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

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Abstract

Overcoming replicative senescence is an essential step during oncogenesis and reactivation of *TERT* via promoter mutations is a common mechanism. *TERT* promoter mutations are acquired in ~75% of melanomas but are not sufficient to maintain telomeres, suggesting additional mutations

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are required. We identified a cluster of variants in the promoter of *ACD*, encoding the shelterin component TPP1. *ACD* promoter variants are present in ~5% of cutaneous melanoma and co-occur with *TERT* promoter mutations. The two most common somatic variants create or modify binding sites for ETS transcription factors, similar to mutations in the *TERT* promoter. The variants increase the expression of *TPP1* and function together with TERT to synergistically lengthen telomeres. Our findings suggest that *TPP1* promoter variants collaborate with *TERT* activation to enhance telomere maintenance and immortalization in melanoma.

One-Sentence Summary:

TPP1 promoter mutations synergize with TERT activation to elongate telomeres in melanoma.

Escaping replicative senescence is an essential step of oncogenesis (1, 2). Telomere shortening limits the proliferative potential of cells and several mechanisms have been identified that permit tumor cells to extend telomeres and increase their replicative capacity (3–8). Somatic mutations in the *TERT* promoter are the most common identifiable mechanism in melanoma and are found in ~75% of cases (5, 9). TERT is the catalytic component of telomerase, the enzyme responsible for *de novo* telomere synthesis and maintenance of telomeres. *TERT* promoter mutations are not sufficient to immortalize some cell types and telomeres continue to shorten in nevi during the transition to melanoma despite acquisition of *TERT* promoter mutations (10). Therefore, while additional mutations are likely required to enable telomere elongation and immortalization, the genomic changes that potentially synergize with *TERT* promoter mutations to achieve sustained telomere maintenance remain unknown (10).

We sought to identify novel mechanisms of telomere maintenance in cancer cells by analyzing somatic mutations that occur in telomere-related genes. We focused our analysis on melanoma as individuals with germline variants in the *TERT* promoter predominantly develop this cancer (5). We examined somatic variants from telomere-related genes in melanoma from the International Cancer Genome Consortium (ICGC) (11). Mutations in TPP1 and POT1, components of the six-protein shelterin complex that coats telomeres, have been reported in familial melanoma (12–14) and we found numerous somatic variants among 749 melanomas samples analyzed (Fig. S1). We noted a cluster of recurrent somatic variants in a conserved region of *ACD*, the gene encoding TPP1 (which we refer to as the *TPP1* gene hereafter for clarity), that co-localize with histone marks typically associated with promoters (Fig 1A). *TPP1* has been reported to have two isoforms, TPP1-long (L) and -short (S), that differ by 86 amino acids in the N-terminus (15, 16). The cluster of variants was positioned such that they would be coding variants in TPP1-L and promoter variants in TPP1-S. To determine which isoform of TPP1 was expressed in melanoma, we examined RNA-seq data from 12 melanoma cell lines and 61 micro dissected nevi and melanoma samples (GSE153592 and GSE112509)(17, 18) and found that TPP1-S was the only isoform expressed (Fig. 1A and Fig. S2). To further validate this finding, we cloned the entire genomic region (3.5 kb including 895 base pairs upstream of the TPP1-S translational start site and 637 base pairs upstream of the TPP1-L translation start site and all exons and introns of both isoforms) of *TPP1* into a plasmid without an additional promoter and incorporated a C-terminal FLAG-tag. Immunofluorescent staining confirmed that the

C-terminally tagged TPP1 co-localized with TRF2 at telomeres (Fig. 1B). We generated cDNA expression constructs for TPP1-L, TPP1-S, and TPP1-L(M87A) which is incapable of expressing TPP1-S as controls (15). Western blot of cells expressing the entire genomic region with (TPP1pro − 108C>T and TPP1pro −75C>T) and without (TPP1pro WT) the two most common variants showed that only TPP1-S was expressed in HEK293 and the melanocytic cell lines LOX and MEL624 (Fig. 1C). Together, these finding support that the cluster of variants we identified are localized to the promoter of TPP1-S. For clarity, we will refer to TPP1-S as TPP1 hereafter.

