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Supplementary Materials for

TPP1 promoter mutations cooperate with TERT promoter mutations to lengthen telomeres in melanoma

Pattra Chun-on, Angela M. Hinchie, Holly C. Beale, Agustin A. Gil Silva, Elizabeth Rush, Cindy Sander, Carla J. Connelly, Brittani K.N. Seynnaeve, John M. Kirkwood, Olena M. Vaske, Carol W. Greider*, and Jonathan K. Alder*

Correspondence to: jalder@pitt.edu and cgreider@ucsc.edu

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Materials and Methods

Cloning of TPP1 expression and reporter constructs

cDNA expressing the long (aa 1-544) and short (aa 87-544) isoforms of TPP1 were cloned with c-terminal flag-tags into pCDNA5/FRT expression vector. The genomic expression constructs were made by amplifying the entire genomic locus of *ACD* (784 base pairs upstream of the translational start site of TPP1-L, GRCh38/hg38 chr16:67,657,609-67,661,115) from a bacterial artificial chromosome (CH17-394I12; BACPAC genomics) and assembling into a pCDNA5 vector that lacked the CMV enhancer and promoter and incorporated a flag-tag at the C-terminus to facilitating imaging and blotting. Luciferase reporter constructs were made by synthesizing specific fragments of decreasing size of the *ACD* proximal promoter (613, 285, 200, and 163 base pairs upstream of the TPP1-S translational start site [GRCh38 chr16:67,660,220] and assembling into the pGL4.10 [luc2] luciferase vector. Site-directed mutagenesis of pGL4.10-TPP1pro(285) fragment was performed using primers with the desired mutations (TPP1pro[-108] C>T, TPP1pro[-75] C>T, relative to the TPP1-S translational start site). All plasmid sequences were verified via Sanger sequencing.

Cell culture, transfection, and luciferase assays

HeLa (ATCC; CCL-2), HEK293FT (ThermoFisher; R70007, HEK293 hereafter), and BJ fibroblasts (ATCC; CRL-2522) cells were cultured in DMEM supplemented with 10% fetal bovine serum (20% fetal bovine serum for BJ fibroblasts) and Penicillin-Streptomycin-Glutamine (100X) and maintained at 37°C in the presence of 5% CO_2 in a humidified incubator. LOX melanoma, MEL624, A375, M308, SK-MEL27, FEMX, MEL526, 938MEL, M255, COLO829, and short term cultures (TPF-14-346 and TPF-11-743 from the Melanoma Center

Biospecimen Repository at the University of Pittsburgh Hillman Cancer Center) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and Penicillin-Streptomycin-Glutamine (100X) and maintained as above. For DNA transfection, HEK293 cells were seeded in 24-well plates and transfected with Lipofectamine 2000 (Life Technologies; cat# 11668-09) following the manufacturer's instructions. Melanoma cell lines were seeded and grown for 48 hours to 75-80% confluence prior to transfection. Cell were trypsinized and transfected using Amaxa Nucleofector II/2b (Lonza) with Amaxa Nucleofection Kit V (Lonza) using the program T-20. For luciferase assays, cells were co-transfected with TPP1 promoter constructs expressing firefly luciferase and a renilla luciferase transfection control plasmid (10:1 reporter/transfection control). Firefly luciferase activity was measured using the Dual-Glo Luciferase Reporter Assay system (Promega, USA) according to the manufacturer's instructions. The promoter activity was calculated from the ratio of firefly to renilla luciferase for each construct, and then normalized to the activity of the wild type TPP1 promoter. For co-transfection with ETS transcription factors, cDNA clones of ETS1 (RC215203L2), ETV4 (RC215093), or ETV5 (RG200366) (OriGene Technologies, Rockville, MD) were subcloned into pCDNA3.1 and co-transfected at a ratio of 5:5:1 (luciferase reporter:pCDNA3.1 expression vector:renilla transfection control). Each experiment was performed in triplicate and repeated at least three times independently (biologic replicates). For growth curves of BJ fibroblasts, cells were transduced and grown in independent cultures (n=3/group) and the cumulative population doubling was plotted as a function of time.

Generation of stable cell lines

Hela stable cell lines were generated using HeLa Flp-In cells (ThermoFisher; cat# R71407). Parental cells were co-transfected with pCDNA5 expression vectors and Flp recombinase

(pOG44, ThermoFisher; cat# V600520). Following transfection, isogenic clones were isolated via selection with hygromycin (550 ug/mL; Invitrogen, cat# 10687010) for two weeks. Stable expression of the transgene was verified by western blotting.

Western blotting

Total protein was isolated from cell pellets lysed in radioimmunoprecipitation assay (RIPA, 25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1 SDS; ThermoFisher) buffer for 20 minutes on ice in the presence of protease inhibitors (cOmplete ULTRA mini tablets, Roche, cat# 05892970001) or sonicated with 25 pulses of a Branson Sonifier. Samples were centrifuge at 12,000g for 10 minutes and total cellular protein quantitated using Bicinchoninic Acid assay (Pierce, Rockford, IL, USA). Proteins were separated using any kD SDS-page gels (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blotted with primary antibodies to FLAG (M2; Sigma, 1:1000), Rhodamine Anti-GAPDH (Bio-Rad, 1:2000), ETS1 (D808A, Cell Signaling, 1:1000), ETV4 (PA5-76825; Thermo Fisher, 1:1000), ETV5 (WH0002119M2; Sigma, 1:1000), TERT (ab32020, Abcam, 1:1000), and TPP1 (A3030-069A; Bethyl Laboratories, 1:1000). Detection was performed using HRP-conjugated secondary antibodies (Bio-Rad, 1:5000). Blots were imaged using a ChemiDoc MP Imaging System.

