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Supplemental Information

The N Terminus of the OB Domain of Telomere Protein TPP1 Is Critical for Telomerase Action

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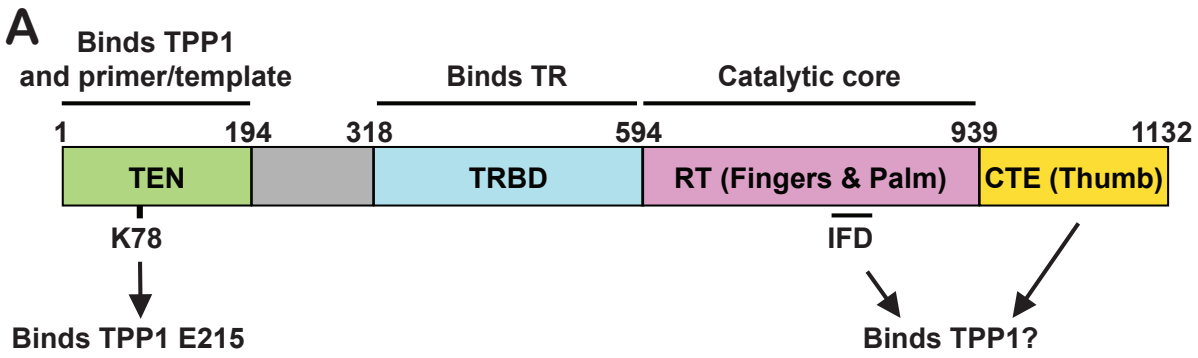
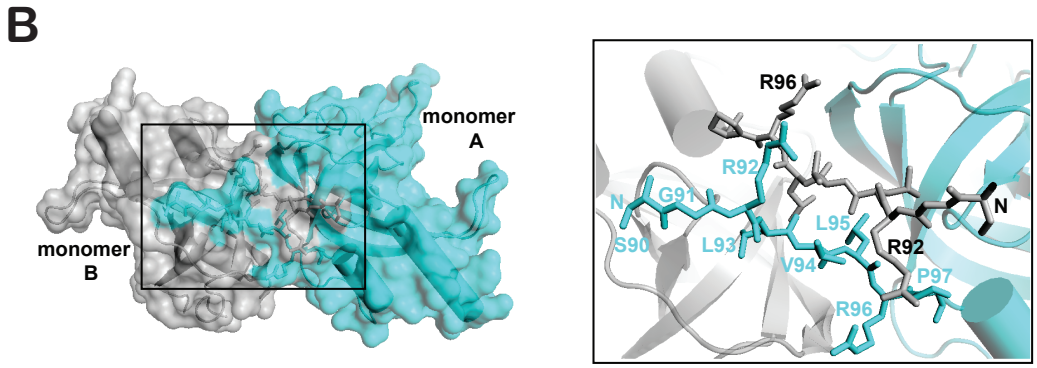
Figure S1





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                160        170        180        190   210        220
Human           EALDTSDWEEKEFGFRGTEGRLLLLQDCGVH....SLLPTEQPRLR
Chimpanzee      EALDTSDWEEKEFGFRGTEGRLLLLQDCGVH....SLLPTEQPRLR
Cow             EALNASDWEEKEFGFRGTEGRILLLRDCKVS....ALLPTEQPREQ
Dog             EALNASDWEEKEFGFRGAEGRLLLLQDCGVR....SLLPTEQPLER
Rat             NAIDTSDWEEKEFGFRGTEGRLLLLQACGLR....NLLPTEQPRVQ
Mouse           NAIDTSDWEEKELGFRGTEGRLLLLQACGLR....NLLPTEQPRIQ
                :*:::*******:****:***:***. * :       *******  .
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Figure S1. Structure of TPP1 OB, and domain diagram and binding properties of human TERT, Related to Figure 1. (A) The arrangement of the TEN, TRBD (telomerase RNA binding domain), RT (reverse transcriptase), and CTE (C-terminal extension) domains in the primary structure of human TERT is shown along with annotations for various (known and putative) binding regions (Gillis et al., 2008; Wang and Feigon, 2017). (B) The TPP1 N-terminal residues 90-96 (NH_2-SGRLVLR-COOH), which include the NOB region, of two OB monomers (labeled A and B) form extended conformations and align roughly anti-parallel to each other to mediate homodimerization in the human TPP1 OB domain crystal structure (PDB accession: 2I46) (Wang et al., 2007). The overall structure is shown as a surface model and the site of dimerization (boxed area) is also shown on the right in stick representation for aa 90-96. (C) The TEL patch is highly conserved across mammalian species. Sequence alignment of selected

regions of indicated TPP1 homologs with TEL patch residues shown in orange. Amino acid numbers represent the human sequence. “ * ” beneath sequence lineups indicate identical residues, “ : ” represent strongly conserved residues, and “ . ” represent weakly conserved residues as described by the MUSCLE algorithm.