Previous studies have investigated the role of non-coding mutations in cancers including melanoma (19–21). Because the annotations of 5`-portion of *TPP1* have changed from coding to noncoding in recent years, (Fig. S2), the region we identified would not have been included as a promoter region in earlier studies. We tested if the proximal 200 base pairs upstream of the *TPP1* translational start site was significantly enriched for somatic variants by examining whole genome sequencing data from 305 patient-derived melanoma samples available from the ICGC and found that the *TPP1* proximal promoter was significantly enriched relative to 59,727 annotated promoters (FDR-corrected p-value 6.57e–14).

We next sought to determine the functional consequences of the somatic variants we identified in the promoter of *TPP1*. The two most common variants were C>T transitions located 75 and 108 base pairs upstream of the translational start site for TPP1. The −108 variant created the core binding sequence TTCC for E-twenty-six (ETS) family of transcription factors. The −75 variant was adjacent to an existing ETS site in the context of a sequence that is enriched for mutations in melanoma (21), created a novel TFIID binding site, and co-localized with the annotated transcriptional start site for the TPP1-S mRNA (Fig. 2 and S3). The identification of two recurrent promoter variants that created or modified ETS transcription factor binding sites bore remarkable similarity to putative activating mutations in the *TERT* promoter (5, 6), although the precise sequences created by the variants are distinct. We found *TPP1* was upregulated in several large databases of cancer gene expression including those with a high frequency of *TERT* promoter mutations (Fig. S4); however, there was insufficient data for the analysis of melanoma with *TPP1* promoter mutations specifically. To further characterize the *TPP1* promoter variant, we generated luciferase reporters of progressively smaller fragments of the TPP1 proximal promoter and found a 285 base pair fragment was sufficient for full basal transcriptional activity (Fig. S3E). Introduction of the −75 or −108 C>T promoter variants had little effect on luciferase expression in HEK293 cells but showed a small but significant increase in luciferase expression in two melanoma cells lines, LOX and MEL624 (Fig. 2A), suggesting that melanoma-specific ETS transcription factors are required for increased activity. RNA-seq data from 426 melanoma samples (11) showed that of 27 ETS family members, ETV5, ETS1, and ETV4 were the most abundantly expressed (Fig. S5). We confirmed this finding using quantitative PCR (qPCR) and western blotting on non-melanoma lines HeLa, BJ fibroblasts, and HEK293 and several melanoma cell lines and short-term primary cultures (Fig. 2B–C). Finally, we overexpressed ETV5, ETV4, or ETS1 in HEK293 cells and found all three robustly increased the activity of the TPP1 promoter only when the promoter variants were present (Fig. 2D). These data suggest that the *TPP1* promoter variants are activated by ETS transcription factors that are abundantly expressed in melanomas.

To determine the cellular consequences of increased TPP1 expression, we generated stable cell lines that overexpress TPP1 in telomerase positive HeLa cells. For completeness we also examined TPP1-L. We examined if cells stably expressing c-terminally tagged TPP1-S and TPP1-L altered telomere length in telomerase-expressing Hela cells. Consistent with previous reports (15, 22), stable -over expression of TPP1-S led to considerable telomere lengthening, whereas TPP1-L caused telomere shortening (Fig. 3A). These data confirm that increased expression of TPP1 can lead to telomere lengthening in telomerase-expressing cells.

We next examined whether over-expression of TPP1 could extend the proliferative capacity of cells that express limiting amounts of telomerase. We expressed TPP1, TERT, or both in primary BJ fibroblasts, and monitored their proliferative capacity for 90 days (Fig. 3B–C). Control untransduced fibroblasts and fibroblasts transduced with TPP1 alone entered replicative senescence after about 40 days. In contrast, cells transduced with TERT and TERT+TPP1 bypassed senescence and were immortalized (Fig. 3B). These findings are consistent with previous reports that have demonstrated that TERT over-expression is sufficient to immortalize primary fibroblasts (23). We next examined telomere length in control and TPP1-transduced fibroblasts and found telomeres were very heterogenous with a median of about 6 kb in length after 15 passages. Introduction of TERT caused telomere lengthening consistent with previous reports (23) and the co-expression of TERT and TPP1 together caused a synergistic effect resulting in significant telomere elongation (Fig 3D). These findings indicate that TERT and TPP1 overexpression is synergistic and lengthens telomeres more than TERT overexpression alone.

