Recurrent TPP1 Promoter Mutations Drive Telomere Maintenance in Melanoma

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Limitless replicative potential is a defining hallmark of cancer. Somatic cells are limited in their replicative potential by telomere shortening. Telomeres are caps on the ends of chromosomes that shorten as cells divide. Telomere maintenance in cancer cells typically depends on the reactivation of telomerase, an enzyme that synthesizes new telomeres, to allow somatic cells to bypass replicative senescence.

TERT is the reverse transcriptase component of telomerase. Mutations in the *TERT* promoter have been found in ~75% of melanomas and create the *de novo* E-twenty six (ETS) transcription factor binding motifs that function to increase *TERT* expression. The acquisition of *TERT* promoter mutations can extend the proliferative capacity of cells but cannot prevent bulk telomere shortening and, therefore, cells remain mortal. Additional somatic alterations are required for cells to become fully immortal.

TPP1 is a component of the shelterin complex that coats telomeres. TPP1 was previously reported to have two isoforms: TPP1-L encompasses 544 amino acids (aa) and TPP1-S, a 458 aa protein that initiates at Met87 of TPP1-L. Here, we identified a cluster of variants at the 5′ region of the *ACD* gene encoding TPP1 in cutaneous melanoma. These variants are TPP1-S promoter variants but not TPP1-L coding variants. The two most common re-occurring variants, -108 C>T and -75 C>T, in the *TPP1* promoter create the *de novo* ETS binding sites and increase TPP1 expression similar to what is seen with the most common *TERT* promoter mutations.

We find that TPP1 acts synergistically with TERT to lengthen telomeres in primary BJ fibroblasts. CRISPR/Cas9-mediated genome editing that introduced *TPP1* promoter variants into human melanomas led to increased TPP1 activity and significantly increased telomerase activity.

Co-occurrence of *TERT* promoter mutations and *TPP1* promoter variants was identified in ~5% of cutaneous melanomas by whole genome sequencing analysis, suggesting that *TPP1* promoter variants cooperate with *TERT* promoter mutations to enhance telomere maintenance and immortalization in melanoma. The findings of this study could potentially be translated and applied in the clinical care of melanoma and other types of cancers.

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Preface

This research was only possible through my mentor and PI's unending generosity and support, Assistant Prof. Jonathan K Alder. Working with Dr. Alder has been an incredible and productive journey as a graduate student who has yet to gain previous knowledge and experience in the lab. He has allowed me to develop my scientific knowledge in basic and translational sciences. I have learned a lot from Dr. Alder, who is my PI and my teacher, friend, and family.

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List of Abbreviations

ACD	ACD shelterin complex subunit and telomerase recruitment factor
AM	Acral Melanoma
ANOVA	Analysis of Variance
ATM	Ataxia-Telangiectasia-Mutated
ATR	Ataxia Telangiectasia and Rad3
BAP1	Breast Cancer Associated Protein-1
CAGE	Cap Analysis Gene Expression
Cas9	CRISPR-Associated Protein 9
CDC	Center of Disease Control and Prevention
CDKN2A	Cyclin-Dependent Kinase 2A
CDK4	Cyclin-Dependent Kinase 4
CHX	Cycloheximide
CMM	Cutaneous Malignant Melanoma
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cryo-EM	Cryo-Electron Microscopy
DDB	DNA Double-stranded Break
DMSO	Dimethyl Sulfoxide
ELK1	ETS transcription factor ELK1
ENCODE	ENCyclopedia Of DNA Elements
hESCs	Human Embryonic Stem Cells
ETS	E-Twenty-Six family of transcription factors

List of Abbreviations (continued)

ETS1	ETS Proto-Oncogene 1, Transcription Factor
ETS2	ETS Proto-Oncogene 2, Transcription Factor
ETV4	ETS Variant Transcription Factor 4
ETV5	ETS Variant Transcription Factor 5
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence In Situ Hybridization
GENCODE	Genome research and part of the ENCODE
GENT2	Gene Expression database of Normal and Tumor Tissues 2
H/ACA	Box H/ACA snoRNA-like domain of hTR
HDR	Homology-Directed Repair
ICGC	International Cancer Genome Consortium
MITF	Microphthalmia-Associated Transcription Factor
NGS	Next-Generation Sequencing
alt-NHEJ	Alternative Nonhomologous End Joining
c-NHEJ	Classical Nonhomologous End Joining
NHP2	Ribonucleoprotein
NOP10	NOP10 ribonucleoprotein
NOB	N-terminus of OB domain
OB	Oligonucleotide and Oligosaccharide-Binding fold domain
PAM	Protospacer Adjacent Motif
PARP1	Poly (ADP-ribose) Polymerase 1

List of Abbreviations (continued)

PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
POT1	Protection Of Telomeres 1
RAMPAGE	RNA Annotation and Mapping of Promoters for the Analysis of Gene Expression
RAP1	Human Ortholog of the Yeast Repressor/Activator Protein 1 encoded by *TERF2IP* gene
RefSeq	Reference Sequence
crRNA	CRISPR RNA
RNP	Ribonucleoprotein Complex
RP	Ribosomal Protein
SEER	Surveillance, Epidemiology, and End Results Program
TCAB1	Telomerase Cajal Body protein 1
TCGA	The Cancer Genome Atlas
TEL patch	TPP1 glutamate (E) and leucine (L)-rich patch
TEN	TERT-unique telomerase Essential N-terminal domain
TERC/TER/hTR	Telomerase RNA Component encoded by *TERC* gene
TERF2IP	Telomeric Repeat Binding Factor 2 Interacting Protein
TERT	Telomerase Reverse Transcriptase
TFIID	Transcription factor II D
TIN2	TRF2- and TRF1-Interacting Nuclear Protein 2

List of Abbreviations (continued)

TNM	Tumor-Node-Metastasis
TPP1	As known as TINT1, PTOP, or PIP1 encoded by *ACD* gene
TPP1-L	TPP1 long isoform
TPP1-S	TPP1 short isoform
TRAP	Telomerase RAP motif
TRF	Southern blot analysis of Terminal Restriction Fragments
TRF1	Telomeric Repeat Binding Factor 1
TRF2	Telomeric Repeat Binding Factor 2
TSS	Transcription Start Site
UM	Uveal Melanoma
UPMC	University of Pittsburgh Medical Center
UVR	Ultraviolet Radiation
WES	Whole-Exome Sequencing
WGS	Whole-Genome Sequencing
XP	Xeroderma Pigmentosum

1.0 Introduction

Advances in medicine and public health have changed life expectancy globally. Life expectancy was 45.51 years in 1950 and has increased to 72.98 years in 2022 (United Nations, 2022). This has led to an era of an aging population resulting in higher burden of age-related diseases and cancer. Despite better prevention strategies and improvement of health policies for older adults in terms of cancer care, the total cancer cases and cancer death rates continue to rise.

The aging process is characterized by the accumulation of malfunctioning cells that prevent normal repair of tissues. Aging cells accumulate changes in their genome that disrupt normal cellular function. Many of these disrupted pathways have been classified as "The Hallmarks of Cancer" (Hanahan & Weinberg, 2011). Somatic cells are typically limited in their capacity to proliferate (replicative senescence). Cells can acquire somatic changes that bypass replicative senescence and enable replicative immortality. Replicative senescence is mediated by telomeres. Telomeres are the regions of repetitive DNA sequences at the ends of chromosomes. They cap the ends of chromosomes to protect the genome from nucleolytic degradation, unwanted recombination, repair, and inter-chromosomal fusion. Each time a cell divides, the ends of linear DNA cannot be replicated completely during lagging strand DNA synthesis. This is termed "The End Replication Problem" (Olovnikov, 1973; Watson, 1972), which results in progressively shorter telomeres, and the eventual loss of protective functions, and entrance into crisis. Telomeric crisis refers to telomeres whose ends have been shortened to the point at which they can no longer protect the chromosome ends. At this point, the cell stops dividing or dies.

Telomeres are maintained by the enzyme telomerase. Telomerase is an RNA dependent DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA. However, its

expression is almost absent in somatic cells. By re-expressing telomerase, a cell is able to overcome replicative senescence and become immortal. However, the full mechanism of how telomerase contributes to tumorigenesis remain incompletely understood.

1.1 Public Health Significance

Melanoma, the skin cancer that causes the majority of skin cancer-related deaths, is rising in the US (American Cancer Society, 2023). GLOBOCAN 2020 database is providing global cancer statistics and estimates of cancer incidence and mortality for 185 countries and 36 types of cancer. GLOBOCAN 2020 estimated there were 324,635 new cases and 57,043 deaths due to melanoma worldwide and predicts cases will continue rising to 510,000 new cases and 96,000 deaths by 2040 worldwide (Arnold et al., 2022; Sung et al., 2021).

Even melanoma is the most serious and deadly skin cancers, but it is highly treatable in the early stages. The five-year survival rate for people whose melanoma is detected and treated before it has spread to the lymph nodes is 99%, for melanoma that has spread to nearby lymph nodes is 68%, and for melanoma that has spread to distant lymph nodes and other organs is 30% (Siegel, Miller, Fuchs, & Jemal, 2022). From this statistics suggest that improvement of early diagnosis and treatment can increase the chance of good clinical outcomes, and also save costs and resources.

Melanoma most commonly occurs in those individuals with an enhanced genetic susceptibility including pigmentation characteristics, response to ultraviolet light, and nevus number. Long-term, intermittent, high-dose UV exposure is the most important environmental risk factor for the development of melanoma, such as seen from sunlight or indoor tanning beds. Immunosuppression also increases the risk of melanoma (Dinh & Chong, 2007).

Familial melanoma is defined as a melanoma patient with either 2 first-degree relatives or 3 or more melanoma patients on the same side of the family (Gandini, Sera, Cattaruzza, Pasquini, Zanetti, et al., 2005). A family history of melanoma is one of the most significant non-modifiable risk factors, with 5-10% of cutaneous melanomas being familial (Florell et al., 2005; Zocchi et al., 2021). Predisposed patients who have family history of melanoma often develop melanoma at a younger age than the general population.

Familial melanoma is largely due to the dysregulation of susceptibility genes. Melanoma susceptibility genes have been associated with high-penetrance melanoma predisposition genes such as *CDKN2A* (cyclin-dependent kinase 2A), *CDK4* (cyclin-dependent kinase 4), *BAP1* (breast cancer associated protein-1), *MC1R* (melanocortin 1 receptor), and *MITF* (microphthalmia-associated transcription factor)(L. G. Aoude, Wadt, Pritchard, & Hayward, 2015; Potrony et al., 2015; Psaty, Scope, Halpern, & Marghoob, 2010), and telomere associated genes including *TERT* (Harland et al., 2016; Horn et al., 2013; Tapper et al., 2015; João Vinagre et al., 2013), *POT1* (Carla Daniela Robles-Espinoza et al., 2014), *TERF2IP* (*TERF2IP* gene encodes RAP1) (Lauren G. Aoude et al., 2014), and *ACD* (*ACD* gene encodes TPP1) (L. G. Aoude, Pritchard, et al., 2015).

The relationship between genetics and environmental factors in cutaneous melanoma is complex. Obviously, the main risk factor is ultraviolet radiation (UVR) from the sun and artificial tanning devices. The evidence of the crucial role of UVR in cutaneous melanoma comes from somatic mutations in the tumor cells. The whole-exome sequencing data from The Cancer Genome Atlas (TCGA) classified primary or metastatic melanomas into 4 genomic subtypes: *BRAF* mutations, *NRAS* mutations, neurofibromatosis type 1 (*NF1*) mutations and triple wild-type *TERT* mutations (Akbani et al., 2015).

Host factors such as pigmentation characteristics, nevi, and genetics can contribute to melanoma susceptibility. Individuals are differentially susceptible to sun exposure and develop different levels of melanoma risk suggesting that genetic factors and environmental factors together play an important role in the development of melanoma.