Immunofluorescence

For TPP1-TRF2 co-localization analysis using co-immunofluorescence (co-IF), 50,000 cells of HeLa Flp-In stable cell lines were seeded on coverslips in a 12-well culture plate. After 24 hours, cells were washed once with PBS and fixed with 2% paraformaldehyde in PBS for 10 min. The

fixative was removed and cells were washed twice with PBS for 10 min each followed by permeabilized with KCM solution (120 mM KCl, 20 mM NaCl, 10 mM Tris pH 7.5, 0.1% triton) for 5 min. Cells were then washed twice with PBS and then incubated in blocking solution (10% normal goat in PBS and 0.1% [v/v] Tween 20 [PBST]) for 30 min. Coverslips were incubated with anti-FLAG (Sigma; M2; 1:1000) antibody and rabbit polyclonal anti-TRF2 (Novus Biologicals; NB110-57130; 1:500 dilution) primary antibodies in IF blocking solution overnight at 4°C. The following day, coverslips were washed three times and incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen cat# A11032) and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen cat# A27040) diluted 1:500 for 1 hour in the dark. The coverslips were then washed three times in PBST and the excess PBST was removed by blotting. Coverslips were incubated with DAPI (Sigma; D9542) in PBS for 5 min and washed with PBS for 5 min. Coverslips were then mounted on microscope slides using Prolong Gold mounting medium (ThermoFisher cat# P36970) and stored overnight prior to imaging. Imaging was performed with an upright Nikon ECLIPSE Ni fluorescent microscope with a charge-coupled device camera (ORCA-Flash4.0LT, Hamamatsu).

Telomere Fluorescence in Situ Hybridization (FISH) Analysis

Telomere FISH was carried as described previously (*26*). Peptide nucleic acid probes for the canonical telomere sequence (TelC-Cy3; F1002; C-rich telomere probe, Cy3 labeled) and a custom TTAGGT probe (Alexa-647 labeled; acctaaacctaaacctaa) were synthesized by PNA Bio (Thousand Oaks, CA). Images were acquired on the Nikon ECLIPSE Ni fluorescent microscope with a charge-coupled device camera. Images were deconvolved and analyzed on NIS-Elements General Analysis 3 Advanced Research software.

Telomere Restriction Fragment (TRF) Southern blot analysis

Genomic DNA was extracted using a Promega Wizard Genomic kit according to the manufacturer's protocol. Approximately 1-2 ug of genomic DNA samples were digested overnight with HinfI and RsaI and resolved on a 0.8% agarose gel. Southern Blots were carried out by modification of the method previously described (*27*). DNA in the gel was denatured for 45 min in 0.5 M NaOH/1.5 M NaCl and then neutralized for 15 min in 1.5 M NaCl/0.5 M Tris-HCL pH 7. The DNA was vacuum transferred in 10X SSC (Sodium Sodium Citrate: 3M NaCl, 0.34M NaCitrate) to a Nylon Membrane (Amersham Hybond N+) and cross-linked with UV Stratalinker (Stratagene). Pre-hybridization was done at 65ºC in Church buffer (0.5M sodium phosphate, pH7.2, 7% SDS, 1% bovine serum albumin, 1 mM EDTA) for 2 hours. 25ng of a 600bp telomeric fragment generated by EcoRI digestion of JHU821 containing 100 repeats of TTAGGG/CCCTAA was radiolabeled along with a 1kb DNA ladder (NEB) with 33mM dATP, dTTP, dGTP and alpha-^{32}P dCTP (3000 Ci/mmol) (Perkin Elmer) using Klenow Fragment (3'-5' exo-, NEB) and random 9-mer oligonucleotides for 8 minutes at 37°C. Unincorporated nucleotides were removed using Micro Bio-Spin P-30 Chromatography Columns (Bio-Rad). Heat denatured radiolabeled probes were added to the pre-hybridization mix at 10^6 counts/ml (telomere probe) or 10^5 counts/ml (ladder) and hybridized overnight at 65°C. Membranes were washed 3 times for 15 minutes in 2X SSC and 0.1%SDS at 65ºC, and 3 times for 15 minutes each in 0.5XSSC and 0.1% SDS at 65ºC, exposed to Storage Phosphor Screens (GE Healthcare) and detected on a Storm 825 Imager (GE Healthcare) using ImageQuant Software (GE Healthcare).

RNA extraction and RT-qPCR for ETS factors and *ACD* mRNA expression

Total cellular RNA was extracted from cell lines using RNeasy™ mini kit (Qiagen). A cDNA was made for each sample using iScript™ cDNA synthesis kit (BIO-RAD). Primers for *ETS1, ETV4, ETV5, GAPDH,* and *B2M* were purchased from Qiagen (QuantiTect Primer Assay). Primers for *ACD, HPRT, GUSB,* and *PPIA* were purchased from IDT (PrimeTime Presdesigned qPCR assay). qPCR was carried out using the CFX96 or CFX384 Real time System, Expression levels of ETS factors mRNA were calculated from threshold cycle values and normalized to *GAPDH* and *B2M* values. Expression of *ACD* was normalized to *HPRT, GUSB,* and *PPIA* using CFX Maestro software (Bio-Rad) .