Figure S2

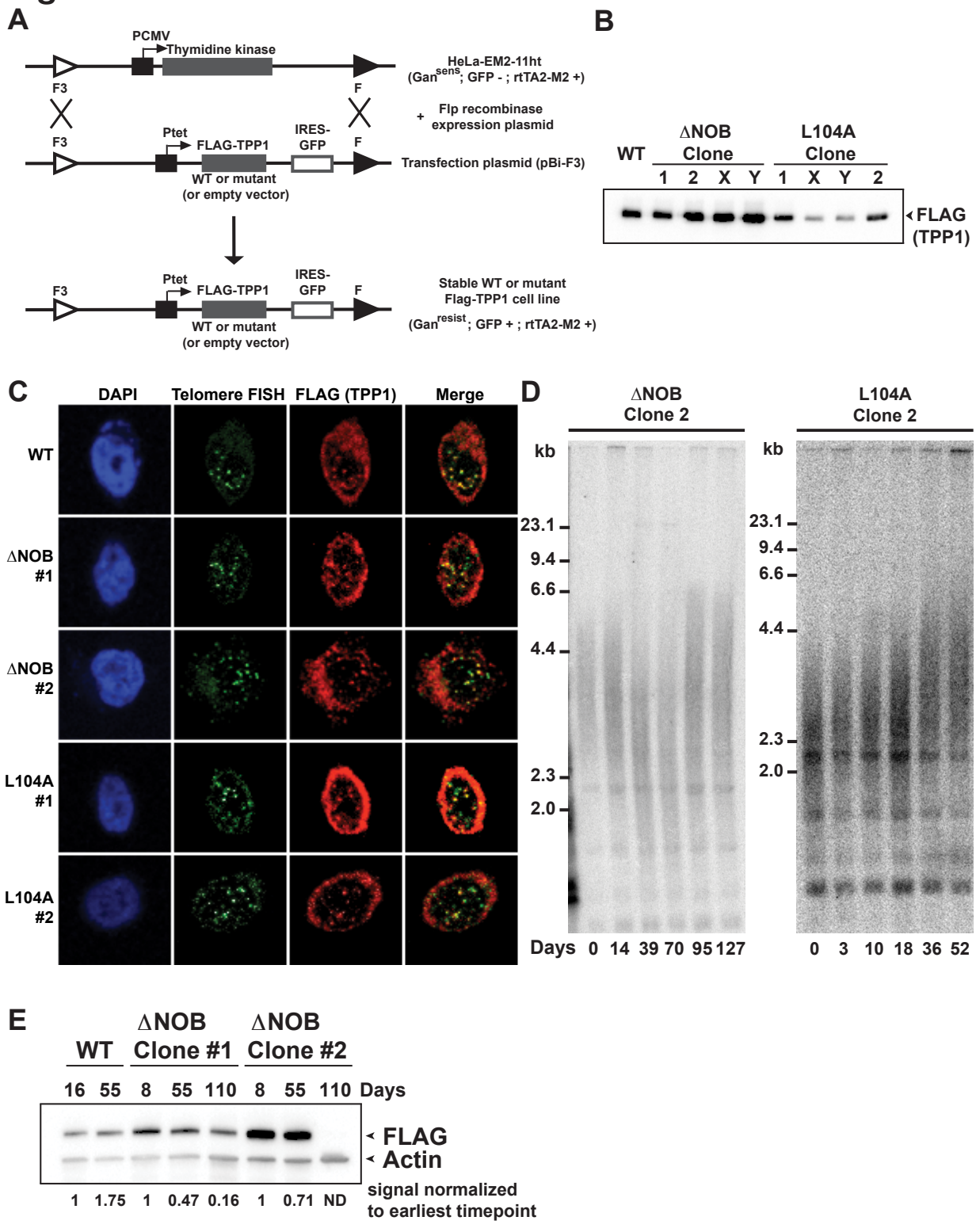

Figure S2. Cell lines stably expressing TPP1 variants for telomere lengthening and telomerase recruitment studies, Related to Figure 3. (A) Scheme for generating cell lines stably integrated with TPP1 WT or mutant, or empty