To determine whether the TPP1 promoter variants were sufficient to increase telomere addition, we introduced the two most common variant (−75C>T and −108C>T) into MEL624 and LOX cells using CRISPR/Cas9 (see supplementary methods) (Fig. S6). Six clones from MEL624 and LOX cells were obtained and sequence verified. We examined the expression of *TPP1* after modifying the endogenous promoter and found that introduction of either the −75 or −108 variants significantly increase expression of *TPP1* (Fig. 4A). The greater increase in expression levels of *TPP1* from the modified endogenous promoter (Fig. 4C) compared to the luciferase assay (Fig. 2A) suggest that additional factors may contribute to *TPP1* expression in melanoma. Telomeres are extremely long in MEL624 and LOX cells (>20 kb), and it is not possible to detect changes in length via a Southern blot. We therefore used fluorescence *in situ* hybridization (FISH) to detect a modified telomere sequence as a surrogate for *in vivo* telomerase activity as previously described (Fig. 4B) (24). We used a telomerase RNA encoding the variant telomere repeat, TTAGGT, which can be incorporated into telomeres and localized with a peptide nucleic acid fluorescent probe. Wild-type or genome-edited MEL624 or LOX cell lines with the most common promoter variants were co-transduced with lentiviruses that express the variant telomerase RNA and hTERT. Using FISH to examine the percentage of telomeres with variant repeats, we found cells that had a modified *TPP1* promoter incorporated significantly more TTAGGT variant repeat sequences on telomeres (Fig. 4C, D and Fig. S7). These findings suggest that TPP1 promoter mutations synergize with hTERT to increase telomere repeat addition in melanoma cells.

We next investigated the co-occurrence of somatic *TERT* and *TPP1* promoter mutation in cancer. *TPP1* promoter variants are found primarily in cancers of the skin but have been reported in several different cancer types (Fig. S8). The rationale for the disproportional number of variants in melanoma is unknown but may be related to the high mutation rate and reliance on telomerase activation in this cancer. In a dataset of deeply sequenced cutaneous melanomas (25), 139 samples were evaluated for the presence of the *TERT* and *TPP1* promoter variants. A large fraction (83%) carried a somatic variant in the *TERT* promoter, as previously reported (5, 9) and 8 samples carried a TPP1 promoter variant (~6%). In all cases except one, the *TERT* and *TPP1* promoter variants were found together in the same tumor (Fig. 4E). However, due to limitations in current WGS data sets, additional studies using targeted resequencing will be required to determine the frequency of *TPP1* and *TERT* promoter mutations in cancers other than melanoma. We find that TPP1 upregulation in the absence of telomerase expression is unlikely to influence telomere length or cellular immortality. Therefore, selection for *TPP1* promoter variants is most likely to occur subsequent to activation of telomerase (Fig 4E). Our data indicate that TPP1 is one of the missing factors that collaborate with *TERT* promoter mutations to facilitate cellular immortalization in melanoma. Identifications of novel pathways that contribute to telomere lengthening and cellular immortalization may have both significant prognostic value and inform the development of possible treatments for patients with cancer and those with diseases of telomere shortening. Our findings further support that multiple non-coding mutations can cooperate to enable cellular immortalization and highlight the importance of understanding the contribution of non-coding variants to cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests:

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

JMK reports advisory/consultancy roles with Amgen Inc., Ankyra Therapeutics, Applied Clinical Intelligence LLC, Axio Research LLC, Becker Pharmaceutical Consulting, Bristol Myers Squibb, Cancer Network, Checkmate Pharmaceuticals, DermTech, Fenix Group International, Harbour BioMed, Immunocore LLC, iOnctura, Istari Oncology, Magnolia Innovations LLC, Merck, Natera Inc, Novartis Pharmaceuticals, OncoCyte Corporation, OncoSec Medical Inc., PathAI Inc., Pfizer Inc., Replimune, Scopus BioPharma, SR One Capital Management,

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Data and materials availability:

All data and materials used in the analysis are available to any researcher for purposes of reproducing or extending our analysis. All data are available in the main text or the supplementary materials. Please contact Jonathan Alder (jalder@pitt.edu) to request any materials.