CRISPR-Cas9 mediated HDR homology-directed repair genome editing

Generation of TPP1 genomic -108C>T and -75C>T knock-in clones was performed using recommended crRNA and HDR temples from IDT (https://www.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool). The following chemically modified HDR templates were used to introduce the -108C>T and -75C>T variants (IDT; Coralville, IA, USA).). -108C>T plus strand: 5'-

TCCTCGGAAGAGGAAGCTCCTTCGCTGGGCGGGGCCGGAGGAAGAGGCCCCGCCCA

CGTACACCCCGCGCCTGCGCACGAGGG -3', -108C>T minus strand: 5'-

CCCTCGTGCGCAGGCGCGGGGTGTACGTGGGCGGGGCCTCTTCCTCCGGCCCCGCCC

AGCGAAGGAGCTTCCTCTTCCGAGGA -3, and -75C>T plus strand: 5'-

CCGGGTTTCCCGCGGGCGCCCAGGCCCCGCCTTTCCTCGGAAAAGGAAGCT

CCTTCGCTGGGCGGGGCCGGAGGAGGAGGCCC -3', Minus strand: 5'- GGGCCTCCT

CCTCCGGCCCCGCCCAGCGAAGGAGCTTCCTTTTCCGAGGAAAGGCGGGGCCTGGG

CGCCCGCGGGAAACCCGG -3'. Alt-R crRNA was synthesized targeting the sequence shown in Fig. S6. Alt-R crRNAs and Alt-R tracrRNA were mixed in an equimolar ratio and heated at 95°C for 5 min and then incubated at room temperature to allow annealing. We use either ATTO 550 labeled tracrRNA or GFP-labeled Cas9 to facilitate identification of transfected cells. For each nucleofection reaction, 104 pmol of Alt-R Cas9 protein (IDT) was complexed with annealed crRNA/tracrRNA in a 1:2.5 molar ratio in PBS (5 μL total volume). Complexes were allowed to form for 15–20 min at room temperature before nucleofection. Cells were reconstituted in Amexa^{TM} Cell Line Nucelofector^{TM} Solution V, according to the manufacturer's instructions (Lonza) and mixed with RNP complexes at a final concentration of 1 or 4 μM. Cells were then supplemented with an equivalent volume of PBS. The supplemented cell solution (final cell concentration of 2×10^6 /mL) was transferred into the Lonza Nucleofector IIb and electroporated using the T-020 program. Recovered cells were cultured for 48 hours prior to sorting into a 96-well plate (one cell per well). Following two weeks of culture, clones were expanded and genomic DNA was isolated to screen for edited clones. Screening was accomplished by PCR amplifying the TPP1 promoter (5`primer – cgcgatgagagtaaacgggc and 3`primer - cctccccgaacctgccat) and then digesting the PCR product with EarI or BseRI. PCR products that were resistant to digest were sequenced to validate the presence of the edited nucleotide.

Lentiviral construction and packaging

Dual promoter lentivirus were constructed that express TPP1 and hTERT under the EF1alpha core promoter and carried selectable markers for blasticidin and neomycin, respectively (under the hPGK promoter). hTERT was codon optimized to facilitate synthesis of the transgene

(sequence available upon request). A lentivirus expressing a modified hTR (TTAGGT) was created using the pLV-IU1-hTR-CMV-Puro vector (a generous gift from Dr. Bradely Stohr). The template region, spanning from r.46 to r.56, was modified to express a variant hTR that encodes TTAGGT repeats rather than the canonical TTAGGG sequence repeats (CUAACCCUAAC -> CUAAACCUAAA). All plasmid sequences were confirmed by Sanger sequence. All lentiviruses were packaged in HEK293 cells by co-transfection of the lentiviral vector and packaging plasmids (pCMV-delta8.9 and pCMV-VSV.G). The following morning, the media was changed to DMEM plus 1% FBS (Gibco) and viral supernatants were isolated 48 hours later. MEL624 and BJ fibroblasts were transduced in the presence of 8ug/mL polybrene overnight and the media was changed on the following morning. Transduced cells were selected 48 hours have by addition of Geneticin (ThermoFisher), Blasticidin (ThermoFisher) or both.

ACD isoform analysis

RNA-seq FASTQ files were downloaded from GSE153592 and GSE112509 (*17*, *18*) trimmed for quality and aligned to the GRCh38 using STAR (*28*). Depth of coverage was determined using Samtools and normalized by dividing the per base coverage by the total number of reads mapping to *ACD* for each sample. The mean coverage of each base was calculated and converted to a Wiggle plot and uploaded as a custom track on the UCSC genome browser. Historic annotations from RefSeq were provided by Mark Diekans at UCSC and screenshots were exported as PDFs and modified in Adobe Illustrator (*29–31*).