vector sequence. (B) Western blot for four clones each of ΔNOB and L104A alongside a WT clone shows high expression for ΔNOB and low expression for L104A. Clones 1 and 2 of each mutant were selected for further analysis of telomerase recruitment and telomere length. (C) IF-FISH to show colocalization of indicated TPP1 constructs with telomeres. "FLAG (TPP1)" shows the FLAG immunofluorescence signal indicative of TPP1 (red), and "Telomere FISH" indicates telomeric DNA probed with a C-rich, Cy3-labeled PNA probe (green). Merge panels reveal colocalization of TPP1 variants with telomeric DNA (yellow). (D) Telomeric restriction fragment (TRF) analysis of clone 2 for HeLa-EM2-11ht cell lines stably expressing the indicated TPP1 constructs (ΔNOB and L104A) for the indicated number of days in culture. (E) FLAG immunoblots from indicated cell lines at indicated days in culture. The FLAG signal was divided by the actin signal in each lane to account for loading differences. The numbers below the blot indicate actin-normalized FLAG signal in that lane divided by the corresponding signal in the earliest time-point for that clone.

Figure S3

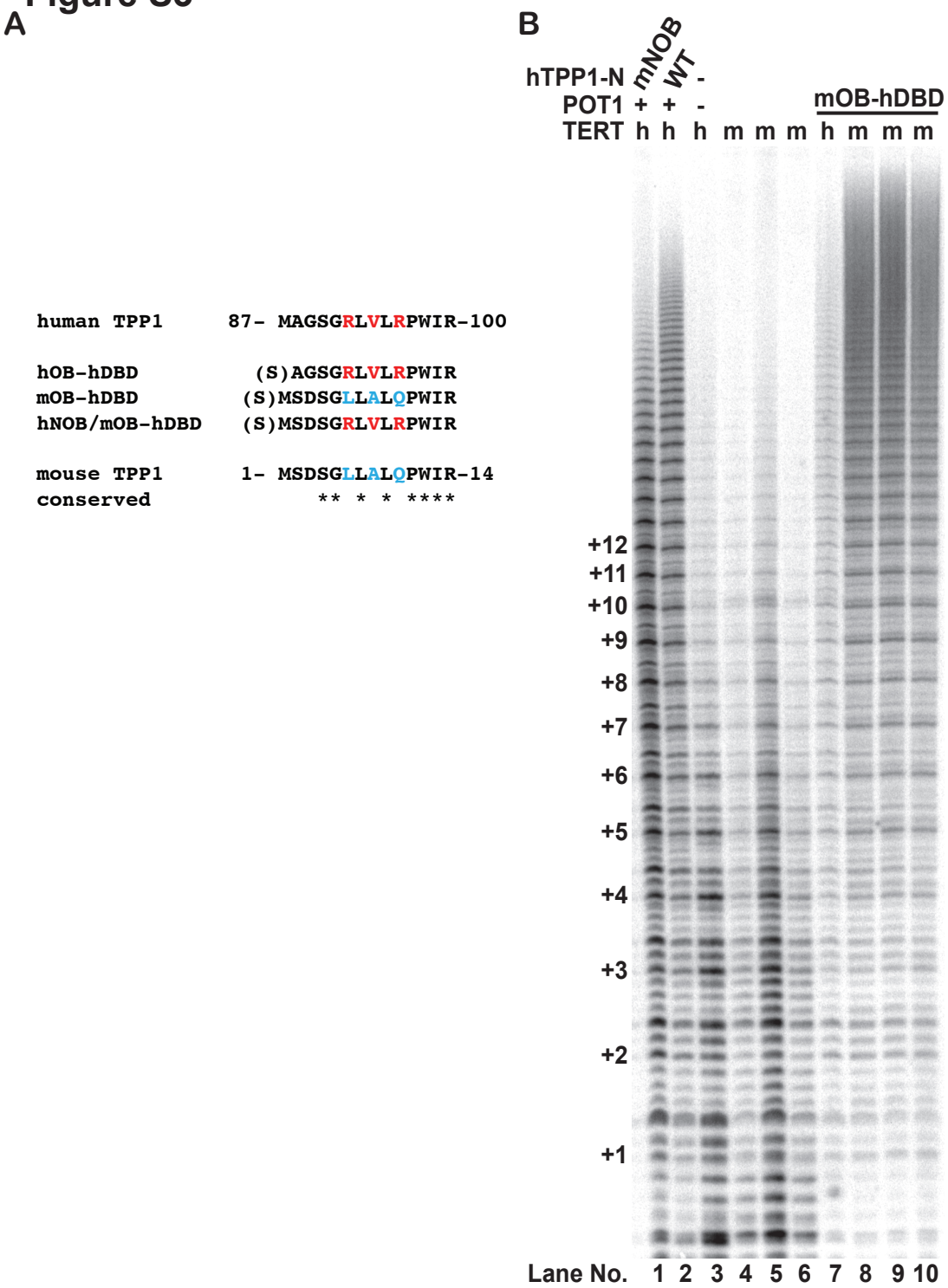

Figure S3. Mouse-human hybrid OB-DBD constructs, Related to Figure 4. (A) Schematic of N-termini for mouse and human OB-DBD variants compared with wild-type sequence for human TPP1 (above) and mouse TPP1 (below). Conserved amino acids between these N-termini are marked with asterisks. (B) Mouse NOB stimulates

reconstituted mouse telomerase but not human telomerase. Direct activity assay using extracts with telomerase reconstituted in HEK 293T cells from hTR and either hTERT (human telomerase) or mTERT (mouse telomerase). Lane 1: human telomerase plus human POT1 and TPP1-N mNOB; lane 2: human telomerase plus human POT1/ TPP1-N WT; lane 3: human telomerase extract alone; lanes 4-6: mouse telomerase reconstituted with differing ratios of transfected mTERT/TR plasmids (mTERT/TR is 1:3 in lane 4, 1:1 in lane 5, and 3:1 in lane 6); lane 7: human telomerase (hTERT/TR = 1:3) plus mOB-hDBD; lanes 8-10: same as lanes 4-6 except that mOB-hDBD was included in the reaction. hTR was used to reconstitute activity with mTERT, because telomerase reconstituted with mTR exhibits very low telomerase processivity, as previously described (Chen and Greider, 2003; Zaug et al., 2010).