Public health strategies in melanoma protection and detection including educational strategies—such as encouraging sun protection behavior with sun protection factor of 15 or more; sun avoidance between 10 am and 4 pm; and physical protection by using umbrellas, clothes, or hats—need to be improved. Primary prevention strategies such as using sunscreen, and focus on reducing ultraviolet light exposure as much as possible. Secondary prevention strategies include skin self-examination and professional skin examinations. Tertiary strategies should focus on the prevention and detection of additional primary skin cancers (Mahon, 2003).

The ABCDEs of skin cancer is a campaign to educate the public about what to look for in skin abnormalities. About half of melanoma cases are self-detected (Avilés-Izquierdo et al., 2016). The ABCDE campaign makes it easy to perform regular skin self-exams to check for signs of skin cancer. Melanoma warning signs include changes in size, shape, or color of a mole or other skin lesion as well as the appearance of a new growth on the skin. Self-examination for early detection of melanoma includes A is for Asymmetry (one half of the spot is unlike the other half), B is for Border (the spot has an irregular, scalloped, or poorly defined border), C is for Color (the spot has varying colors such as shades of tan, brown or black, or areas of white, red, or blue), D is for Diameter (melanomas are usually greater than 6 millimeters), and E is for Evolving (the spot looks different from the rest or is changing in size, shape, or color) (Abbasi et al., 2004; Rigel, Friedman, Kopf, & Polsky, 2005). Self-skin examination regularly can improve self-awareness and facilitate the early detection, diagnosis, and treatment which leads to better prognosis in melanoma patients.

In summary, skin cancer interventions provide both health and economic benefits. According to Center of Disease Control and Prevention (CDC), the benefits of using proven community skin cancer prevention programs could reduce skin cancer risk in people and save millions a year in health care costs. Communities and decision makers can help implement skin cancer prevention programs. Skin cancer prevention efforts such as monitoring national surveillance data; conducting research; examining the policy, health system, and environmental factors; and confirming partners and the public have accurate information can help can aid the early diagnosis of melanoma in communities.

1.2 Melanoma Overview

Melanoma, the most serious type of skin cancer, originates from melanocytes (skin pigment-producing cells). Melanoma is classified based on the clinical and histological characteristics of the primary lesion from which it arises. The four major subtypes of melanoma include cutaneous melanoma, which arises in non-glabrous skin; acral melanoma, which originates from the skin of palms, soles, and nail beds; mucosal melanoma, which arises from the mucosal lining; and uveal melanoma, which develops from melanocytes in the uveal tract of the eye (Figure 1A-D) (Rabbie, Ferguson, Molina-Aguilar, Adams, & Robles-Espinoza, 2019). Apart from clinical and histological differences, there are genetic differences between melanoma subtypes. For example, *TERT* promoter mutations were more common (83%) in cutaneous melanoma but less common (23%) in acral melanoma and mucosal melanoma (Hayward et al., 2017).

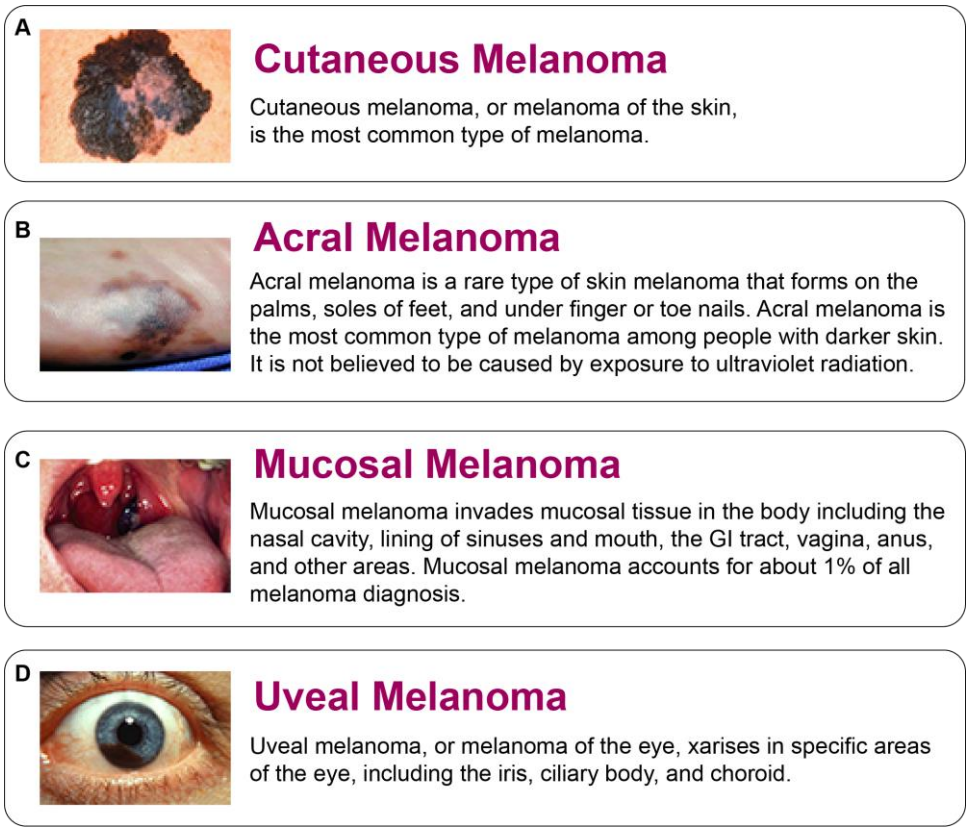


Figure 1. Melanoma Subtypes.
(A) Cutaneous melanoma arises in non-glabrous skin. (B) Acral melanoma originates from skin of the palms, soles, and nail beds. (C) Mucosal melanoma arises from the mucosal lining. (D) Uveal melanoma develops from melanocytes in the uveal tract of the eye. Photo credit: Melanoma Research Alliance.

Melanoma staging, which can be very complex, is based on the American Joint Committee on Cancer (AJCC) staging system that uses three key pieces of information for assigning Tumor-Node-Metastasis (TNM) scores as well as additional prognostic factors. Melanoma staging could be different in real clinical practice. For example, the pathologic stage, also called the surgical stage, which is determined by examining tissue removed during an operation. If surgery is not an option, the cancer will be given a clinical stage instead. The goal of melanoma staging is that melanomas of the same stage will have similar characteristics, treatment options, and outcomes (Black & Brockway-Lunardi, 2013).

1.3 Cutaneous Malignant Melanoma

Cutaneous malignant melanoma (CMM) or melanoma of the skin, is the most common type of melanoma worldwide. UVR—from the sun and/or indoor tanning—is a primary cause of CMM, which is characterized by a high incidence among populations with fair skin pigmentation. Melanomas have a higher mutation burden than other cancer cell types. The mutations seen in CMM are associated with a signature of UVR exposure, specifically C>T mutations (Hodis et al., 2012; Krauthammer et al., 2012; Martincorena & Campbell, 2015).

UV exposure, the most critical risk factor for cutaneous melanoma, induces somatic mutations and initiates malignant transformation in melanocytes (De Fabo, Noonan, Fears, & Merlino, 2004). A typical nevus, also known as a mole, is a benign pigmented tumor. Most moles will never cause any problems, but someone with many moles is more likely to develop melanoma (Gandini, Sera, Cattaruzza, Pasquini, Abeni, et al., 2005). Atypical moles or dysplastic nevi can look like normal moles but also have some features of melanomas (Silva, de Sá, de Ávila, Landman, & Neto, 2011). These moles often run in families (Boyle, Maisonneuve, & Doré, 1995). People with this condition have a very high risk of melanoma and need to have regular skin exams by a dermatologist. Around 10% of all people with melanoma have a family history of the disease (Boyle et al., 1995). The increased risk in people who have family history of melanoma might be due to a shared family lifestyle of frequent sun exposure, a family tendency to have fair skin, certain gene mutations that run in a family, or a combination of these factors (Yoon et al., 2002).

People with weakened immune systems from certain diseases or medical treatments are more likely to develop many types of skin cancer, including melanoma. Being older and being male also increase the risk of melanoma. Melanoma is also found in younger people in Australia (Nicholls, 1973) compared to in the United Kingdom (Mackie, English, Aitchison, Fitzsimons, &

Wilson, 1985) which may explain by the ambient sunlight exposure (Tsao, Bevona, Goggins, & Quinn, 2003).

Xeroderma pigmentosum (XP) is an inherited condition characterized by extreme sun sensitivity. People with XP have a high risk of developing melanoma and other skin cancers especially on sun-exposed areas (Landi et al., 2020).

Mutations affecting the *TERT* promoter are the most frequent mutations (>75%) in cutaneous melanomas (Hayward et al., 2017; Horn et al., 2013; Huang et al., 2013) suggesting that there is an association between cutaneous melanomas and the telomere maintenance mechanism. This relationship is the basis for our focus on cutaneous melanoma in this study. However, recent studies have shown that mutational activation of *TERT* is not sufficient to immortalize melanocytes. Specifically, telomeres continue to shorten in nevi with *TERT* promoter mutations. The complex relationships between alternative telomere maintenance mechanisms, telomere length, and immortalization in melanoma development are the subject of this thesis.

1.4 Telomeres and Telomerase

Telomeres are the protective elements at the ends of chromosomes that act to maintain the integrity of eukaryotic genomes. Telomeres are composed of $(5'\text{-TTAGGG-}3')_n$ repeats that form a specific DNA architecture comprising two structures: a 5-15 kilobase double-stranded DNA (dsDNA) region and a 150-200 nucleotide single-stranded 3′ tail known as the G-overhang (Makarov, Hirose, & Langmore, 1997). Telomeres play a critical role in dealing with two unavoidable biological threats: the end-protection problem (McClintock, 1939; MULLER, 1938) and the end-replication problem (Wynford-Thomas & Kipling, 1997).

The end-protection problem is the state in which the ends of the linear chromosomes are mistakenly recognized as DNA double strand breaks, which would activate the DNA damage response (DDR) machinery leading to chromosome end-fusions and genome instability. This end protection problem shows that chromosome ends must be protected from DDR pathways. If the DDR pathways are activated, cells will undergo cell cycle arrest and attempt to repair the chromosome ends resulting in genome instability. The solution to the end-protection problem depends on the telomere's unique architecture to prevent the activation of the DNA damage signaling pathways.

Mammalian cells have two independent signaling pathways activated by double- and single-stranded breaks in DNA: the ATM (ataxia-telangiectasia-mutated) kinase pathway, which is activated by double-strand breaks, and the ATR (ataxia telangiectasia and Rad3-related) kinase pathway, which is activated by single-strand DNA. At the telomeres, mammalian cells also need to block the DNA repair pathways that usually repair a broken chromosome: homology-directed repair (HDR) and nonhomologous end joining (NHEJ) (T. de Lange, 2009). Therefore, the end-protection problem of mammalian chromosome must escape these pathways, otherwise activation of the DDR will result in cell cycle arrest, chromosome end-to-end fusions, or sequence exchanges.

Telomeres solve the end-protection problem via the six protein complex known as shelterin. Deprotected telomeric DNA is the target of illicit DNA end-joining and cell cycle checkpoint activation events that results in inappropriate processing of chromosome ends by DNA-repair enzymes such as nucleases and ligases. Inappropriate activation of DDR pathways is repressed by shelterin proteins. For example, TRF2 represses ATM (Celli & de Lange, 2005; Karlseder, Broccoli, Dai, Hardy, & de Lange, 1999), while POT1 repress the ATR pathway (Denchi & de Lange, 2007; Lange, 2018). TRF2 and POT1 also block HDR and NHEJ. TRF2

plays the main role in repressing NHEJ at telomeres (Konishi & de Lange, 2008) whereas TRF2 and POT1 contribute to block HDR (Hockemeyer, Daniels, Takai, & de Lange, 2006; Wu et al., 2006).