Promoter enrichment analysis

Promoter enrichment analysis was conducted using Gencode (*32*) version 40 gene models lifted to hg19 from https://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/wgEncodeGencodeBasicV40lift37.txt.gz downloaded on 6/10/2022. Promoters were defined as 200 bases upstream from each transcription start site. Overlapping promoters were merged, resulting in 59,727 promoter regions. Genome-wide mutation calls from 305 tissue donors with melanoma were obtained via the International Cancer Genome Consortium Data Portal (*11*): 37 donors from the TCGA-ICGC sequencing project with variants called with the PCAWG Consensus SNV-MNV caller, downloaded as final_consensus_snv_indel_tcga.controlled.tgz from https://dcc.icgc.org/releases/PCAWG/consensus_snv_indel; 100 donors from the SKCA-BR ICGC project with variants called using varscan downloaded as simple_somatic_mutation.controlled.SKCA-BR.tsv.gz from https://dcc.icgc.org/releases/current/Projects/SKCA-BR; 168 donors from the MELA-AU ICGC project with variants called using PCAWG and GATK, download as simple_somatic_mutation.controlled.MELA-AU.tsv.gz from https://dcc.icgc.org/releases/current/Projects/MELA-AU. All downloads required permission to access ICGC controlled data. Cell lines were excluded from the MELA-AU dataset and variants from multiple tumors from the same individual were combined so no mutation was reported more than once per individual. Promoters enriched for mutations were identified using MutEnricher (*33*) version 1.3.3 with default settings. We report significance values from the Fisher_FDR column, which combines the results of the region analysis, the weighted average proximity procedure and hotspot analysis, and is corrected for multiple hypotheses testing using

the Benjamini-Hochberg FDR method. Additional analysis using locally calculated background mutation rate, as opposed to global mutation rate, was also highly significant (not shown).

Comparison of tumor and normal *TPP1* expression

Gene expression data from GENT2 (*34*) and OncoDB (*35*) were downloaded on 07/10/2022 and replotted using GraphPad Prism.

Statistical Analysis

A p-value less than 0.05 was considered significant after correction for multiple testing. Data was analyzed using GraphPad Prism version 9.3.1. Statistical tests used are listed in the figure legends where the data is shown.

Fig. S1

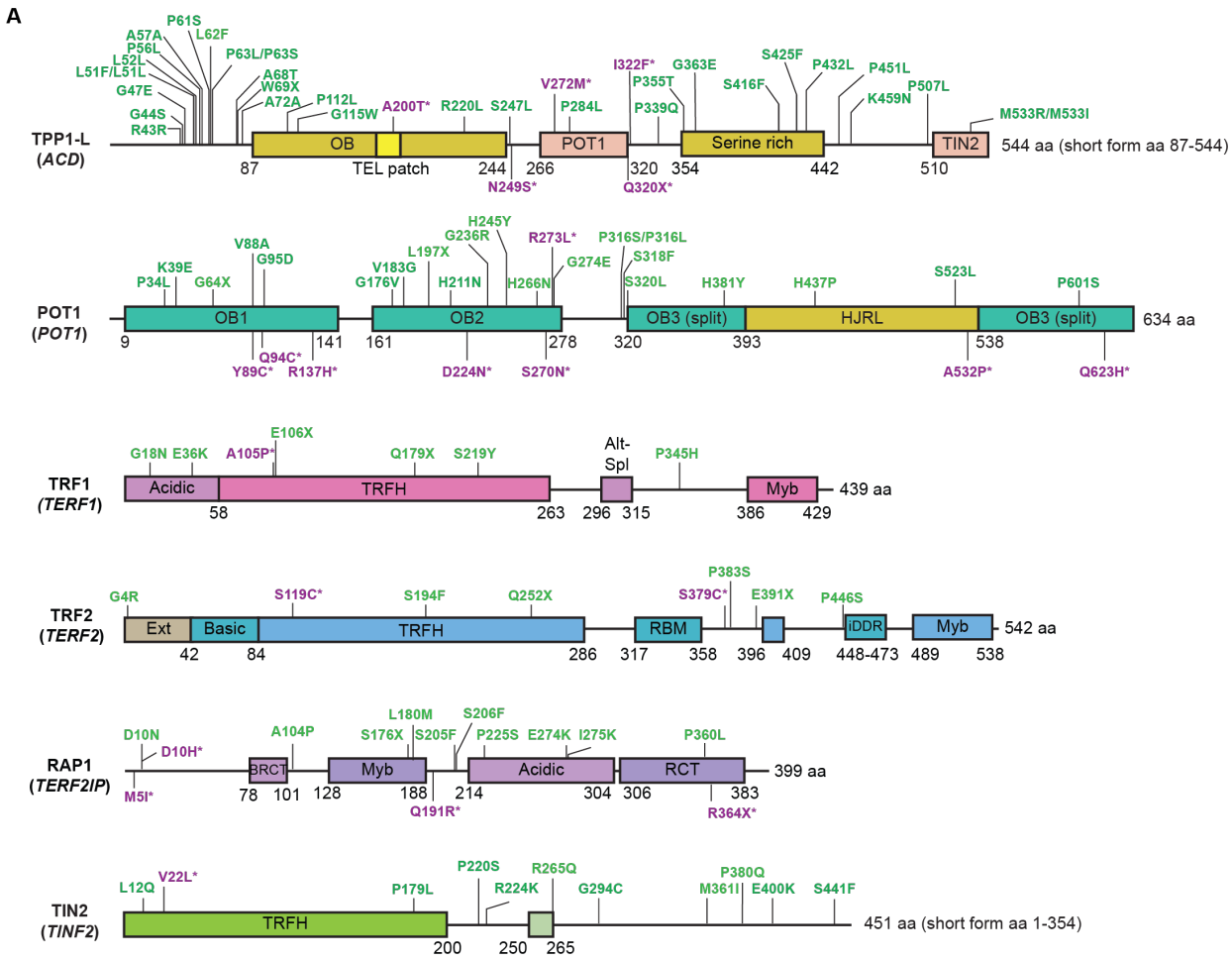


Fig. S1. Somatic variants in shelterin components. (A) Schematic of the six shelterin proteins and somatic variants from the ICGC data portal. Variants were extracted on March 1, 2022 from exome and genome sequencing data from skin cancer (n=749, predominantly cutaneous melanoma). Variants from the ICGC are annotated in green while a subset of variant reported in previous publications are reported in purple with an asterisks (***12–14***, ***36***, ***37***). The long isoforms of TPP1 and TINF2 are depicted along with several of the previously characterized domains in each of the proteins.