Figure S4

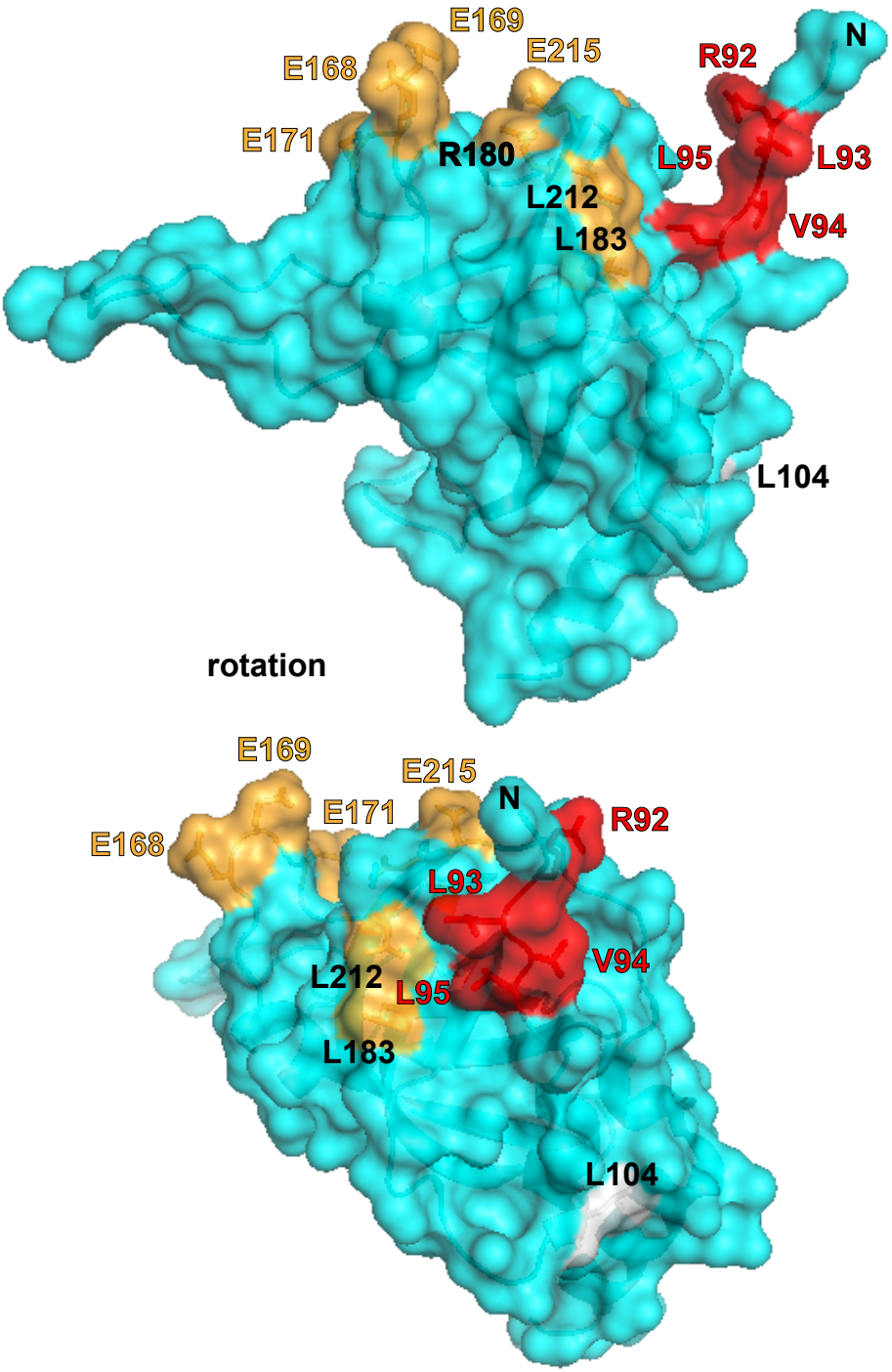

Figure S4. Relative locations of the TEL patch, NOB, and L104 regions on the surface of the TPP1 OB domain, Related to Figures 1 and 4. Two separate surface views of TPP1 OB with TEL patch residues shown in mustard, NOB residues in red, and L104 in grey.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plasmid constructs and mutagenesis: The following parental constructs used in this study have been described elsewhere: pFBHTb-Smt3star-hPOT1 for purification of insect cell-expressed human POT1 (Kocak et al., 2014); pET-Smt3-TPP1-N WT and L104A for bacterial expression of TPP1-N (aa 90-334) (Kocak et al., 2014; Nandakumar et al., 2012; Wang et al., 2007); pET-Smt3 vector for bacterial expression of constructs newly described in this study (MTA with Cornell University) (Mossessova and Lima, 2000); pTERT-cDNA6/myc-HisC and phTR-BluescriptIISK(+) constructs for overexpression of telomerase in HEK 293Tcells (Nandakumar et al., 2012); pTet-IRES-eGFP-BI4 vector, p3x-FLAG-TPP1-BI4 plasmid for Tet-inducible expression of FLAG-tagged human TPP1 (aa 87-544) in HeLa-EM2-11ht cells, and pd1gfpPtetmiR vector for Flp recombinase-mediated stable clone generation (MTA with Tet System Holdings GmbH & Co KG) (Nandakumar et al., 2012).