The end replication problem results because chromosome ends shorten in every cell division due to incomplete lagging-strand DNA replication by DNA polymerases (Titia de Lange, 2004). This can lead to chromosomal instability and the loss of genetic information (Nandakumar & Cech, 2013; L. Xu, Li, & Stohr, 2013). One solution to the end replication problem is the use of telomerase, an enzyme that can add nucleotides to the ends of telomeres and counteract their shortening.

Telomerase is a ribonucleoprotein complex (RNP) responsible for telomeric DNA synthesis. Telomerase contains TERT, responsible for the reverse transcriptase activity (Y.-S. Cong, Wright, & Shay, 2002; Greider & Blackburn, 1985; Harrington et al., 1997; Lingner et al., 1997; Meyerson et al., 1997) and hTR (or TERC) (Feng et al., 1995), its RNA component that serves as a template for the telomere. The cryo-EM structure of human telomerase at ~8 Å resolution revealed that it has a bilobed structure composed of a catalytic core RNP of TERT and hTR and an H/ACA RNP (Nguyen et al., 2018). The H/ACA RNP is responsible for telomerase RNA maturation and trafficking, ultimately essential for telomere biogenesis (Schmidt & Cech, 2015). H/ACA proteins (including dyskerin, NOP10, NHP2, and GAR1) function to recognize H/ACA-box sequences and recruit hTR into the intranuclear foci called Cajal bodies (H. Li, 2008; Mitchell, Cheng, & Collins, 1999; Vulliamy et al., 2006; Y. Zhu, Tomlinson, Lukowiak, Terns, & Terns, 2004) (Figure 2). Telomerase switching activity depends on the RNA conformation and is shaped by the Telomerase Cajal Body protein 1 (TCAB1) holoenzyme, which in turn controls conformation of a distal three-way junction CR4/5 (L. Chen et al., 2018). The shelterin complex

recruits telomerase to the telomeres (Jayakrishnan Nandakumar et al., 2012; F. L. Zhong et al., 2012). The heterotrimeric CTC1–STN1–TEN1 (CST) complex mediates the termination of the telomere extension process and recruits DNA polymerase α-primase (pol α-primase) to the newly synthesized telomeric tail for C-strand fill-in (Casteel et al., 2009; L. Y. Chen, Redon, & Lingner, 2012; Surovtseva et al., 2009). Collectively, telomerase function and activity are controlled by telomerase reconstitution and trafficking. Genomic alterations in telomere-related genes can dysregulate telomerase causing telomere-related diseases and cancers.

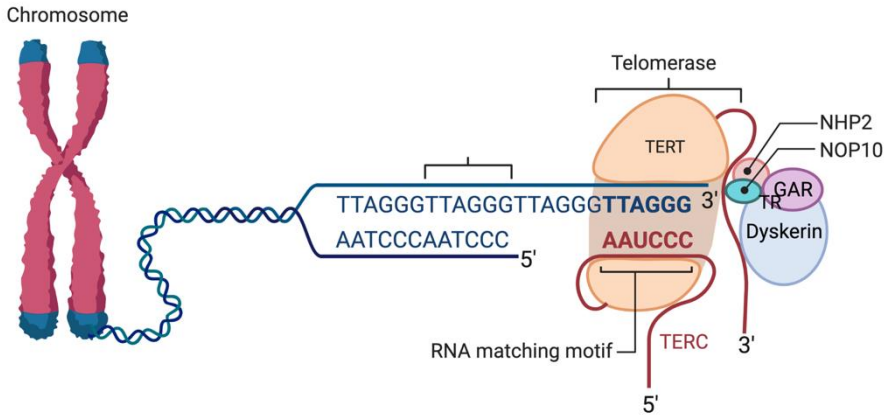


Figure 2. Telomere and Telomerase Components.
Telomeres consist of tandem repeats of six nucleotides (5'-TTAGGG-3')$_n$ found at both ends of each chromosome. Telomerase is a ribonucleoprotein complex comprising the scaffolding non-coding human telomerase RNA (TERC), the enzyme telomerase reverse transcriptase (TERT) and associated cofactors. TERT binds to hTR through its telomerase RNA binding domain. Telomerase extends the telomere by TERT catalytic activity or processivity with hTR as a telomeric template. The left hand figure shows a chromosome in red with the telomeres shown in blue. The right hand figure represents the actions of the telomerase and dyskerin complex at the telomere. The dyskerin complex (comprising dyskerin, NOP10 and NHP2) binds to the H/ACA domain of hTR.[1] [2]

[1] Created with BioRender.com.

[2] Modified from de Lange, 2018; Stanley & Armanios, 2015.

1.5 Shelterin Components

As noted earlier, shelterin is the protein complex that serves as a cap on the end of a chromosome. The shelterin cap is formed by six telomere-specific proteins that associate with the tandem repeat sequence 5′-TTAGGG$_n$-3′ in human telomeres. The six proteins in the shelterin complex are TRF1, TRF2, TIN2, TPP1, POT1, and RAP1. Multiple shelterin complexes bind to the long repetitive telomeric DNA and sense changes in telomere length (J. S. Z. Li et al., 2017; Marcand, Gilson, & Shore, 1997). Two models for regulating the elongation of telomeres by telomerase were proposed; 1) Protein-counting model: shelterin proteins block the telomerase accessibility to the end of telomere, the long telomere with more bound shelterin proteins have a more repressive effect than the short telomere, and 2) Replication fork model: telomerase functions to add telomeric DNA at fork replication, which passes through nucleosome and bound shelterin proteins that can cause telomerase dissociation from the fork and stop adding new telomeric DNA (Greider, 2016). Putting together, shelterin is crucial for both telomere protection and telomerase regulation.

Protein complexes bind differentially to telomeric DNA. TRF1 and TRF2 homodimers bind to double-stranded telomeric DNAs while POT1-TPP1 heterodimers bind to single-stranded telomeric DNAs. TIN2, the linchpin of the shelterin complex, simultaneously interacts with TRF1, TRF2, and TPP1 to mediate the stable assembly of this complex. RAP1 interacts with TRF2 to modulate and increase the binding specificity of TRF2 for 5′-TTAGGG-3′ repeats (Bianchi, Smith, Chong, Elias, & de Lange, 1997; Broccoli, Smogorzewska, Chong, & de Lange, 1997; Y. Chen et al., 2008; Fairall, Chapman, Moss, de Lange, & Rhodes, 2001; Gaullier et al., 2016; Janoušková et al., 2015; C. J. Lim & Cech, 2021; Necasová, Janoušková, Klumpler, & Hofr, 2017; J. Z. Ye, J.

R. Donigian, et al., 2004). Shelterin contributes to several functions including DNA damage suppression and telomerase regulation.

DNA damage suppression by shelterin regulates the activities of three DNA damage response enzymes: ATM, ATR, and poly (ADP-ribose) polymerase 1 (PARP1); inhibits three double-stranded breaks (DSB) repair pathways: classical NHEJ (c-NHEJ), alternative (alt)-NHEJ, and HDR, and prevents telomeric hyper-resection (T. de Lange, 2018).

Shelterin can also function as a regulator of telomerase activity by both recruiting telomerase to the telomere (Jayakrishnan Nandakumar et al., 2012; Sexton, Youmans, & Collins, 2012; Franklin L. Zhong et al., 2012) and facilitating telomerase processivity (Ci Ji Lim, Zaug, Kim, & Cech, 2017; Feng Wang et al., 2007). The TEL patch on the TPP1 OB (Oligonucleotide and Oligosaccharide-Binding fold) domain in the TPP1-POT1 heterodimer recruits telomerase to the telomere (Latrick & Cech, 2010; J. Nandakumar et al., 2012; Sexton et al., 2012; F. L. Zhong et al., 2012). The POT1-TPP1 heterodimer enhances telomerase processivity by decreasing the primer dissociation rate and increasing the translocation efficiency (Latrick & Cech, 2010). Collectively, the shelterin complex modulates telomere architecture, recruits telomerase to accessible telomeres, and regulates telomerase to synthesize new telomeric DNA. It is essential for chromosome end-capping and maintaining genome stability.

1.6 Mechanisms of Telomere Maintenance in Cancer Cells

Telomere length influences both cellular lifespan and tumor formation. The entire length of the telomeres cannot be fully replicated so telomeres become shorter with each cell division. When telomeres reach a critical shortness, known as telomere crisis, they can no longer form a

structure to protect the ends of chromosomes. These short telomeres are recognized by the DNA repair machinery as a DNA double-stranded breaks (DDB) and further cell division is blocked resulting in crisis and cellular senescence. To bypass this crisis, cells often acquire additional mutations that may promote their ongoing growth. Normal cells may become immortal cells if they can reactivate telomerase, which allows them to maintain their telomere length while they keep dividing. Telomerase reactivation in cancer cells occurs in over 85% of all malignant cells (Akincilar, Unal, & Tergaonkar, 2016).

Telomerase reactivation can be induced by genetic mechanisms including *TERT* promoter mutations (Barthel et al., 2017; Horn et al., 2013; Huang et al., 2013; P. J. Killela et al., 2013; X. Liu et al., 2013; Nault et al., 2013; J. Vinagre et al., 2013), *TERT* amplifications (Barthel et al., 2017; K. T. Hwang et al., 2008; Piscuoglio et al., 2016; Yamamoto et al., 2007; A. Zhang et al., 2000; C. Q. Zhu et al., 2006) and *TERT* rearrangements (Valentijn et al., 2015; Yoo et al., 2019; Zhao, Wang, Popova, Grigoryev, & Zhu, 2009), as well as epigenetic mechanisms such as DNA methylation of the *TERT* promoter (Castelo-Branco et al., 2013; Castelo-Branco et al., 2016; Donghyun D. Lee et al., 2019), histone acetylation/deacetylation (Krajewski, 2002; Masahiro Takakura et al., 2001), and expression of non-coding RNAs (Gala & Khattar, 2021; Kang et al., 2021; Nelson & Shippen, 2015). With respect to tumor progression, one study of the genetic evolution of melanomas demonstrated that 77% of intermediate lesions and melanomas *in situ* harbored *TERT* promoter mutations suggesting that these mutations are selected at an early stage of neoplastic progression (Shain et al., 2015).

The mechanism by which tumor cells activate telomerase is usually tumor-type specific. For example, *TERT* amplification/gene overexpression occurs in 1-2% of cases of lung adenocarcinoma, breast invasive ductal carcinoma, small cell lung carcinoma, squamous cell lung

carcinoma, and bladder urothelial carcinoma (Consortium et al., 2017). High-throughput sequencing shows somatic copy-number alterations in the *TERT* gene (*TERT* amplification) are significantly enriched (~15%) in triple wild-type (BRAF-, RAS-, and NF1-wild-type) cutaneous melanomas (Akbani et al., 2015) *TERT* rearrangements have been reported in melanomas (3 cases from the total of 74 skin cutaneous melanoma cases) and are thought to increase the expression of *TERT* due to the overlapping between super-enhancers and juxtaposed *TERT* coding region which leads to massive chromatin remodeling and transcription activation (Barthel et al., 2017; Yuan, Larsson, & Xu, 2019).

Epigenetic mechanisms that regulate *TERT* gene transcription also play a role in *TERT* reactivation. *TERT* gene transcription is regulated by the assembly of transcription factors at promoter and enhancer regions. DNA methylation, histone acetylation, methylation, and phosphorylation have been shown to affect the *TERT* transcription regulation (Ge, Liu, Björkholm, Gruber, & Xu, 2006; C. Liu et al., 2007; D. Xu et al., 2001; Yuan et al., 2019).