Fig. S2

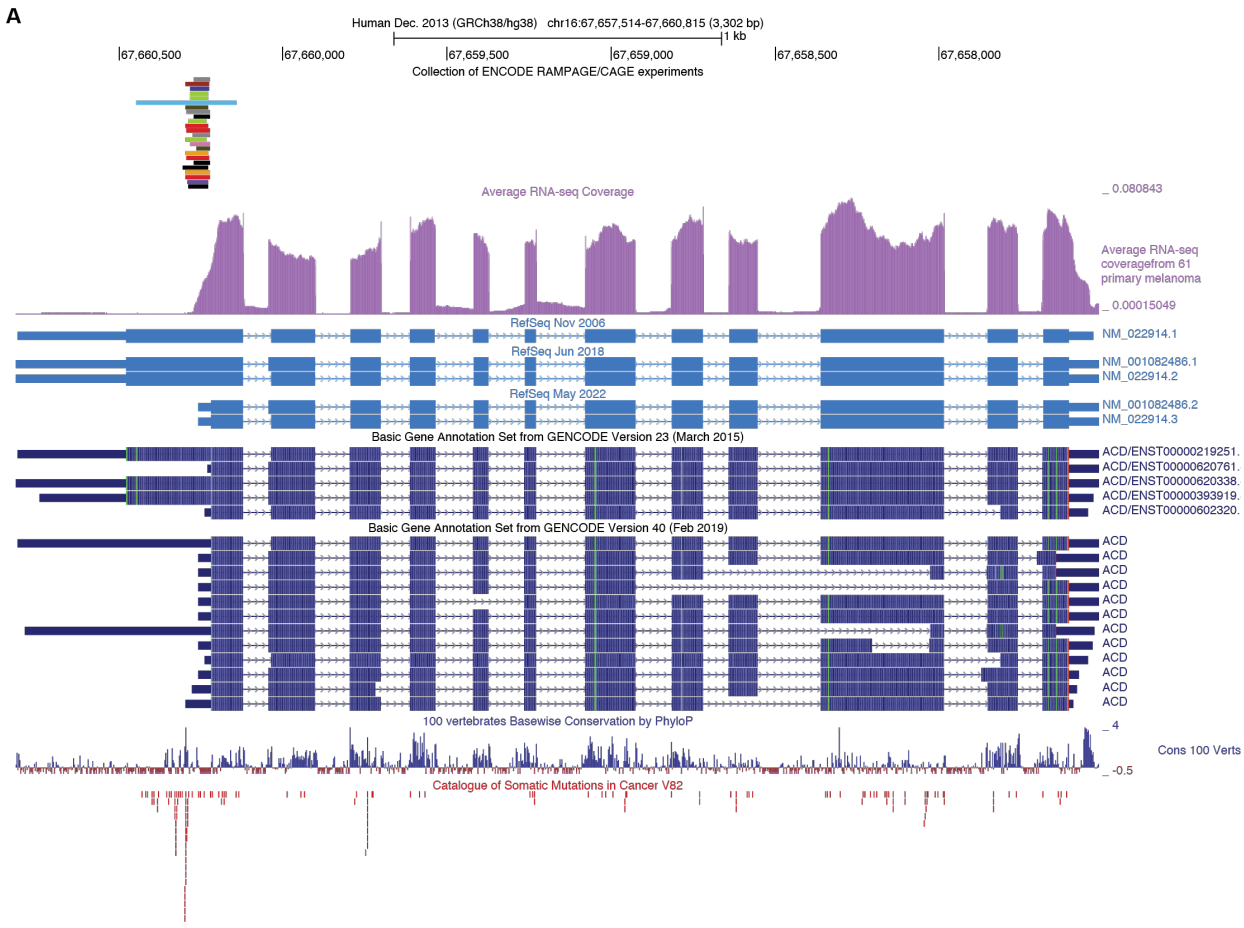
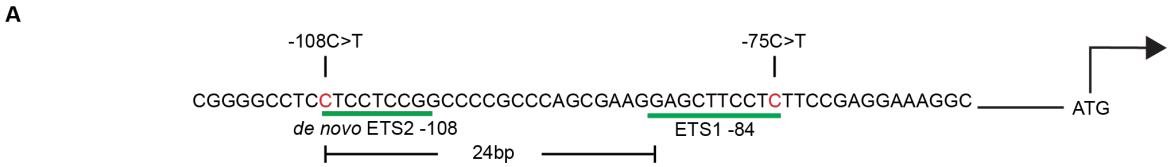


Fig. S2. The short isoform of *TPP1* is the only transcript in primary melanoma samples. (A) Screen capture from UCSC genome browser (https://genome.ucsc.edu) showing the historic annotation of *ACD* from RefSeq and GENCODE. RNA-seq coverage from 61 primary nevi and melanoma samples support that the short isoform is expressed in melanoma. RAMPAGE/CAGE data from several tissues from ENCODE further support that transcription initiation occurs in proximity to the cluster of variants we identified and that the short isoform is the only transcript in most tissues (top panel). Vertebrate conservation and the location of COSMIC variants is shown in the bottom panels.