For expression of N-terminal 3X-FLAG-tagged mTERT in human cell lines, we substituted the mTERT cDNA sequence (pCiteE-mTERT plasmid was a kind gift from Julian Chen, Arizona State University) for the human TERT cDNA sequence in pTERT-cDNA6/myc-HisC using Gibson Assembly (New England Biolabs; NEB).

3X-FLAG-tagged TPP1 ΔNOB and L104A were cloned into the pTet-IRES-eGFP-BI4 and p3X-FLAG-TPP1-F3 vectors as described previously (Nandakumar et al., 2012). Myc-tagged POT1 and TIN2 were similarly amplified from plasmids containing the respective cDNA sequences (Kocak et al., 2014; Nandakumar et al., 2012) and cloned into the pTet-IRES-eGFP-BI4 vector to furnish p6X-Myc-POT1-BI4 and p6X-Myc-TIN2-BI4, respectively.

For bacterial expression of TPP1-N variants ΔNOB, 92-95A, 92-93A, 94-95A, and mNOB, appropriate TPP1-N sequences were cloned into the in pET-Smt3 vector using standard restriction endonuclease-mediated cloning. TPP1-N variants 96A and 100-101A were generated by QuikChange site-directed mutagenesis (Agilent technologies) using pET-Smt3-TPP1-N WT as template.