Several transcription factors—including the MYC proto-oncogene, ETS, and the p53 tumor suppressor—bind to the *TERT* promoter (Y. S. Cong, Wen, & Bacchetti, 1999; M. Takakura et al., 1999). One experiment demonstrated that ETV5 and c-Myc cooperate to activate *TERT* transcription via two ETS/E-box motifs (F. Zhang, Wang, & Zhu, 2020). The *TERT* promoter is generally unmethylated in normal cells, but methylation is required for *TERT* expression and telomerase activation in cancer cells (D. D. Lee et al., 2019; Lewis & Tollefsbol, 2016; L. Liu et al., 2017; Svahn et al., 2018). From this regard, hypermethylation in *TERT* promoter mutations contributes to *TERT* activation in cancer cells.

In conclusion, the telomere maintenance mechanism in cancer cells, a critical driver of cancer cell immortality, is regulated by multiple pathways that may be targets for cancer treatment.

1.7 Non-coding Mutations in Cancer

Only about one percent of the human genome is made up of protein-coding genes; the other 99% is noncoding (Dunham et al., 2012). Non-coding DNA contains sequences that act as regulatory elements, which determine when and where genes are turned on and off, such as promoters, enhancers, silencers, and insulators. Non-coding mutations can potentially effect cancer driver genes by several mechanisms including altering transcriptional regulation, deregulating mRNA translation and stability, altering regulatory elements that control splicing, disrupting the structure of chromatin domains to alter gene expression, and altering the function of regulatory non-coding RNAs.

Exome-based sequencing analysis has been the predominant technique used for the identification of tumor mutations. Exome sequencing, however, is targeted primarily at protein-coding mutations, and does not capture information about non-coding changes. Whole genome-based analyses have been more useful since both protein-coding and non-coding mutations are identified. Many non-coding mutations include promoters, 5′ untranslated regions (5′ UTR), 3′ untranslated regions (3′ UTR), enhancers, long non-coding RNAs (lncRNA), precursor-miRNAs (pre-miRNA), and small RNAs, which associate significantly with cancer development have been identified from International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA)(Rheinbay et al., 2020). These repositories store whole-genome sequencing (WGS) data from thousands of cancer genomes in 38 cancer types that were generated by more than 1,300 scientists in 37 countries (Campbell et al., 2020). WGS allows comprehensive cancer genome analyses including mutational signatures and cancer genomic information that provide a comprehensive view of cancer genomes. WGS approaches also allow exploration of the landscape of driver mutations (causative mutations) and passenger mutations (mutations that arise during

tumorigenesis, but do not contribute to oncogenesis) (Alexandrov et al., 2013; Degasperi et al., 2022; Helleday, Eshtad, & Nik-Zainal, 2014).

Despite the large number of non-coding mutations that have been identified in cancers, there are limited data regarding the proportion of these non-coding mutations that actually alter gene expression and contribute to disease. Significant additional research will be required to understand how non-coding variants contribute to carcinogenesis. As sequencing prices continue to decrease, WGS will likely become more common, including its use in the clinical setting.

1.8 TPP1 and the Mechanism of Telomere Maintenance

TPP1 plays a central role in telomere maintenance by both end protection and end replication. It interacts with the other shelterin proteins to protect chromosome ends and regulates telomerase by recruiting and promoting its catalytic activity (Figure 3B) (Chu, D'Souza, & Autexier, 2016; Sherilyn Grill et al., 2021; Schmidt, Dalby, & Cech, 2014; Sexton et al., 2012; Tesmer, Smith, Danciu, Padmanaban, & Nandakumar, 2019; F. L. Zhong et al., 2012). TPP1 or TINT1-PTOP1-PIP1 (human gene name: *ACD* located on chromosome 16q22.1) (T. de Lange, 2005; Houghtaling, Cuttonaro, Chang, & Smith, 2004; Dan Liu et al., 2004; J. Z. Ye, D. Hockemeyer, et al., 2004) is one of six shelterin proteins that interact to form numerous complexes with telomeric DNA (T. de Lange, 2018; C. J. Lim & Cech, 2021). TPP1 binds POT1 through the TPP1 OB domain that contains two critical structures: the NOB domain and the TEL patch. The NOB, short for the N-terminus of the OB domains, interacts with telomerase to promote enzymatic activity (S. Grill, Tesmer, & Nandakumar, 2018; B. Liu et al., 2022; J. Nandakumar et al., 2012; Sexton et al., 2012; F. L. Zhong et al., 2012). The seven conserved and surface-exposed TPP1 OB

residues (E168, E169, E171, R180, L183, L212, and E215) are referred to as the TPP1 glutamate (E)- and leucine (L)- rich patch (TEL patch). A K170Δ mutation in the TEL patch of TPP1 was identified in two patients—one patient with bone marrow failure (Guo et al., 2014) and another patient with Hoyeraal-Hreidarsson syndrome (Kocak et al., 2014)—who presented with short telomeres. These findings demonstrate the importance of the TEL patch in telomerase regulation.

Human TPP1 has been reported to have two isoforms (Figure 3A). The TPP1 long isoform (TPP1-L) encompasses 544 amino acids (Houghtaling et al., 2004; D. Liu et al., 2004; J. Z.-S. Ye et al., 2004). The short form of TPP1 (TPP1-S) initiates at Met87 (H. Hwang, Buncher, Opresko, & Myong, 2012; Jayakrishnan Nandakumar et al., 2012; Xin et al., 2007) to express a 458 amino acid protein. TPP1-S is the major isoform in human cells and is conserved in other species. TPP1-S expression in stem cells and most somatic cells suggests that TPP1-S contributes to the maintenance of telomere length rather than TPP1-L. Furthermore, TPP1-S and TPP1-L isoforms have opposing regulatory functions. Both TPP1-S and TPP1-L are capable of binding telomeres, promoting end protection, and recruiting telomerase to telomeres (Sherilyn Grill et al., 2019). Functionally, only the overexpression of TPP1-S causes hyper-elongation of telomeres. TPP1-L expression blocks telomere extension by telomerase (Sherilyn Grill et al., 2019).

Mutations in TPP1 were identified in both sporadic and familial forms of melanoma (L. G. Aoude, Pritchard, et al., 2015; Horn et al., 2013; C. D. Robles-Espinoza et al., 2014) but no functional studies have been done. Interestingly, data from WGS and whole exome sequencing (WES) of melanomas demonstrate the somatic variants of TPP1 are clustered, which is not seen in the other shelterin genes. However, the impact of these cluster variants in TPP1 with respect to telomere maintenance needs to be investigated.

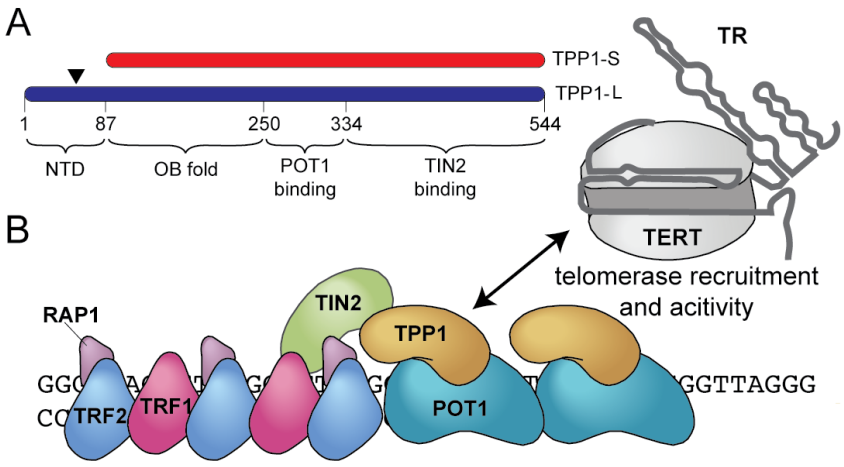


Figure 3. Schematic of the TPP1 Shelterin Protein in Humans.
(A) The two isoforms of TPP1 reported in human cells contain an N-terminal domain (NTD), an oligonucleotide and oligosaccharide-binding fold domain (OB), a POT1 binding domain, and a C-terminal TIN2 binding domain. TPP1-L and TPP1-S differ by 86 amino acids at the N-terminus. (B) TPP1 plays an important role in telomere maintenance in both capping and telomerase regulation. POT1 binds the telomeric overhang with high affinity in a specific manner, a process mediated by its two N-terminal OB folds that inhibits telomere extension. The POT1-TPP1 heterodimer acts as a switch to promote telomerase activity. TPP1 can facilitate telomerase recruitment and processivity via its OB fold which contains the TPP1 glutamate (E)- and leucine (L)- rich (TEL) patch and the N-terminal domain of TERT (TEN).

1.9 *TERT* Promoter Mutations in Cutaneous Melanoma

In 2013, two breakthrough studies discovered highly recurrent mutations in gene regulatory regions. Huang *et al.* found that 51 of 70 melanomas harbored the two most common recurrent mutations in the promoter region of the *TERT* gene (Huang et al., 2013) and confirmed that the mutations—located at positions chr5:1295228 (-124 C>T) and chr5:1295250 (-146 C>T)—are mutually exclusive. At the same time, these same mutations and the A>C mutation at -57 bp from the ATG start site of the *TERT* gene were identified as germ-line *TERT* promoter mutations in melanoma patients from melanoma-prone families (Horn et al., 2013). These mutations create *de novo* ETS transcription binding motifs that induced a two- to four-fold increase in transcription in luciferase reporter assays. GABP was identified as a specific ETS transcription factor that binds

to the mutated *TERT* promoter in gliomas (R. J. Bell et al., 2015) and melanomas (Makowski et al., 2016).

The less frequent -138/139 CC>TT tandem mutation most often seen with *BRAF/NRAS* mutations is associated with the worst prognosis for stage I and stage II melanomas (Andrés-Lencina et al., 2019). The presence of *TERT* promoter mutations and *BRAF/NRAS* mutations are associated with poor disease prognosis in melanomas (Nagore et al., 2016). Somatic *TERT* promoter mutations were also frequently identified in melanomas and many other cancers with a low rate of self-renewal (P. J. Killela et al., 2013) indicating that telomerase reactivation is an essential mechanism for enabling replicative immortality in cancer.

To understand the role of the *TERT* promoter in tumorigenesis and the regulation of telomere length and genome stability, Chiba *et al*. elucidated that *TERT* promoter mutations acquired in benign nevi can cause the transition to a malignant melanoma but these mutations did not maintain telomere length (Chiba et al., 2017b). They demonstrated that *TERT* promoter mutations were sufficient to prolong cellular life span but not sufficient to immortalize melanocytes. These mutations induced *TERT* expression that did not prevent telomere shortening but promoted the repair of the shortest telomeres. This observation raised the question of how cells with critically short telomeres can become fully immortal and have the capacity to lengthen their telomeres. Moreover, it is unclear whether reactivation of telomerase through *TERT* promoter mutation is required only for early stages of tumorigenesis or is also necessary for sustaining neoplastic growth. This work begins to study the novel insights into mechanisms of telomere maintenance in cancers, and the application of our findings may improve disease outcomes.

2.0 Materials and Methods

This chapter presents the materials used and methods used in this thesis. Standard laboratory equipment, reagents, and instruments are not mentioned. It is important to note that the molecular genetic analyses used by the Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, School of Medicine, and its standard protocols have been used in this research.

2.1 Experimental Models and Methods

2.1.1 Cloning of TPP1 Expression and Reporter Constructs

cDNAs expressing the long (AA 1-544) and short (AA 87-544) isoforms of TPP1 were cloned with C-terminal flag-tags into the pCDNA5/FRT expression vector. According to a previous study, N-terminal modifications of TPP1 impair its function (Sandhu, Wei, Sharma, & Xu, 2019), therefore, we put a C-terminal Flag-tag on TPP1 plasmid constructs. 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 assembled into a pCDNA5 vector that lacked the CMV enhancer and promoter and incorporated a flag-tag at the C-terminus to facilitate imaging and blotting. Luciferase reporter constructs were constructed by synthesizing fragments of the *ACD* proximal promoter of decreasing size (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 the 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.