Fig. S3



B

***De novo* ETS binding motifs created by -108C>T TPP1pro variant**

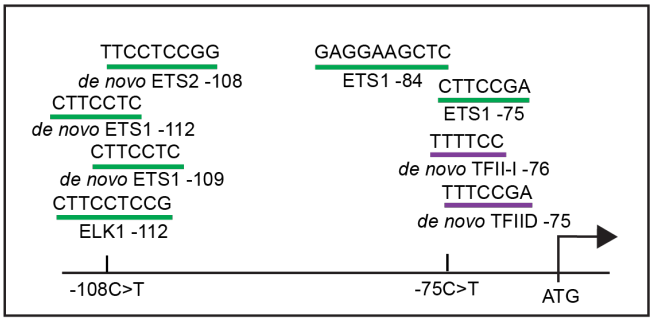
Factor name	Start position	End position	Sequence
ETS2	-108	-100	TTCCTCCGG
ETS1	-112	-103	CCTCTTCCTC
ETS1	-109	-103	CTTCCTC
ETS1	-84	-75	GAGCTTCCTC
ETS1	-75	-69	CTTCCGA
ELK1	-112	-103	CTTCCTCCG

C

***De novo* TFII binding motifs created by -75C>T TPP1pro variant**

Factor name	Start position	End position	Sequence
TFII-I	-76	-71	TTTTCC
TFIID	-431	-425	TAATAAA
TFIID	-75	-69	TTTCCGA

D



E

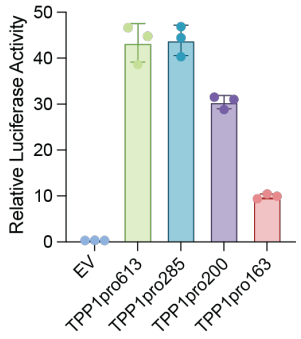


Fig. S3. Recurrent variants in the TPP1 promoter create ETS transcription factor binding sites. (A) Schematic of the TPP1 promoter showing the sequence context and location of the most common variants and their position relative to the TPP1-S translational start site. (**B-D**) Promoter analysis using PROMO ***(38, 39)*** showed that the -108 variant created a core ETS binding site while the -75 variant modified and existing ETS transcription factor binding site. The -75 variant fell in the region of the annotated transcriptional start site for TPP1-S and created several TFIID binding motifs (purple). (**E**) Luciferase reporter with specific fragments of the TPP1 proximal promoter containing 613, 285, 200, and 163 base pairs of the proximal promoter relative to the TPP1-S translational start site. The 285 base pair fragment was the minimal sequence sufficient for full basal activity of the reporter. Mean and standard deviation from three independent experiments is shown.

Fig. S4

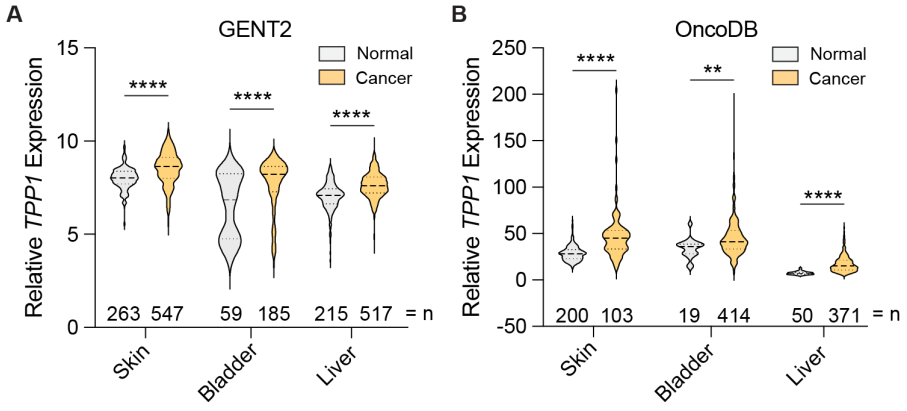


Fig. S4. *TPP1* expression is elevated in several cancers. (**A**) Gene expression data from the Gene Expression database of Normal and Tumor Tissues 2 (GENT2; http://gent2.appex.kr). (**B**) Normal and tumor gene expression data from OncoDB. Numbers below each of the violin plots indicate the number of samples included. These two database were selected because their differing methodologies decreased the probability of including overlapping datasets. GENT2 uses exclusively microarray data whereas OncoDB uses RNA-seq. Mann-Whitney test was used to compare each of the groups of samples. **P < 0.01 and ****P < 0.0001.