The construct pET-Smt3-hOB-hDBD was created for expression of hOB-hDBD WT, which consists of the OB domain of TPP1 (aa 88-250) and the DNA binding domain (DBD) of human POT1 (aa 2-303), linked by a 29 aa glycine and serine-rich linker NH_2-VDGSGGSSGSGGSGSSGSSGGSKLAAALD-COOH. This was established by

first splitting the multiple cloning site of the pET-Smt3 vector by inserting the cDNA sequence coding for the glycine-serine stretch (IDT) between the *SalI* and *HindIII* restriction sites. The human OB domain was cloned between the upstream *BamHI* and *SalI* sites, while the DBD was cloned between the downstream *HindIII* and *XhoI* sites. A similar strategy was used to create the mOB-hDBD and the hNOB/mOB-hDBD constructs (the N-termini of these proteins are specified in Fig. S3A). hOB-hDBD mutants (ΔNOB, 92-95A, and EE-AA) were generated by QuikChange site-directed mutagenesis (Agilent technologies) using hOB-hDBD WT as template.

For insect cell expression of TPP1-N we engineered a pFBHTb-sumostar-TPP1-N plasmid using the strategy described for engineering $(His)_6$-SUMOstar human POT1 (Kocak et al., 2014).

All oligonucleotides used in this study were purchased from IDT.

Purification of protein expressed in insect cells: Full-length human POT1 was expressed as a SUMOstar-$(His)_6$-POT1 fusion protein in baculovirus-infected High Five cells (Thermo Fisher Scientific), as described previously (Kocak et al., 2014). Briefly, POT1 was purified from the soluble cellular lysate with Ni-NTA agarose resin (Qiagen). After tag cleavage with SUMOstar protease (LifeSensors), untagged protein further purified by Superdex 200 size-exclusion chromatography (GE Healthcare Life Sciences) in 25 mM Tris-Cl (pH 8), 150 mM NaCl, and 2 mM DTT. The POT1-TPP1-N heterodimeric complex was similarly prepared from insect cells co-infected with viruses separately expressing $(His)_6$-SUMOstar-POT1 and $(His)_6$-SUMOstar-TPP1-N fusion proteins. Typical final yields were 2 and 6 mg per liter of culture for POT1 and the POT1-TPP1-N heterodimer, respectively.

Purification of protein expressed in bacteria: WT and variants of TPP1-N and OB-hDBD were expressed in *E. coli* BL21(DE3) strain as Sumo-$(His)_{10}$-fusion proteins. For protein purification, cells were harvested, sonicated, and centrifuged to clarify the soluble cellular lysates. Fusion proteins were purified with Ni-NTA agarose resin (Qiagen) and eluted with buffer containing 300 mM imidazole. Following tag cleavage with Ulp1 protease (MTA with Cornell University) (Mossessova and Lima, 2000) TPP1-N or OB-hDBD was further purified by Superdex 75 size-exclusion chromatography (GE Healthcare Life Sciences) in 25 mM Tris-Cl (pH 8), 100 mM NaCl, and 2 mM DTT. Typical final yields were 2 mg and 1-2 mg per liter of culture for TPP1-N and the OB-DBD constructs, respectively.

Primer extension assay: Super telomerase extracts were prepared from HEK 293T cells transiently transfected with constructs to express hTR and 3X-FLAG-tagged hTERT (or 3X-FLAG-tagged mTERT), as previously described (Cristofari and Lingner, 2006). Extension assays (Blackburn et al., 1989) supplemented with α^{32}P-dGTP were performed for an hour at approximately 30ºC and included super telomerase extract, 1 μM primer a5 [5'-TTAGGGTTAGCGTTAGGG-3'; (Wang et al., 2007)] and 500 nM of the purified TPP1-N variants, POT1 or OB-hDBD chimeras following a previously detailed method (Nandakumar et al., 2012). Extension products were precipitated with ethanol, dissolved in formamide loading dye and resolved on a 10% polyacrylamide/ 7 M urea/ 1x TBE sequencing gel. The data were imaged using a Phosphorimager (Storm; GE Healthcare Life Sciences) and analysis was performed with ImageQuant TL software (GE Healthcare Life Sciences) using 1D analysis and the rolling ball method for background correction. Activity was scored by quantitating the signal for the entire lane. Processivity values were obtained by dividing the total intensity from bands representing addition of 9 or more hexad repeats by the total intensity in the lane (omitting repeats 1 and 2) using the rolling ball method to define background.