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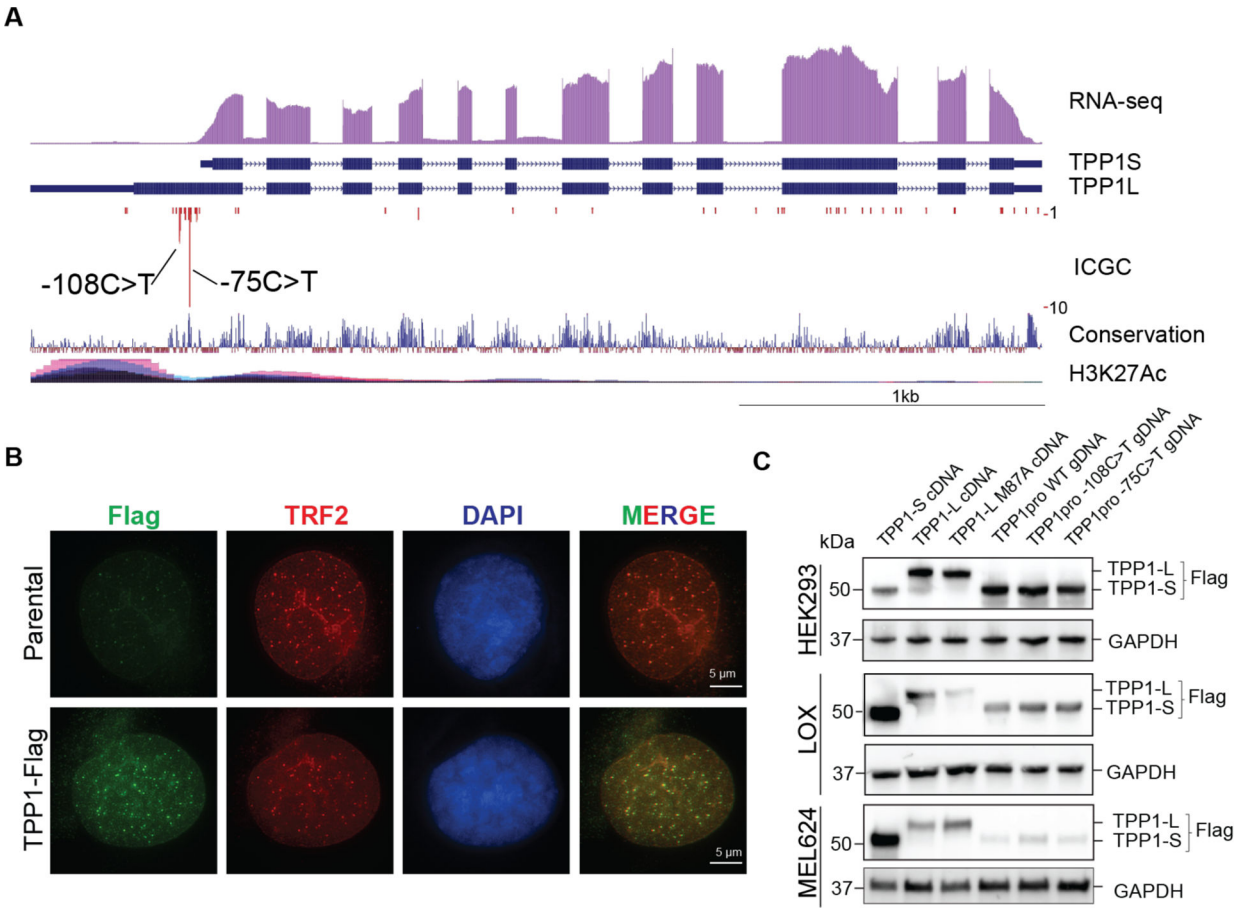

Fig. 1. Identification of a cluster of somatic promoter variant in *TPP1*.

(**A**) The genomic locus of the *ACD* gene is depicted from UCSC genome browser data with dark blue rectangles indicating the exon for TPP1-S and TPP1-L respectively. The red bars below the gene tract show the location of the somatic variants identified in the ICGC database with taller bars corresponding to the number of melanomas found with a specific variant. RNA-seq data (GSE153592) is shown above the gene tract in purple along with vertebrate conservation and H3K27 acetylation marks from multiple cell lines, indicating the location of likely regulatory regions. (**B**) HeLa stable cell lines expressing a C-terminally FLAG-tagged TPP1 were stained for the shelterin component TRF2 and the FLAG epitope. Colocalization of TPP1 with TRF2 suggest that the C-terminal FLAG-tag does not disrupt localization of TPP1 to the telomere. (**C**) Western blot of HEK293, LOX, and MEL624 cells transfected with plasmids encoding the cDNAs for TPP1-S and TPP1-L, and TPP1-L-M87A that is incapable of expressing TPP1-S together with plasmids expressing the entire genomic locus of TPP1 with and without the most common promoter variants.