Fig. S5

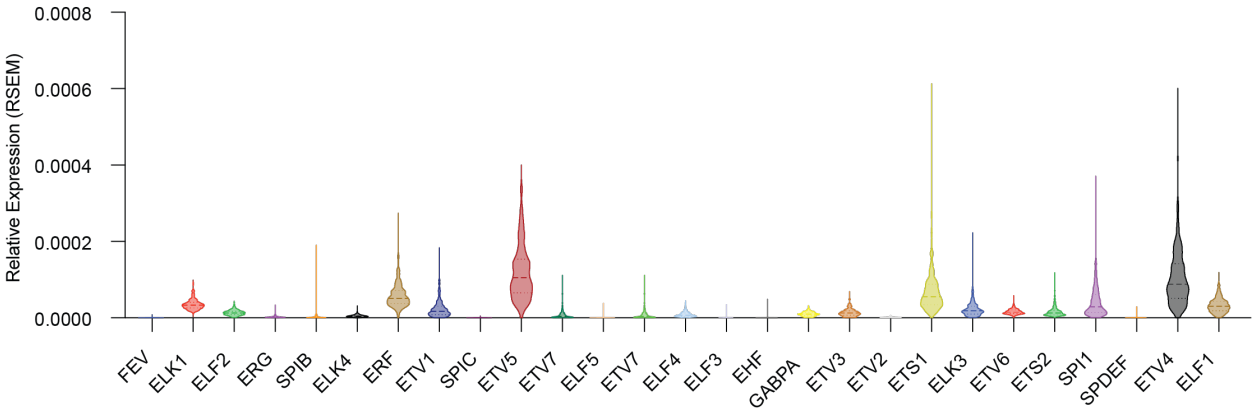


Fig. S5. Expression of ETS transcription factors in melanoma. RNA-seq data was obtained from 426 melanoma samples from the ICGC data portal. Relative expression of 27 ETS family members is shown.

Fig. S6

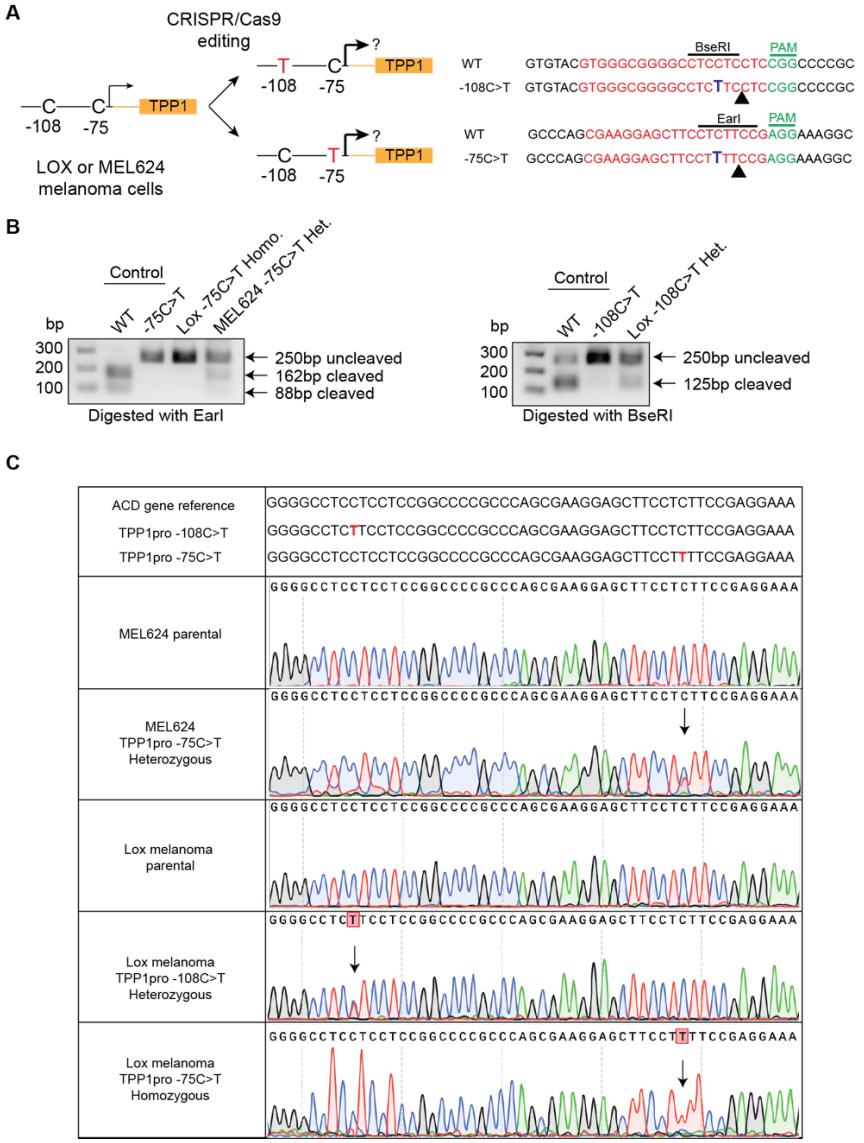


Fig. S6. Verification of genome editing of melanoma cell lines. (**A**) Schematic of experimental approach for CRISPR/Cas9 genome editing in LOX and MEL624 cells. Sequences used for targeting (crRNAs, sense strand) are shown (red) in the context of the targeted nucleotides (blue) and the protospacer adjacent motif (PAM, green). (**B**) Representative restriction digests of screen PCRs performed on successfully edited clones. (**A**) Representative chromatograms showing sequence verification of the indicated modified melanoma cell lines.

