induced cellular senescence. (*A*) TRF analysis of the *TELR/TELR* or *TEL-P/TEL-P* homozygotes overexpressing GFP, TPP1, or telomerase. Pre-senescence *TELR/TELR* (at PD 37) and *TEL-P/TEL-P* cells (at PD 0) were infected with lentivirus expressing a GFP control, Flag-tagged TPP1, or telomerase (TERT + TR). Infected cells were pooled, continuously passaged, and collected for telomere length analysis and cell counts. (*B*) Growth curves of the *TELR/TELR* or *TEL-P/TEL-P* homozygotes overexpressing GFP, TPP1, or telomerase. (*C*) Telomerase enzymatic activity examined by the TRAP assay. Whole cell extracts from 100 and 25 cells were analyzed for each cell line. IC: internal PCR control.

cell population, the survivor cells exhibited increased telomerase enzymatic activity as indicated by the TRAP assay results (*SI Appendix*, Fig. S6 *A* and *B*). This suggests that the bypass of the senescence was prompted by telomerase-dependent, but not ALT-dependent, telomere maintenance. In line with this observation, overexpression of telomerase in *TELR/TELR* homozygotes was found to extend telomeres and sustain cell proliferation (Fig. 6 *A* and *B*). Although the TELR mutations impaired telomerase processivity, telomerase recruitment was normal in the *TELR/TELR* cells. Overexpression of telomerase produced excess amount of telomerase molecules. The decreased length of extension per telomerase binding event could be compensated by multiple rounds of extension by different telomerase molecules. As a result, telomeres were extended in these cells. Overexpression of telomerase in the *TEL-P/TEL-P* mutant cells, in contrast, failed to extend telomeres. This is expected since telomerase recruitment was completely abrogated in the *TEL-P/TEL-P* cells.

Lastly, our data suggest that the TELR equivalent region on the *S. cerevisiae* Est3 protein may likewise contribute to telomere length maintenance through enhancing telomerase processivity. Although significant differences exist in the composition and structure of telomerase accessory factors in budding yeast and mammals, this study further highlights the possibility that telomerase action at telomeres is regulated through several conserved modes during evolution.

Materials and Methods

Cell lines. The HCT116 parental cells were obtained from the American Type Culture Collection. HeLa1.2.11 cells were kindly provided by Dr. Titia de Lange (Rockefeller University). All cell lines were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum.

Plasmids. The pSpCas9(BB)-2A-Puro (PX459) vector (Addgene plasmid #62988) (39) and the guide RNA (gRNA) cloning vector (Addgene plasmid #41824) (40) were gifts from Drs. Feng Zhang (Broad Institute) and George Church (Harvard University), respectively. The pHR'CMV lentiviral expression system was kindly provided by Dr. Didier Trono (The École polytechnique fédérale de Lausanne). Flag-TPP1, GFP-TPP1, GFP-POT1, and Flag-TERT lentiviral constructs contain respective cDNA driven by a CMV promoter, followed by an internal ribosome entry site and a hygromycin resistance gene. For full-length TPP1 expression, TPP1 coding sequence from amino acids 87 to 544, as previously defined (24, 41), was used. Telomerase RNA (hTR) expression was driven by an IU1 promoter (42). Lentivirus was prepared as described previously (43).

Antibodies. Immunoprecipitation and immunoblotting were carried out according to standard protocols with the following antibodies: rat anti-Flag L5 antibody (BioLegend; 637301), rabbit anti-GFP antibody (Novus; NB600-308), and mouse anti-TPP1 (Abnova; H00065057-M02). Immunofluorescence staining and FISH were carried out as described previously (44) with the following antibodies: rabbit anti-GFP antibody (Novus; NB600-308), mouse anti-TRF1 antibody (GeneTex; GTX70304), mouse anti-TRF2 antibody (Millipore; 05–521), and rabbit anti-coilin antibody (GeneTex; GTX112570).

Telomerase recruitment assay. Cells were transiently transfected with plasmid constructs for hTERT and hTR using Lipofectamine 2000 (Life Technologies) or the jetPrime reagent (Polyplus transfection). After 24 h, transfected cells were trypsinized and seeded onto sterile coverslips placed in a six-well plate. Two days after seeding, combined immunofluorescence staining-telomerase RNA FISH was carried out as described (44). To assess endogenous telomerase recruitment, untransfected cells were subjected to immunofluorescence staining-telomerase RNA FISH analysis. For immunostaining of telomeres, the cells were incubated with anti-TRF1 and anti-TRF2 primary antibodies, followed by an Alexa Fluor 488 (Molecular Probes) conjugated secondary antibody. For immunostaining of GFP-TPP1, an anti-GFP primary antibody was used. Subsequent telomerase RNA FISH was performed with a mixture of three Cy3-conjugated telomerase RNA probes (45). Cell images were acquired using a Nikon Ti-U microscope with a 100x objective and collected as a stack of 0.2 μm increments in the *z*-axis. After deconvolution using the AutoQuant X3 software (Media Cybernetics), images were viewed with the maximal projection option on the *z*-axis. All image files were randomly assigned coded names to allow blinded scoring for spots colocalization.

CRISPR-Cas9–mediated genome editing, screening of knock-in clones, direct telomerase activity assay, TRF analysis, and TRAP telomerase activity assay were performed using published protocols (24, 32, 41). See *SI Appendix, SI Materials and Methods* for full details.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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