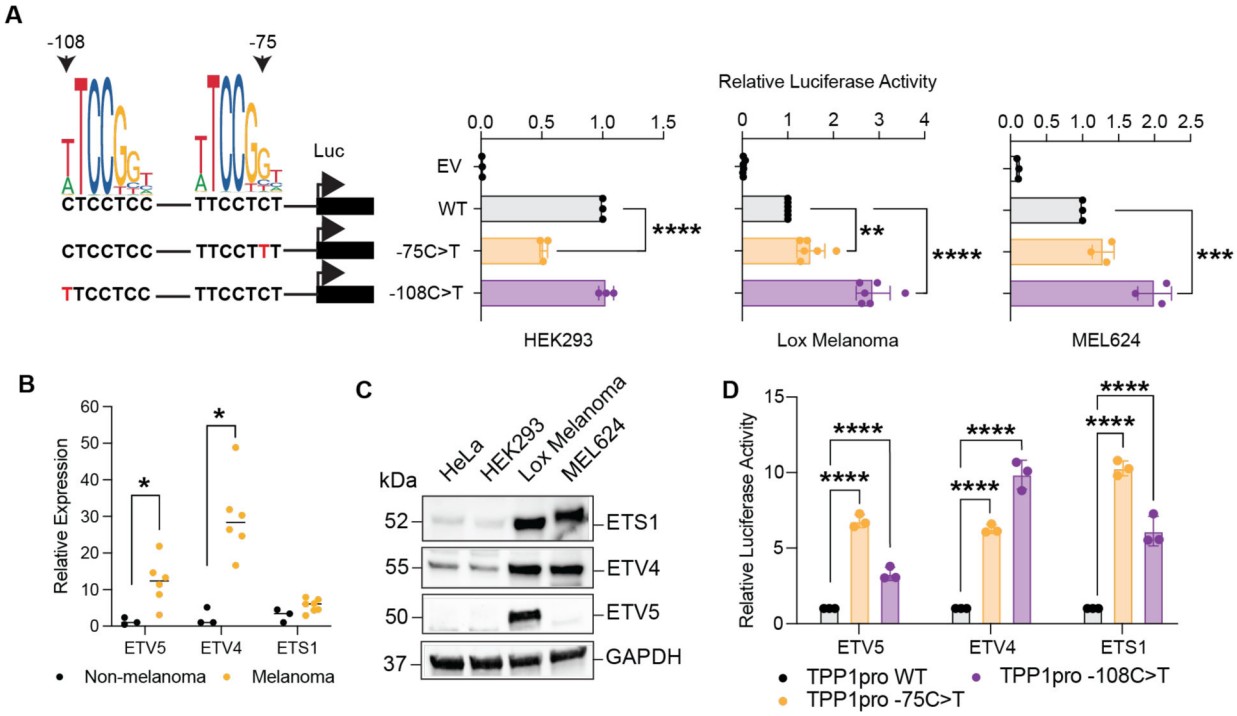

Fig. 2. ETS transcription factors activate the variant TPP1 promoter.

(**A**) Luciferase assays were performed with a 285 base pair fragment of the TPP1 proximal promoter in melanoma and non-melanoma cell lines. The TPP1 promoter variants had little effect on the transcriptional activity in non-melanoma cells lines (HEK293), but increased reporter activity in two melanoma derived lines. (**B**) Quantitative PCR examining the levels of three ETS transcription factor family members in non-melanoma (HeLa, HEK293, and BJ fibroblast; n=3) and melanoma cell lines (MEL624 and LOX) and short-term primary cultures (n=6–7). Medians are shown and groups were compared using Mann-Whitney test. (**C**) Western blot showing high expression of ETS transcription factors in LOX melanoma and MEL624 lines. (**D**) Luciferase assays comparing activity of the TPP1 promoter reporter in the presence of three transfected ETS transcription factors in HEK293 cells. Cells were co-transfected with a pGL4 reporter and pCDNA3.1 expression plasmid with one of the three ETS transcription factors. Mean and standard deviation are shown from at least three independent experiments in (A) and (D) and groups were compared with a one-way ANOVA followed by Tukey's multiple comparison test for pairwise comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