HeLa culture and stable cell line generation: Tetracycline-inducible protein expression was performed in HeLa-EM2-11ht cells that constitutively express a tetracycline-controlled transcriptional activator and contain a thymidine kinase gene that can be excised with Flp recombinase for facilitating cassette exchange (Nandakumar et al., 2012; Weidenfeld et al., 2009). HeLa-EM2-11ht cells were cultured at 37°C in the presence of 5% CO_2 and propagated in modified DMEM (Dulbecco's Modified Eagle Medium; Gibco 11995-065) medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. Stable clones for doxycycline-inducible expression of TPP1 (ΔNOB and L104A) were engineered by the same procedure used to create the TPP1 WT line (Nandakumar et al., 2012). Briefly, cells were co-transfected with Lipofectamine 2000 (Thermo Fisher Scientific) and 1 ug each of the p3X-FLAG-TPP1-F3 (TPP1ΔNOB or TPP1-L104A) and a Flp recombinase-expressing plasmid also encoding puromycin resistance. One day of positive selection was performed with puromycin (5 ug/mL; Sigma-Aldrich), followed by 10 days of negative selection in fresh medium adjusted to 50 μM ganciclovir (Sigma-Aldrich). Individual clones were picked and expanded, and two positive clones of each TPP1 variant were selected based on GFP fluorescence (From IRES-GFP locus downstream of TPP1 construct) and Western blot analysis of FLAG-

TPP1 signal after overnight induction with doxycycline (200 ng/mL). Protein expression of POT1, TIN2, and TPP1 from transiently transfected p6X-Myc-POT1-BI4 and p6X-Myc-TIN2-BI4 plasmids and from the stable cell lines expressing FLAG-TPP1 (WT, ΔNOB, and L104A) were induced with doxycycline (200 ng/mL).

Co-immunoprecipitation: HeLa-EM2-11ht cells were transfected with 1 ug each of plasmids containing FLAG-TPP1 WT, FLAG-TPP1 ΔNOB, Myc-POT1, or Myc-TIN2 plasmid. 48 h after transfection/induction with doxycycline, cells were washed with PBS, trypsinized, and dislodged with medium containing 50% fetal bovine serum to inactivate the trypsin. Cell pellets were resuspended in 400 µL of lysis buffer [50 mM Tris-Cl (pH 7.4), 20% glycerol, 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.02% SDS, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche)] and kept on ice. Next, 33 µL of 4 M NaCl was added followed by 433 µL of water before centrifuging at 16,000 x g for 10 min. Supernatants were used directly in immunoprecipitation. A portion of the lysate (50 µL) was kept aside to serve as an "input" sample. 30 µL of pre-washed anti-FLAG M2 affinity beads slurry (Sigma; A2220) was added to the remaining supernatant and the samples were rocked overnight at 4°C. The beads were washed three times with 0.5X lysis buffer and the bound proteins were eluted in 60 µL of 2X SDS gel loading buffer. All samples were heated for 10 min at 95°C and analyzed by SDS-PAGE followed by immunoblotting with HRP-conjugated anti-FLAG or anti-myc antibodies.

Immunoblotting: Proteins from cellular lysates or immunoprecipitation were resolved by SDS-PAGE, transferred to nitrocellulose (BioRad), and blocked with StartingBlock (TBS) blocking buffer (Thermo Scientific). Immunoblotting was performed following standard procedures and 1:10,000 dilution of the following antibodies: mouse monoclonal anti-FLAG M2-HRP conjugate (Sigma; A8592), mouse monoclonal anti-c-Myc (Developmental Studies Hybridoma Bank; 9E10) HRP conjugate (Santa Cruz; sc-40 HRP), and mouse monoclonal anti-β-actin antibody (Sigma; A5441) in conjunction with secondary horseradish peroxidase-conjugated goat antibody against mouse IgG (Santa Cruz Biotechnology). Antibodies were detected by chemiluminescence with ECL plus reagents (Pierce ECL Western Blotting Substrate; Thermo Scientific). The data were visualized using a gel-documentation system (ChemiDoc™ MP System; BioRad). Quantitation of actin or FLAG band intensity was performed using ImageJ software.