2.1.2 Cell Culture, Transfection, and Luciferase Assays

HeLa, HEK293FT (HEK293 hereafter), and BJ fibroblast cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (20% FBS for BJ fibroblasts) and Penicillin-Streptomycin-Glutamine (1x) and maintained at 37°C in the presence of 5% CO_2 in a humidified incubator. Melanoma cell lines (see Appendix A.1) were cultured in RPMI 1640 supplemented with 10% FBS and Penicillin-Streptomycin-Glutamine (1X) 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.

2.1.3 Generation of Stable Cell Lines

Hela stable cell lines were generated using HeLa Flp-In cells (ThermoFisher; cat# R71407). The Flp-In system is based on the integration of a single copy of an exogenous plasmid at a single FRT "docking site" in the genome in the presence of Flp recombinase. Each cell line generated is isogenic (the exogenous construct integrates at the exact same site in each clonal line) and facilitates the comparison between variant constructs. 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.

2.1.4 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 12,000xg 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; ThermoFisher, 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.

2.1.5 Protein Half-life Analyses

Cells stably expressing TPP1-S, TPP1-L, and TPP1 genomic 3xFlag constructs were treated with solvent (DMSO) or 20 μM of MG132 (Sigma-Aldrich) for 2 h followed by addition of cycloheximide (CHX) to a final concentration of 100 μg/ml. Cells were then directly resuspended in RIPA buffer and boiled for 5 min at 95 °C followed by SDS-PAGE and Western blot analysis.

2.1.6 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 permeabilization 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 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).

2.1.7 Telomere Fluorescence in Situ Hybridization Analysis

Telomere fluorescence in situ hybridization (FISH) was carried as described previously (Cesare, Heaphy, & O'Sullivan, 2015). 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; 5′acctaaacctaaacctaa3′) were synthesized by PNA Bio (Thousand Oaks, CA). Images were acquired on the Nikon ECLIPSE Ni fluorescent microscope with a CCD camera.

Images were deconvolved and analyzed on NIS-Elements General Analysis 3 Advanced Research software.

2.1.8 Telomere Restriction Fragment 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 (Morrish & Greider, 2009). DNA was denatured in the gel 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 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. A 600 bp telomeric fragment generated by EcoRI digestion of JHU821 containing 100 repeats of 5′TTAGGG3′/3′CCCTAA5′ was radiolabeled (25 ng) along with a 1 kb DNA ladder (NEB) with 33 mM 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 0.5X SSC 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) and TeloTool Software (Göhring, Fulcher, Jacak, & Riha, 2013).

2.1.9 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 synthesized for each sample using an 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 Predesigned qPCR assay). qPCR was carried out using the CFX96 or CFX384 Real time System. Expression levels of ETS mRNA were calculated from threshold cycle values and normalized to GAPDH and B_2M values. Expression of ACD was normalized to HPRT, GUSB, and PPIA using CFX Maestro software (Bio-Rad).

2.1.10 CRISPR-Cas9 Mediated Homology-Directed Repair Genome Editing

Generation of TPP1 genomic -108 C>T and -75 C>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′TCCTCGGAAGAGGAAGCTCCTTCGCTGGGCGGGGCCGGAGGAAGAGGCCCCGCCC ACGTACACCCCGCGCCTGCGCACGAGGG3′,

-108C>T minus strand:

5′CCCTCGTGCGCAGGCGCGGGGTGTACGTGGGCGGGGCCTCTTCCTCCGGCCCCGCC

CAGCGAAGGAGCTTCCTCTTCCGAGGA3′,

and -75C>T plus strand:

5′CCGGGTTTCCCGCGGGCGCCCAGGCCCCGCCTTTCCTCGGAAAAGGAAGCTCCTTC

GCTGGGCGGGGCCGGAGGAGGAGGCCC3′,

Minus strand:

5′GGGCCTCCTCCTCCGGCCCCGCCCAGCGAAGGAGCTTCCTTTTCCGAGGAAAGGCG

GGGCCTGGGCGCCCGCGGGAAACCCGG3′.

Alt-R crRNA was synthesized targeting the sequence shown in Figure 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 used either ATTO550 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 Amaxa™ Cell Line Nucelofector™ 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 amplification of the *TPP1* promoter (5′primer –

cgcgatgagagtaaacgggc and 3′primer - cctccccgaacctgccat) and digestion of the PCR product with EarI or BseRI. PCR products that were resistant to digestion were sequenced to validate the presence of the edited nucleotide.

2.1.11 Lentiviral Construction and Packaging

Dual promoter lentiviruses 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 (5′TTAGGT3′) was created using the pLV-IU1-hTR-CMV-Puro vector (a generous gift from Dr. Bradley Stohr). The template region, spanning from r.46 to r.56, was modified to express a variant hTR that encodes 5′TTAGGT3′ repeats rather than the canonical 5′TTAGGG3′ sequence repeats (5′CUAACCCUAAC3′ -> 5′CUAAACCUAAA3′). All plasmid sequences were confirmed by Sanger sequencing. 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 8 ug/mL polybrene overnight and the media was changed on the following morning. Transduced cells were selected 48 hours have by addition of geneticin (ThermoFisher Scientific), blasticidin (ThermoFisher Scientific) or both.

2.1.12 Biospecimens

We collaborated with Dr. John Kirkwood, director of the Melanoma Center at UPMC Hillman Cancer at the University of Pittsburgh for melanoma patient samples and clinical data. All cancer samples analyzed in this study were collected and sequenced under an Institution Review Board-approved protocol (IRB: MOD19080226-001) at the University of Pittsburgh. Clinical and histologic data were retrieved retrospectively from the patient records of the University of Pittsburgh Hillman Cancer Center. Biospecimens from resected primary and/or metastatic melanomas were obtained from patients with appropriate informed consent and institutional review board or ethics board approval. Biospecimens were classified as either primary or metastatic based on the available clinical and pathological information. Melanoma cell lines were established from melanoma tumors.

2.1.13 DNA Isolation

A pathologist identified tumor regions in micro-dissected tumor slides derived from paraffin-embedded blocks. Genomic DNA was extracted from the cell lines and formalin-fixed, paraffin-embedded tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen).

2.1.14 *ACD* Isoform Analysis

RNA-seq FASTQ files were downloaded from GSE153592 (Motwani et al., 2021) and GSE112509 (M. Kunz et al., 2018), trimmed for quality and aligned to the GRCh38 using STAR (Dobin & Gingeras, 2015). 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 Diekhans at UCSC and screenshots were exported as PDFs and modified in Adobe Illustrator.

2.1.15 Identification of the Cluster of *TPP1* Promoter Variants and Prediction of Transcription Factor Binding Sites

We identified a cluster of the *TPP1* promoter variants in somatic mutations from melanoma using COSMIC (Tate et al., 2019), ICGC (International Cancer Genome Consortium et al., 2010), and TCGA (Cancer Genome Atlas Research Network et al., 2013) databases and analyzed the sequences of the wild type and variant promoters using the TRANSFAC database. TRANSFAC is a database of DNA sequence motifs and the transcription factors that bind them (Farré et al., 2003; Wingender et al., 1996).

2.1.16 Promoter Enrichment Analysis

Promoter enrichment analysis was conducted using GENCODE 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; 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 SKCABR 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, downloaded 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 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).

2.1.17 Comparison of Tumor and Normal *TPP1* Expression

Gene expression data from GENT2 and OncoDB were downloaded on 07/10/2022 and replotted using GraphPad Prism.

2.1.18 Statistical Analysis

A *P*-value < .05 was considered significant after correction for multiple testing. Data were analyzed using GraphPad Prism version 9.3.1. Statistical tests used are listed in the figure legends where the data are shown.

2.2 Key Resources Tables

Table 1. Reagents and Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
ETS1 (NM_005238) Human Tagged ORF Clone (pCMV6-ETS1)	Origene	Cat. No. RC215203L2
ETV5 (NM_004454) Human Tagged ORF Clone (pCMV6-AC-GFP-ETV5)	Origene	Cat. No. RG200366
pBS-hTERT-PGK-Neo	Gene Universal	Cat. No. 722561-2
pBS-hTPP1-PGK-Blast	Gene Universal	Cat. No. 722561-1
pcDNA-3xFLAG-NLS-TPP1	Addgene	Cat. No. 53585
pcDNA5/FRT	Invitrogen	Cat. No. V601020
pcDNA5/FRT-EGFP	(Scott et al., 2018)	N/A

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pcDNA5/FRT-hTPP1-genomic -108C>T-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-genomic -75C>T-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-genomic WT-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-L L51F-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-L L62F-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-L M87A-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-L-2xFlag	This project	N/A
pcDNA5/FRT-hTPP1-S-2xFlag	This project	N/A
pcDNA3.1 (+) Mammalian Expression Vector	Invitrogen	Cat. No. V79020
pCMV-dR8.91 (delta 8.9)	Lifescience Market	Cat. No. PVT2323
pCMV-VSV.G	Addgene	Cat. No. 8454

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pea3 (ETV4) (NM_001986) Human Tagged ORF Clone (pCMV6-ETV4)	Origene	Cat. No. RC215093
pGL4.10-hERTpro WT	This project	N/A
pGL4.10-hERTpro -124C>T	This project	N/A
pGL4.10-hTPP1pro163 WT	This project	N/A
pGL4.10-hTPP1pro200 WT	This project	N/A
pGL4.10-hTPP1pro285 -108C>T	This project	N/A
pGL4.10-hTPP1pro285 -75C>T	This project	N/A
pGL4.10-hTPP1pro285 WT	This project	N/A
pGL4.10-hTPP1pro613 WT	This project	N/A
TPP1 genomic clone1	BACPAC genomics	BAC CH17 394I12
TPP1 genomic clone2	BACPAC genomics	BAC CH17 425N4
Antibodies		
Alexa Fluor™ 647 goat anti-rabbit IgG (H+L)	Invitrogen	Cat. No. A27040
Alexa Fluor™ 594 goat anti-mouse IgG (H+L)	Invitrogen	Cat. No. A11032

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-GAPDH hFAB Rhodamine Antibody	Bio-Rad	Cat. No. 12004167
ETS-1 (D8O8A) Rabbit mAb	Cell Signaling Technology	Cat. No. 14069
ETV4 Polyclonal Antibody	Invitrogen	Cat. No. PA5-76825
Goat Anti-Mouse IgG (H+L)-HRP Conjugate	Bio-Rad	Cat. No. 170-6516
Goat Anti-Rabbit IgG (H+L)-HRP Conjugate	Bio-Rad	Cat. No. 170-6515
hFAB™ Rhodamine Anti-Tubulin Primary Antibody	Bio-Rad	Cat. No. 12004165
K48-linkage Specific Polyubiquitin (D9D5) Rabbit mAb	Cell Signaling Technology	Cat. No. 8081
Monoclonal Anti-ETV5 antibody produced in mouse	Millipore Sigma	Cat. No. WH0002119M2-100UG
Monoclonal ANTI-FLAG M2 antibody	Sigma-Aldrich	Cat. No. F1804
Phospho-Cyclin D1 (Thr286) (D29B3) XP® Rabbit mAb	Cell Signaling Technology	Cat. No. 3300
Rabbit anti-TPP1 Antibody	Bethyl Laboratories	Cat. No. A303-069A
Rabbit polyclonal anti-TRF2	Novus Biologicals	Cat. No.NB110-57130