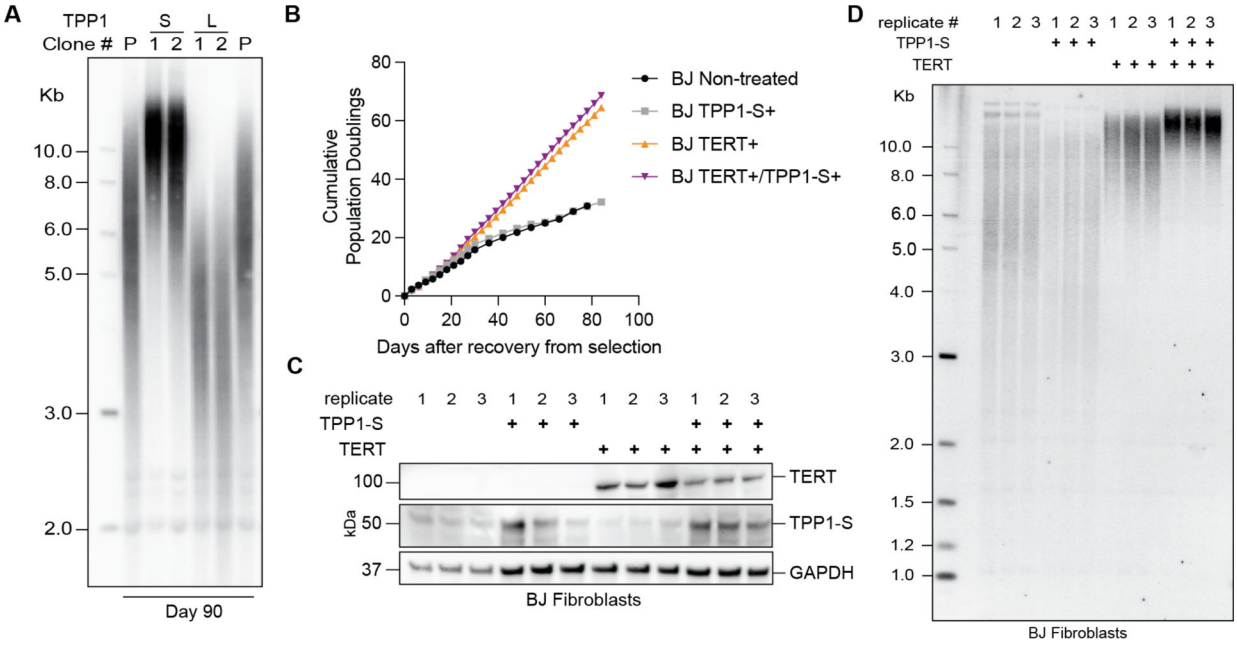

Fig. 3. TPP1-S over expression causes telomere lengthening and is synergistic with TERT overexpression.

(**A**) Southern blot of telomeres in HeLa cell lines that stably expressed TPP1-S or TPP1-L for 90 days. Two independent clones of each are shown. P – parental cell line that was used to establish each of the modified clones. (**B**) Growth curves of cumulative population doublings of BJ fibroblasts expressing TPP1-S or TERT (average of 3 independent transduction for each group). (**C**) Western blot showing expression of each of the transgenes in cells collected from (B). (**D**) Southern blot of telomere lengths of BJ fibroblasts in (B) 15 passages after transduction showing synergistic telomere lengthening in cells exogenously overexpressing TPP1 and TERT.