Fig. S7

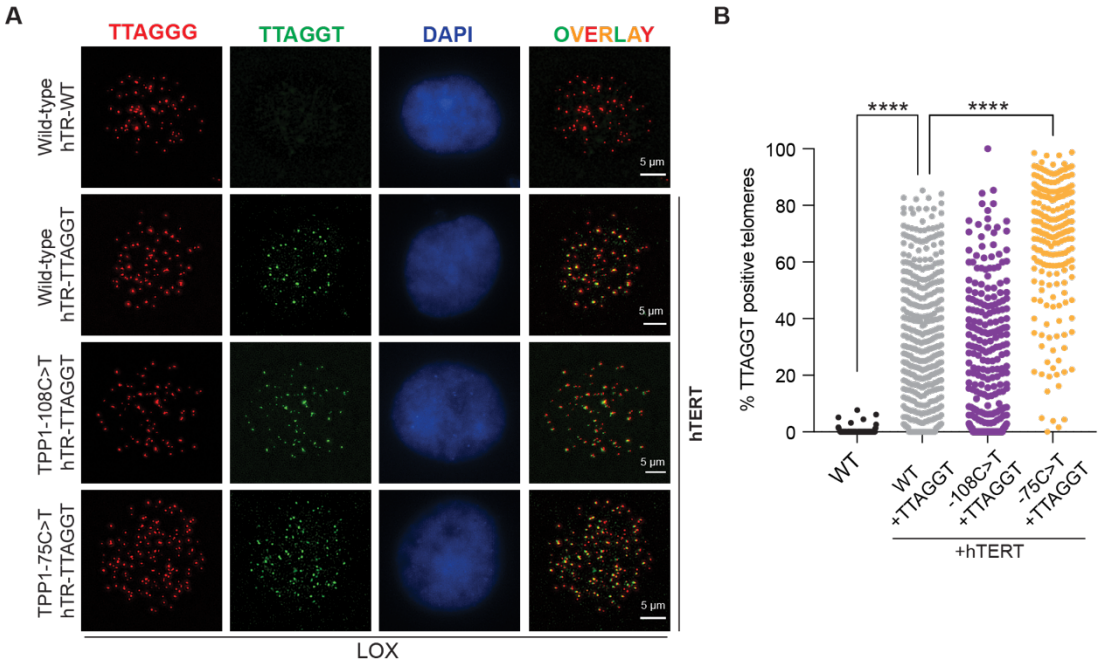


Fig. S7. Increased addition of variant telomere sequences in cells with *TPP1* promoter mutations. (**A**) Fluorescent in situ hybridization for the WT (TTAGGG; red) and variant (TTAGGT; green) in parental or genome edited LOX cells. Images were taken 7 days after transduction with lentiviruses. (**B**) Quantitation of the fraction of telomeres that had both TTAGGG and TTAGGT signals. Groups were compared using ANOVA with Dunnett's correction for multiple comparison. ****P < 0.0001.

Fig. S8

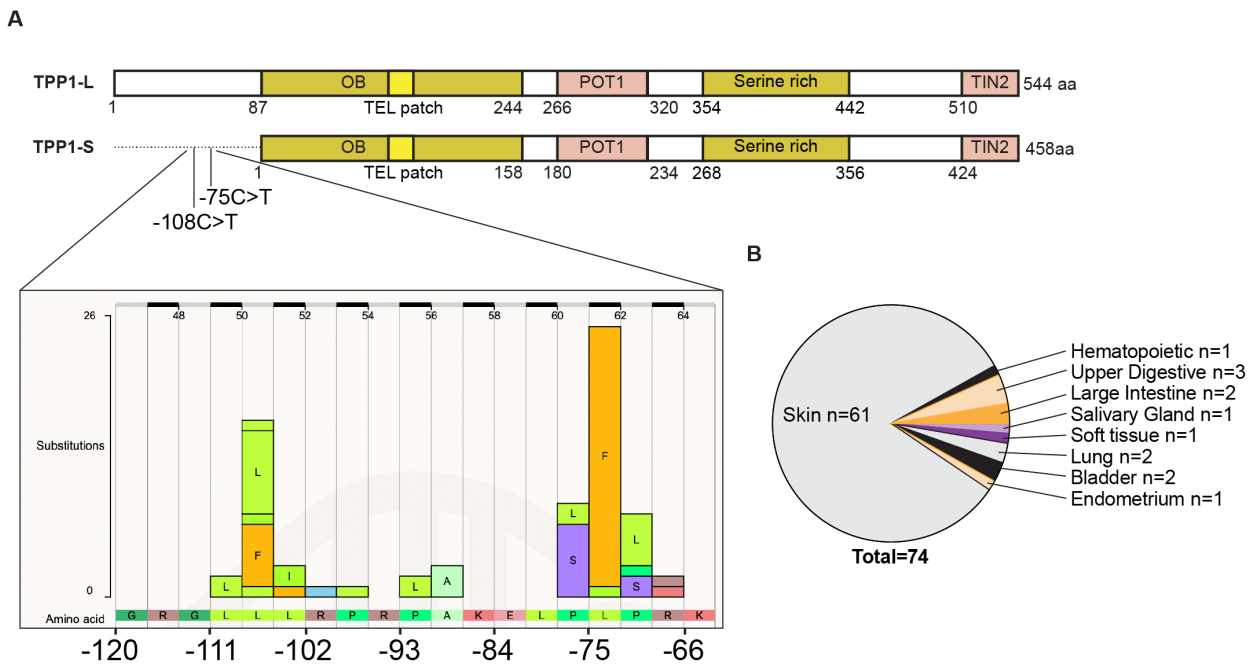


Fig. S8. Spectrum of cancers harboring *TPP1* promoter mutations. (**A**) Schematic of the TPP1-L and TPP1-S isoforms with screen capture of the cluster of mutations in the *TPP1* promoter from the Catalogue of Somatic Mutations in Cancer database (COSMIC; http://cancer.sanger.ac.uk/cosmic). The COSMIC database uses GENCODE basic annotations which prioritizes longer transcripts and therefore has annotated all of the promoter variants as coding variants in TPP1-L. We have also included the nucleotide position relative to the TPP1-S translational start site. Note that several of the variants are synonymous changes supporting that these variants to not function in TPP1-L. (**B**) Distribution of reported TPP1 promoter variants across major cancer types in the COSMIC database (v96).