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant Anti-Telomerase reverse transcriptase antibody [Y182]	Abcam	Cat. No. ab32020
Ubiquitin (P37) Antibody	Cell Signaling Technology	Cat. No. 58395
Bacterial and Virus Strains		
STBL3	Homemade	N/A
TOP10	Homemade	N/A
Cell Culture Media		
Dulbecco's Modified Eagle Medium (DMEM)	Gibco Life Technologies	Cat. No. 11-995-065
Fetal Bovine Serum (FBS)	Gibco Life Technologies	Cat. No. 26-140-079
Penicillin-Streptomycin-Glutamine (100X)	Gibco Life Technologies	Cat. No. 10-378-016
Roswell Park Memorial Institute (RPMI) 1640	Gibco Life Technologies	Cat. No. 11-875-093
Trypsin EDTA (1x)	Gibco Life Technologies	Cat. No. 25-200-114
Cell lines		
HEK-293FT	ThermoFisher Scientific	Cat. No. R70007
HEK-293T	ATCC	Cat. No. CRL-3216
BJ fibroblast	ATCC	Cat. No. CRL-2522
HeLa T-rex	ThermoFisher Scientific	Cat. No. R71407

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
hTERT RPE-1	ThermoFisher Scientific	Cat. No. CRL-4000
Lox melanoma	(Fodstad et al., 1988)	N/A
MEL624	(Ji et al., 2016)	N/A
Chemicals and reagents		
1 kB Plus DNA Ladder	Invitrogen	Cat. No. 10787018
10x Tris/Glycine/SDS	Bio-Rad	Cat. No.1610732
2x Laemmli Sample Buffer 4x Laemmli Sample Buffer	Bio-Rad	Cat. No. 161-0737EDU Cat. No. 161-0747
Acetic Acid, Glacial	Fisher Chemical	Cat. No. A38S-500 CAS No. 64-19-7
Agarose UltraPure	Invitrogen	Cat. No. 16500500
Alt-R™ S.p.Cas9-GFP V3, 100 µg	Integrated DNA Technologies	Cat. No. 10008100
Alt-R® S.p. Cas9 Nuclease V3, 100 µg	Integrated DNA Technologies	Cat. No. 1081058
Ampicillin (Sodium), USP Grade	Gold Biotechnology	Cat. No. A-301-5 CAS No. 69-52-3
AnykD™ Criterion™ TGX™ Precast Midi Protein Gel	Bio-Rad	Cat. No. 5671125
Betaine solution	Sigma-Aldrich	Cat. No. B0300 CAS No. 107-43-7

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Blasticidin S HCl (10 mg/mL)	Gibco Life Technologies	Cat. No. A1113903
Bovine Serum Albumin (BSA) DNase- and Protease-free Powder	Fisher BioReagents	Cat. No. BP9706100
Carbenicillin (Disodium), USP Grade	Gold Biotechnology	Cat. No. C-103-5 CAS No. 4800-94-6
CENPB-Alexa488	PNA Bio	Cat. No. F3004
Chloramphenicol, USP Grade	Gold Biotechnology	Cat. No. C-105-5 CAS No. 56-75-7
cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail	Roche	Cat. No. 04693116001
Cy5-(ACCTAA)3 probe	PNA Bio	Cat. No. F1003
Cycloheximide	Cell Signaling Technology	Cat. No. 2112S CAS No. 66-81-9
DAPI (4′,6-diamidino-2-phenylindole)	Cell Signaling Technology	Cat. No. 4083 CAS No. 28718-90-3

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
deoxynucleotide (dNTP) solution mix	New England Biolabs	Cat. No. N0447L
Dimethyl Sulfoxide (DMSO)	Fisher BioReagents	Cat. No. BP231-100 CAS No. 67-68-5
Ethanol 200 Proof (500 mL)	Decon	Cat. No. BP2818500 CAS No. 64-17-5
Geneticin Selective Antibiotic (G418 Sulfate) (50 mg/mL) (20 mL)	Gibco Life Technologies	Cat. No. 10131035 CAS No. 108321-42-2
Gibco KaryoMAX Colcemid Solution in PBS	Gibco Life Technologies	Cat. No. 15212012
Hyclone molecular grade water	Gibco Life Technologies	Cat. No. SH3053802 CAS No. 7732-18-5
Hydrochloric acid, 1N	Fisher Chemical	Cat. No. SA48-1 CAS No. 7647-01-0
Hygromycin B (50 mg/mL)	Invitrogen	Cat. No. 10-687-010 CAS No. 31282-04-9
IDTE pH 7.5 (1X TE Solution) 300 mL	Integrated DNA Technologies	Cat. No.11-05-01-15

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Octylphenoxy poly (ethyleneoxy) ethanol, branched (IGEPAL)	Sigma-Aldrich	Cat. No. 18896 CAS No. 68412-54-4
Isopropanol, Molecular Biology Grade	Fisher BioReagents	Cat. No. BP26181 CAS No. 67-63-0
Kanamycin monosulfate, USP Grade	Gold Biotechnology	Cat. No. K-120-5 CAS No. 8063-07-8
LB Agar (Granulated) - Lennox	Fisher BioReagents	Cat. No. BP9724-500
LB Broth (Powder) - Lennox	Fisher BioReagents	Cat. No. BP1427-500
Lipofectamine 2000	Invitrogen	Cat. No. 11668019 CAS No. 158571-62-1
Methanol (Certified ACS) 4 L	Fisher Chemical	Cat. No. A412-4 CAS No. 67-56-1
Milk, Non-fat, Dry	LabScientific	Cat. No. M0842 CAS No. 8049-98-7
7.5% Mini-PROTEAN® TGX™ Precast Gels (12 wells)	Bio-Rad	Cat. No. 4561025

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Opti-MEM™ Reduced Serum Medium	Gibco	Cat. No. 31-985-062
Phosphate Buffered Saline Solution (PBS)	Gibco	Cat. No. 10-010-072
Pepsin	MP Biomedicals	Cat. No. 195367 CAS No: 9001-75-6
Polybrene	Sigma Aldrich	Cat. No. TR1003 CAS No. 28728-55-4
Precision Plus Protein All Blue Prestained Protein Standards	Bio-Rad	Cat. No. 1610373
ProLong Diamond Antifade Mountant	Invitrogen	Cat. No. P36930
Puromycin Dihydrochloride	Gibco	Cat. No. A1113803 CAS No. 58-58-2
RIPA Lysis and Extraction Buffer	ThermoFisher Scientific	Cat. No. 89900
RNase A	Qiagen	Cat. No. 19101 CAS No. 9001-99-4
S.O.C. medium	New England Biolabs	Cat. No. B9020

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TelC-Cy3	PNA Bio	Cat. No. F1002
TRIS HCl	Sigma Aldrich	Cat. No. 10812846001 CAS No. 1185-53-1
Trypan Blue Stain (0.4%)	Gibco	Cat. No. 15250061 CAS No. 72-57-1
Tween20	Fisher BioReagents	Cat. No. BP337-500 CAS No. 9005-64-5
DNA Modifying Enzymes, Restriction Endonucleases and Buffers		
AgeI-HF	New England Biolabs	Cat. No. R3552S
BamHI-HF	New England Biolabs	Cat. No. R3136S
BseRI	New England Biolabs	Cat. No. R0581S
DpnI	New England Biolabs	Cat. No. R0176S
EarI	New England Biolabs	Cat. No. R0528S
EcoRI-HF	New England Biolabs	Cat. No. R3101S
HindIII-HF	New England Biolabs	Cat. No. R3104S
KasI	New England Biolabs	Cat. No. R0544S
KpnI-HF	New England Biolabs	Cat. No. R3142S
MluI-HF	New England Biolabs	Cat. No. R3198S
NotI-HF	New England Biolabs	Cat. No. R3189S
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat. No. M0530S

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
5x Phusion® GC PCR Reaction Buffer	New England Biolabs	Cat. No. B0519S
ThermoFisher Scientific	ThermoFisher Scientific	ThermoFisher Scientific ThermoFisher Scientific
Proteinase K	New England Biolabs	Cat. No. P8107S CAS No. 39450-01-6
SmaI	New England Biolabs	Cat. No. R0141S
T4 DNA Ligase	New England Biolabs	Cat. No. M0202T CAS No. 9015-85-4
T4 Polynucleotide Kinase	New England Biolabs	Cat. No. M0201S CAS No. 37211-65-7
rCutSmart Buffer	New England Biolabs	Cat. No. B6004S
NEBuffer r2.1	New England Biolabs	Cat. No. B6002S
NEBuffer r3.1	New England Biolabs	Cat. No. B6003S
Kits		
Applied Biosystems PowerUp SYBR Green Master Mix	Applied Biosystems	Cat. No. A25741
Cell Line Nucleofector Kit V	Amaxa/Lonza	Cat. No. VCA-1003
Dual-Glo Luciferase Reporter Assay system	Promega	Cat. No. E2920

Table 1. Reagents and Resources (continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
iScript™ cDNA Synthesis Kit	Bio-Rad	Cat. No. 1708890
iTaq Universal SYBR Green Supermix	Bio-Rad	Cat. No. 1725121
Monarch® DNA Gel Extraction Kit	New England Biolabs	Cat. No. T1020L
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientific	Cat. No. 23225
Puregene Cell Kit (8×10^8)	Qiagen	Cat. No. 158767
QIAGEN Plasmid Maxi	Qiagen	Cat. No. 12163
Qubit dsDNA BR assay	Life Technologies	Cat. No. Q32850
Qubit RNA BR assay	Life Technologies	Cat. No. Q10210
RNeasy Mini Kit	Qiagen	Cat. No. 74104
Trans-Blot Turbo RTA Mini 0.2 µm PVDF Transfer Kit, for 40 blots	Bio-Rad	Cat. No. 1704272
*T*elo*TAGGG* Telomere Length Assay	Roche	Cat. No. 12209136001
WesternBright Quantum HRP substrate	Advansta	Cat. No. K-12042-C20

Table 2. Oligonucleotides

Oligonucleotide	Sequence
-108 HDR template-	5′CCCTCGTGCGCAGGCGCGGGGTGTACGTGGGCGGGGCCTCTTCCTCCGGCCCCGCCCAGCGAAGGAGCTTCCTCTTCCGAGGA3′
-108 HDR template+	5′TCCTCGGAAGAGGAAGCTCCTTCGCTGGGCGGGGCCGGAGGAAGAGGCCCCGCCCACGTACACCCCGCGCCTGCGCACGAGGG3′
-75 HDR template-	5′GGGCCTCCTCCTCCGGCCCCGCCCAGCGAAGGAGCTTCCTTTTCCGAGGAAAGGCGGGGCCTGGGCGCCCGCGGGAAACCCGG3′
-75 HDR template+	5′CCGGGTTTCCCGCGGGCGCCCAGGCCCCGCCTTTCCTCGGAAAAGGAAGCTCCTTCGCTGGGCGGGGCCGGAGGAGGAGGCCC3′
hEST1_GA_Fwd	5′GGGTTTGCCGCCAGAACACAGGACCGGTTCTAGAGCCACCATGAAGGCGGCCGTCGATC3′
hEST1_GA_Rev	5′GCTTGTTTCAGCAGAGAGAAGTTTGTTGCGCCGGATCCCTCGTCGGCATCTGGCTTGACG3′
Hs_ACD_1_SG	NM_022914, NM_001082487, NM_001082486, XM_005256115
Hs_B2M_1_SG	NM_004048, XM_006725182, XM_005254549
Hs_ETS1_1_SG	NM_001143820, NM_005238, XM_005271428

Table 2. Oligonucleotides (continued)