Immunofluorescence (IF) and fluorescence in situ *hybridization (FISH) microscopy:* Stable cell lines expressing either TPP1 WT, ΔNOB, or L104A were induced with doxycycline for 3 days and then ~100,000 cells were seeded on coverslips in a 12 well plate with growth medium containing doxycycline. Twenty-four hours post-seeding the medium was removed and the cells were washed with PBS. All subsequent steps were performed at room temperature. Cells were fixed with 4% formaldehyde in PBS for 10 min and washed three times for 5 min in PBS before permeabilization in PBS containing 0.5% Triton-X 100 for 10 min. IF-FISH experiments for telomerase recruitment were performed as described previously (Nandakumar et al., 2012). Briefly, IF was first performed to visualize FLAG-TPP1 proteins using mouse monoclonal anti-FLAG M2 (Sigma; F1804; 1:500) in combination with Alexa Fluor 568-conjugated anti-mouse IgG (Life Technologies). Subsequently a mixture of Cy5-conjugated probes complementary to TR was used at a concentration of 30 ng per probe per coverslip to detect TR by FISH (Abreu et al., 2010). The cells were washed three times in PBS and mounted on microscope slides using ProLong Gold mounting medium with DAPI (Life Technologies). Coverslips were sealed with transparent nail polish and stored at -20°C until the time of imaging. A laser scanning confocal microscope (SP5; Leica, Germany) equipped with a 100x oil objective was used to image IF-FISH experiments. The images were processed with ImageJ and Adobe Photoshop, and colocalizations were quantified manually by two separate individuals.

Telomere restriction fragment length analysis: Genomic DNA was purified by the GenElute kit (Sigma, G1N350-1KT) from stable cell lines overexpressing either FLAG-TPP1 constructs or a vector control. DNA (2 µg) was digested with *HinfI* and *RsaI* and incubated overnight at 37°C. The digested DNA was run on a 25 cm long 0.8% agarose-1X TBE gel along with a lambda DNA-HindIII digest ladder (NEB) at a constant 50 V for 21-23 h. The gel was imaged with a fluorescent ruler and then dried at 55°C for one hour prior to denaturation in 0.5 M NaOH for 30 min. The gel was then rinsed with water, neutralized with 1.5 M NaCl and 0.5 M Tris-Cl (pH 7.5) for 30 min, and prehybridized in Church buffer [0.5 M sodium phosphate buffer (pH 7.2), 1% bovine serum albumin, 1 mM EDTA, and 7% SDS] for 30 min at 65°C in a rotating hybridization oven. T4 polynucleotide kinase was used to 5' ^{32}P-label a $(TTAGGG)_4$ oligonucleotide, which was added at 20 million cpm to the gel. Hybridization was continued overnight at 55°C. The gel was then washed three times with 2X SSC for 10 min at 55°C and exposed to a phophorimager screen. The gel was analyzed using the Imagequant TL software and calibrated using the molecular

weights of the lambda DNA-*HindIII* digest ladder. The mean telomere length for each lane was plotted as a function of days in culture for each cell line.

Telomere localization of TPP1 constructs: Telomeres were visualized by fluorescence *in situ* hybridization (FISH) using a fluorescent telomeric PNA probe. Briefly, cells were fixed for 10 min with 4% formaldehyde in PBS, washed, then permeablized for 10 min in PBS containing 0.5% Triton X-100. Cells were then washed twice in PBS and soaked in 2X SSC, 50% formamide for 5 min. 40 μl of hybridization solution containing 0.3 μg/ml Cy3-conjugated PNA-$(CCCTAA)_3$ probe was placed on a microscope slide, and a coverslip containing cells from each engineered stable cell line (WT, ΔNOB, L104A) was inverted on the hybridization solution. DNA was denatured by heat for 6 min at 80°C followed by hybridization for two hours at room temperature. After hybridization the slides were washed twice at room temperature with 50% formamide in 2X SSC for 30 min. Slides were then washed with PBS and used for subsequent immunofluorescence (IF). IF was performed to visualize FLAG-TPP1 constructs to determine telomere localization. For IF, cells were blocked for 30 min with 1 mg/ml BSA, 3% goat serum, 0.1% Triton X-100, and 1 mM EDTA (pH 8.0) in PBS. FLAG-TPP1 constructs were visualized using mouse monoclonal anti-FLAG M2 (Sigma; F1804; 1:500) in combination with Alexa Fluor 633-conjugated anti-mouse IgG (Life Technologies; A21052). Cells were washed three times with PBS and mounted on microscope slides using ProLong Gold mounting medium with DAPI (Life Technologies). Imaging and analysis was performed as described above.

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