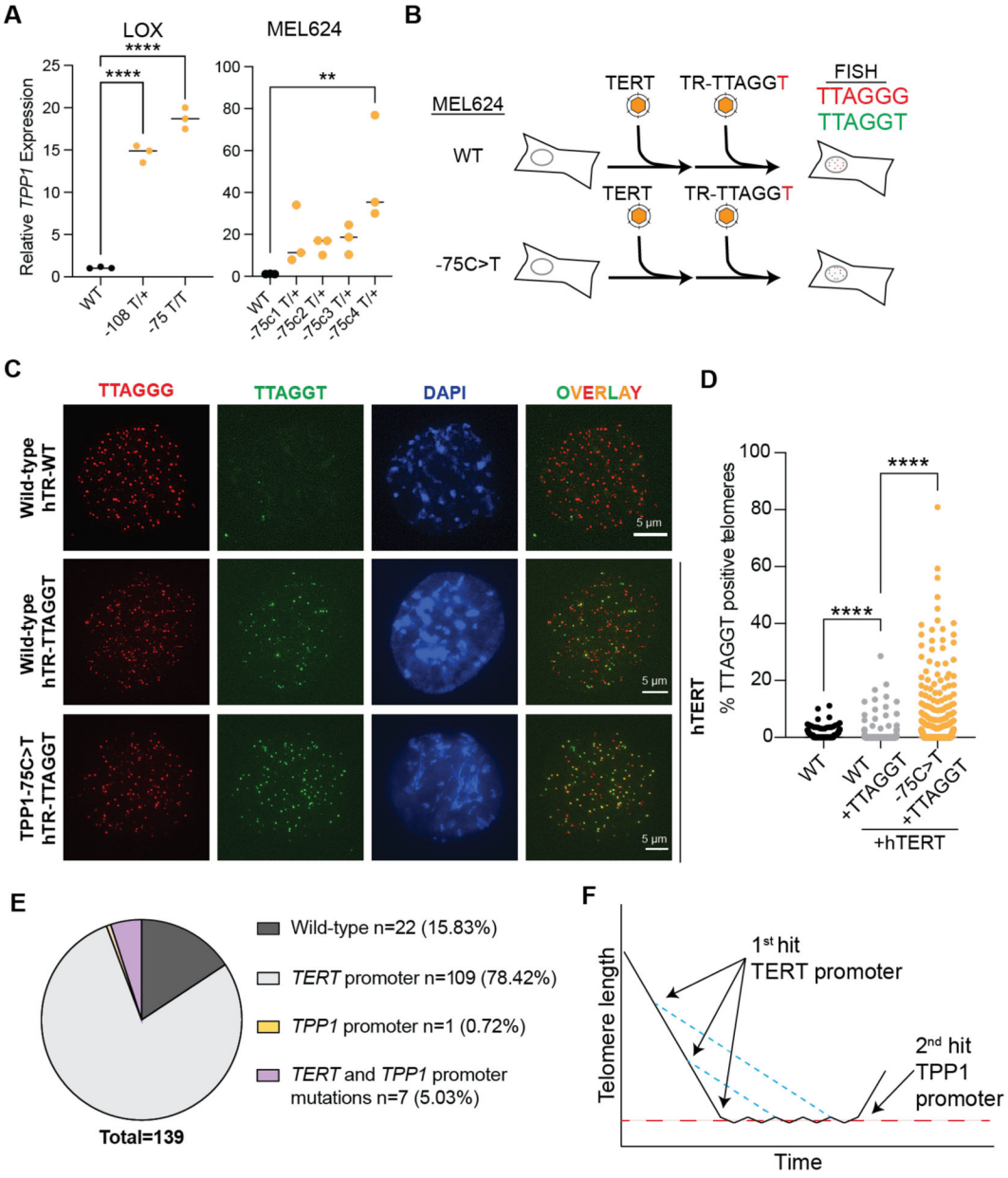

Fig. 4. TPP1 promoter mutations increase expression of the endogenous transcript and co-occur with *TERT* promoter mutations.

(A) Quantitative PCR of *TPP1* expression following introduction of promoter mutations in LOX and MEL624 cells. Labels below the graph indicate the presumed zygosity based on sequencing. The media is shown from three independent measurements from each clone and groups were compared using one-way ANOVA followed by Dunnett's multiple comparison test. (B) Schematic of the experimental approach to measure telomerase activity in genetically modified cells. Cells are transduced with a *TERT*-expressing lentivirus to increase the rate of variant telomere incorporation. Following introduction of the mutant telomerase RNA (encoding TTAGGT), cells are passaged and the canonical and variant telomeres are quantitated. (C) Fluorescent in situ hybridization for the WT (TTAGGG; red) and variant (TTAGGT; green) in parental or genome edited MEL624 cells. Images

were taken 7 days after transduction with lentiviruses. (D) Quantitation of the fraction of telomeres that had both TTAGGG and TTAGGT signals from a single clone. Groups were compared using ANOVA with Dunnett's correction for multiple comparison. **P<0.01 and ****P < 0.0001. (E) Proportion of cutaneous melanomas that had *TERT, TPP1*, or *TERT+TPP1* variants from Hayward et. al. (25) (F) Model of telomere length dynamics in melanoma progression. *TERT* promoter variants likely occur early and slow telomere attrition but are not sufficient to prevent telomere shortening (blue dashed lines in model). Telomere shortening continues until cells enter crisis (red dashed line). Additional mutations, like the *TPP1* promoter, are likely required to fully maintain telomeres and escape crisis (2^{nd} hit).