Oligonucleotide	Sequence
Hs_ETV4_1_SG	NM_001986, NM_001079675, NM_001261437, NM_001261438
Hs_ETV5_va.1_SG	NM_004454
Hs_GAPDH_1_SG	NM_002046, NM_001256799, NM_001289746, NM_001289745
Hs.PT.58v.45621572_HPRT1	NM_000194 (1)
Hs.PT.58v.27737538_GUSB	NM_000181 (1)
Hs.PT.58v.38887593.g_PPIA	NM_021130 (1)
hTPP1_crRNA	5′CGAAGGAGCTTCCTCTTCCG3′
hTPP1_crRNA2	5′GTGGGCGGGGCCTCCTCCTC3′
hTPP1-cDNA-qPCR-FWD1	5′ctccgctcttgcgtcatcacg3′
hTPP1-cDNA-qPCR-FWD10	5′ccctgctctgtctgggaaccc3′
hTPP1-cDNA-qPCR-FWD11	5′gtccaagctgtcaggcttcctc3′
hTPP1-cDNA-qPCR-FWD2	5′ggcagctgcttgaggtactacag3′
hTPP1-cDNA-qPCR-FWD3	5′acctcggactgggaggagaag3′
hTPP1-cDNA-qPCR-FWD4	5′ggcggcgcgcccgcagag3′
hTPP1-cDNA-qPCR-FWD5	5′ggtgcctggttgcaaccaag3′
hTPP1-cDNA-qPCR-FWD6	5′actgccttgaggagcacctttc3′
hTPP1-cDNA-qPCR-FWD7	5′cctcgtccaatgcaggcctatc3′
hTPP1-cDNA-qPCR-FWD8	5′aggccacgggagaagctg3′
hTPP1-cDNA-qPCR-FWD9	5′cagaggacacagggaacccc3′

Table 2. Oligonucleotides (continued)

Oligonucleotide	Sequence
hTPP1-gDNA-Seq-FWD1	5′gtccacgtttcctaggaaacggag3′
hTPP1-gDNA-Seq-FWD2	5′ccctggattcgggagctgattc3′
hTPP1pro_GA_pGL4_Fwd1	5′CATTTCTCTGGCCTAACTGGCCGGTACCccccgcccctg gcggtag3′
hTPP1pro_GA_pGL4_Rev1	5′GGCTTTACCAACAGTACCGGATTGCCAAGCTTcccc acggctacacccag3′
hTPP1pro_PCR_fwd2	5′GGACCAGCCCTTGTTCTGT3′
hTPP1pro_PCR_rev1	5′GGACTGGAGGGTGTCTCTGA3′
hTPP1pro_PCR_rev2	5′CTCGGCGTCCTGTAGTACCT3′
hTPPpro_PCR_fwd1	5′TGCTGAAATGGTTTCGAGTC3′
pGL4_TPP1-163_GA_fwd	5′TGGCCTAACTGGCCGGTACCatgagagtaaacgggccagcatc 3′
pGL4_TPP1-163_L62F_GA_Rev	5′CCAACAGTACCGGATTGCCAAGCTTTCCTCGGAA AAGGAAGCT3′
pGL4_TPP1-163_P61S_GA_Rev	5′CCAACAGTACCGGATTGCCAAGCTTTCCTCGGAA GAGAAAGCT3′
pGL4_TPP1-163_WT_GA_Rev	5′CCAACAGTACCGGATTGCCAAGCTTtcctcggaagaggaa gct3′
pGL4-hTPP1pro200_GA_fwd	5′TTTCTCTGGCCTAACTGGCCGGTACCgccgcgatgagagt aaacgg3′

Table 2. Oligonucleotides (continued)

Oligonucleotide	Sequence
pGL4-hTPP1pro200_GA_Rev	5′GGCTTTACCAACAGTACCGGATTGCCAAGCTTgggtttcccgcgggc3′
PrimeTime qPCR Primers ACD Exon 10-11a	NM_001082486 (2)
RVprimer3	5′CTAGCAAAATAGGCTGTCCC3′
RVprimer4	5′GACGATAGTCATGCCCCGCG3′
TPP1_GA_2xflag_Rev1	5′GGTTTAAACGGGCCCTCTAGACTCGAGCGGCCGCTCACTTCTCGTCATCGTCTTTATAATCCTTGTCGTCATCGTCTTTGTAGTCCATCGGAGTTGGCTCAGACCC3′
TPP1-gDNA_PCR_Fwd1	5′GAGACCAGCAGTGGAGG3′
TPP1-gDNA_PCR_Fwd2	5′GGAGGAGCCCTTACTTTGCT3′
TPP1-gDNA_PCR_Fwd3	5′CGCGATGTACGGGCCAGATATACGCGTGGAGGAGCCCTTACTTTGCT3′
TPP1-gDNA_PCR_Rev1	5′CCTGCCGCATGAGATTATTT3′
TPP1-gDNA_PCR_Rev2	5′GCACTGGAGGTGGAAGAGAG3′
TPP1-L-L51F (c.151 C-T).FOR	5′GTACGTGGGCGGGGCCTCTTCCTCCGGCCCCGCCCAGCG3′
TPP1-L-L51F (c.151 C-T).REV	5′CGCTGGGCGGGGCCGGAGGAAGAGGCCCCGCCCACGTAC3′
TPP1-L-L62F (c.184 C-T).FOR	5′CCAGCGAAGGAGCTTCCTTTTCCGAGGAAAGGCGGGGCC3′

Table 2. Oligonucleotides (continued)

Oligonucleotide	Sequence
TPP1-L-L62F (c.184 C-T).REV	5′GGCCCCGCCTTTCCTCGGAAAAGGAAGCTCCTTCGCTGG3′
TPP1-L-M87A.FOR	5′CTGGGTGTAGCCGTGGGGGCAGCAGGTTCGGGGAGGCTG3′
TPP1-L-M87A.REV	5′CAGCCTCCCCGAACCTGCTGCCCCCACGGCTACACCCAG3′
TPP1L_GA_Fwd1	5′CGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCGCCACCATGCCTGGCCGCTGTCAGAGTG3′
TPP1pro250_Fwd1	5′CCTGGCCGCTGTCAGAGTG3′
TPP1pro250_Fwd2	5′CGCGATGAGAGTAAACGGGC3′
TPP1pro250_Fwd3	5′CATCCCGTGCACCAGCGG3′
TPP1pro250_Rev1	5′CCTCCCCGAACCTGCCAT3′
TPP1pro250_Rev2	5′TCAGCTCCCGAATCCAGGG3′
TPP1pro700_Fwd1	5′CGGGAGTCTGCACACAGG3′
TPP1pro700_Fwd2	5′GAGCAGGACGCCCTCGTG3′
TPP1pro700_Rev1	5′CAAGGCAGTCTCACCACTCAC3′
TPP1pro700_Rev2	5′GTCACGAAGAGTCATGCC3′
TPP1S_GA_Fwd1	5′CGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCGCCACCATGGCAGGTTCGGGGAGGCTG3′

Table 3. Buffers and Solutions

Buffers and Solutions	Composition
10x Tris/Glycine/SDS	30.29 g Tris-base (25 mM) 144.13 g Glycine (192 mM) 50 ml of 20% SDS (0.1% SDS) pH to 8.3 ddH_2O to 1 liter
1x Tris/Glycine/SDS	100 ml 10x Tris-Glycine-SDS ddH_2O to 1 liter
2% paraformaldehyde (PFA)	Prior to use, dilute 4% paraformaldehyde 1:1 in PBS for a final concentration of 2%.
4% paraformaldehyde (PFA)	Weigh out 40 g paraformaldehyde Add 800 ml distilled, deionized water Add 5 M NaOH (500 μL) dropwise Stir and heat on a hot plate (let paraformaldehyde dissolve; up to 60°C, do not boil!) Add 100 ml 10× PBS Cool down to room temperature Adjust pH to precisely 7.4 Make up to 1 liter with ddH2O Store up to 1 year at −20°C in aliquots as necessary

Table 3. Buffers and Solutions (continued)

Buffers and Solutions	Composition
ABDIL	20 mM Tris·Cl, pH 7.5 2% (w/v) bovine serum albumin (BSA) 0.2% (w/v) fish gelatin (e.g., Sigma) 150 mM NaCl 0.1% (v/v) Triton X-100 0.1% (w/v) sodium azide Store up to 1 year at 4°C
Antibody diluent (WB)	non-fat milk powder, ddH_2O (5% milk)
Fixative (3:1 methanol: glacial acetic acid)	75% (v/v) methanol 25% (v/v) glacial acetic acid Prepare fresh each time and cool in freezer or in ice-bucket
Freezing media	10% DMSO in complete media prior to use
HCl solution	0.25 M HCl For a 200 cm^2 blot, approximately 250 ml of solution is needed Store at +15 to +25°C
KCl 75mM	279 mg KCl in 50ml of ddH_2O
KCM	120 mM KCl 20 mM NaCl 10 mM Tris·Cl, pH 7.5 0.1% (v/v) Triton X-100 Store up to 1 year at room temperature

Table 3. Buffers and Solutions (continued)

Buffers and Solutions	Composition
LB agar	32 g LB + agar ddH_2O to 1 liter autoclave and store at 4°C
LB medium	20 g LB ddH_2O to 1 liter autoclave and store at room temperature
Neutralization solution	0.5 M Tris-HCl, 3 M NaCl, pH 7.5 For a 200 cm^2 blot, approximately 500 ml of solution is needed Store at +15 to +25°C
PBST (PBS with Tween 20)	Phosphate-buffered saline containing: 0.1% (v/v) Tween 20 Store up to 1 year at room temperature
Phosphate-buffered saline containing 250 μg/ml RNase A	10 ml phosphate-buffered saline (PBS) 125 μL 20 mg/ml RNase A Store at 4°C
PNA hybridization solution	70% (v/v) formamide (deionized) 0.25% (w/v) Blocking Reagent 10 mM Tris·Cl, pH 7.5 Store up to 6 months at −20°C
PNA wash A	70% (v/v) formamide (deionized) 10 mM Tris·Cl, pH 7.5 (Prepare fresh)

Table 3. Buffers and Solutions (continued)

Buffers and Solutions	Composition
PNA wash B	50 mM Tris·Cl, pH 7.5 150 mM NaCl 0.8% (v/v) Tween 20 Store at room temperature
PNA wash buffer	140 ml formamide (deionized) 58 ml deionized distilled water 2 ml 1 M Tris·Cl, pH 7.5 Store up to 1 year at room temperature
Pre-extraction buffer (10×)	0.5% Triton X-100 20 mM HEPES-KOH, pH 7.9 50 mM NaCl 3 mM MgCl2 300 mM sucrose Sterilize by autoclaving and store indefinitely Dilute to 1× with ddH_2O prior to use
TBS buffer (10x)	24 g of Tris base (200 mM) 88 g NaCl pH to 7.6 ddH_2O to 1 litter
TBS buffer (1x)	100 ml 10x TBS buffer ddH_2O to 1 liter

Table 3. Buffers and Solutions (continued)

Buffers and Solutions	Composition
TBS-T	0.05 % Tween®20 1 liter 1x TBS
Transfer Buffer 1x (WB)	20 ml Trans-Blot Turbo 5x Transfer Buffer, 20 ml Ethanol, 60 ml ddH_2O
Tris-acetate-EDTA (TAE) (1x)	160 ml 50x TAE buffer ddH_2O to 5 liters
Tris-acetate-EDTA (TAE) (50x)	242 g Tris-base (2 M) 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA ddH_2O to 1 liter
Washing buffer, 1x	Thaw Washing buffer, 10x conc. and mix homogeneously Dilute an appropriate volume of Washing buffer, 10x conc. 1:10 with autoclaved, ddH_2O Store at +15 to +25°C

Table 4. Instruments

INSTRUMENT	SOURCE	MODEL
Autoclave	Getinge	Getinge HS Lab Steam Sterilizer
Blotting instrument	Bio-Rad	Trans-Blot Turbo Transfer System
Centrifuge (Cell culture)	Beckman Coulter	Avanti J15 Benchtop Centrifuge
Charge-coupled device camera	Hamamatsu	ORCA-Flash4.0LT
CO_2 incubator for cell culture	ThermoFisher Scientific	Heracell VIOS 160i
Dry Bath Incubator Heat Block	ThermoFisher Scientific	Multi-Block Heater
Fluorescence microscope	Nikon	Nikon ECLIPSE Ni fluorescent microscope
Fluorometer	Life Technologies	Qubit 2.0
Gel chamber for SDS-Page	Bio-Rad	Mini-PROTEAN Tetra, Criterion Vertical Electrophoresis Cell
Gel documentation device	Bio-Rad	ChemiDoc Imaging System
Gel System with Built-In Recirculation	ThermoFisher Scientific	Owl EasyCast B3
Incubator for bacteria	Boekel Scientific	Incubator, Digital, 0.8 cf capacity
Incubator with shaking device for bacteria	New Brunswick	Innova 42 - Stackable Incubator Shaker

Table 4. Instruments (continued)

INSTRUMENT	SOURCE	MODEL
Microcentrifuge	ThermoFisher Scientific	Sorvall Legend Micro 21 Microcentrifuge
Microscope	Nikon	Nikon ECLIPSE TS100
Microwave	GE	Countertop Turntable
Multi-Mode Reader	Agilent BioTek	Synergy H1 Hybrid Multi-Mode Reader
Nucleofector	Amaxa/Lonza	Amaxa Nucleofector II/2b
Orbital Shaker	Corning	LSE Orbital Shaker, without platform
pH Meter	Mettler-Toledo	SevenExcellence pH meter S400
Pipet Tip	Olympus Plastics	Reach Ergonomic Filter Pipet Tips 10 µL , 20 µL , 200 µL , 1000 µL
Portable Pipet	Drummond Pipettes	Drummond Portable Pipet Aid XP
Power Supply for agarose gel and western blot	ThermoFisher Scientific	EC 300 XL Power Supply
quantitative real-time PCR instrument	Bio-Rad	CFX96 Real-Time system
Rocking Shaker	Reliable Scientific	11"x14" Double Platform Rocking Shaker

Table 4. Instruments (continued)

INSTRUMENT	SOURCE	MODEL
Safety Cabinet	ULINE	Flammable storage cabinet and Clear-view storage cabinet
Serological Pipets	GenClone Brand	5.0 ml, 10 ml, 25 ml, 50 ml
Single Channel Manual Pipettes	Mettler-Toledo Rainin	Pipet-Lite XLS: 0.1-2 µL, 2-20 µL, 20-200 µL, 100-1000 µL
Small Benchtop Centrifuge	ThermoFisher Scientific	Sorvall ST 8R centrifuge
Spectrophotometer	Molecular Devices	SpectraMax Spectrophotometers for UV-Vis Absorbance Detection
Spectrophotometer	ThermoFisher Scientific	NanoDrop OneC Microvolume UV-Vis Spectrophotometer
Thermocycler	Bio-Rad	T100 Thermo Cycler
Vacuum tool	Argos Technologies	EW-13050-31
Vortexer	Thermo Scientific	LP Vortex Mixer
Water bath	Fisher Scientific	Isotemp
Weighing device	Mettler-Toledo	Bench Scales

Table 5. Software

SOFTWARE	MANUFACTURER	PURPOSE
Adobe Illustrator Version 25.1	Adobe	Scientific illustration
CFX Manager	Bio-Rad	Control console and analyses of qPCR data
GraphPad Prism 9 for macOS Version 9.4.1 (458)	GraphPad Software	Statistical analyses
Gen5 Software Features for Detection	Agilent BioTek	Assays in microplates, Petri and cell culture dishes and cuvettes
ImageJ	https://imagej.nih.gov/ij/ (Schindelin et al., 2012)	Immunofluorescence Imaging Analysis
Image Lab Version 6.0.1 build 34	Bio-Rad	Caption and analyses digital image data from electrophoresis gels and blots
MutEnricher	https://github.com/asoltis/MutEnricher	Analyses of somatic tumor enrichment
NIS Elements Advanced Research software	Nikon Instruments Inc	Immunofluorescence and Telomere Fluorescence in Situ Hybridization (FISH) Analysis
Samtools	http://www.htslib.org/	Depth of coverage analysis of next generation sequencing data

Table 5. Software (continued)

SOFTWARE	MANUFACTURER	PURPOSE
SnapGene Version 6.1.1	GSL Biotech LLC	Analyses of obtained sequences
SoftMax Pro 7 Software	Molecular Devices	Assays in microplates, Petri and cell culture dishes and cuvettes
SRA toolkit	https://hpc.nih.gov/apps/sratoolkit.html	Download of SRA files and conversion to fastq files
STAR 2.5	https://github.com/steve tsa/STAR	Aligner for spliced transcripts
TeloTool	https://github.com/jagoe hring/TeloTool (Göhring et al., 2013)	Telomere length measurement from terminal restriction fragment analysis

Table 6. Online Databases and Algorithms

Name	URL	REFERENCE
The Catalogue of Somatic Mutations In Cancer (COSMIC)	https://cancer.sanger.ac.uk/cosmic	(Tate et al., 2018)
Gene Expression database of Normal and Tumor Tissues 2 (GENT2)	http://gent2.appex.kr/gent2/	(Park, Yoon, Kim, & Kim, 2019)
The International Cancer Genome Consortium Data Portal (ICGC Data Portal)	https://dcc.icgc.org/	(J. Zhang et al., 2019)
OncoDB	http://oncodb.org	(Tang, Cho, & Wang, 2022)
The Cancer Genome Atlas (TCGA) Program	https://www.cancer.gov/tcga	TCGA Research Network
UCSC genome browser	https://genome.ucsc.edu/	(Kent et al., 2002)

3.0 Results

3.1 A Cluster of Somatic Variants in the *TPP1* Promoter is Found in Melanomas

Highly recurrent *TERT* promoter mutations in human tumors suggest telomerase reactivation plays a major role in tumorigenesis. The somatic point mutations at positions 228 (C228T or -124C>T) and 250 (C250T or -146C>T) reactivate telomerase. These mutually exclusive mutations are typically heterozygous in a variety of human tumors including >75% of melanomas (T. Liu, Yuan, & Xu, 2016). Previous studies of these *TERT* promoter mutations have shown that they do not prevent telomere attrition (Chiba et al., 2017b). Therefore, telomeres shorten and cells enter crisis (Chiba et al., 2017a). Melanomas with *TERT* promoter mutations have shorter telomeres than nevi and melanomas with wild-type *TERT* promoters (Chiba et al., 2017a; Hayward et al., 2017). Chiba *et al.*, proposed a two-hit model of immortalization: the first hit is the acquisition of *TERT* promoter mutations that reactivate telomerase activity, which allow the cell to maintain short telomeres after crisis. A second hit is needed to boost telomerase function in order to lengthen telomeres. The second hit is still unknown.

To investigate a novel mechanism of telomere maintenance in melanoma, we analyzed somatic variants across telomere-related genes in the 749 cutaneous melanoma samples in the ICGC (Figure 4). We identified several somatic variants in the six shelterin genes. Two variants located in the 5′ region of the *TPP1* gene occurred at a high frequency, but mutations were random in the other genes.

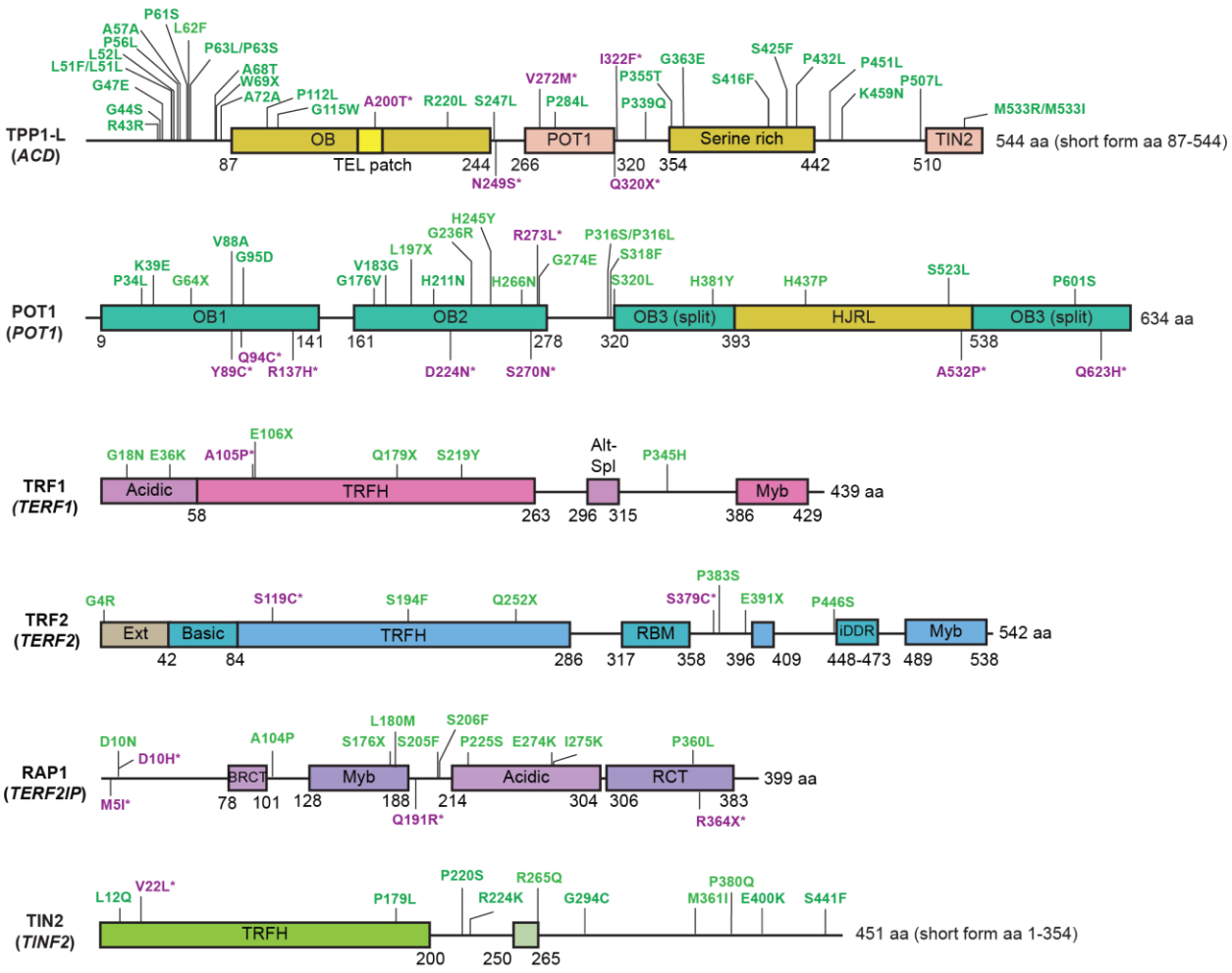


Figure 4. Somatic Variants in Shelterin Components.
Schematic of the six shelterin proteins and the somatic variants from the ICGC data portal. Variants were extracted on March 1, 2022 from exome and genome sequencing data of cutanoeus melanomas (n=749). Variants from the ICGC are annotated in green while a subset of variants reported in previous publications are indicated in purple with an asterisk. The long isoforms of TPP1 and TINF2 are depicted along with several of the previously characterized domains in each of the proteins.

TPP1 has been reported to have two isoforms: TPP1-L (544 aa) and TPP1-S (458 aa, initiates at Met87 of TPP1-L). TPP1-S is conserved in humans and other species, but TPP1-L has only been identified in male germ cells as a transcript mapping to N-terminus region of TPP1 (Sherilyn Grill et al., 2019). The two recurrent somatic variants were C>T transitions located 75 and 108 base pairs upstream of the translational start site for TPP1-S (-75 C>T and -108 C>T in

non-coding variants or p.L62F and p.L51F for TPP1-L in coding variants). These variants co-localize with histone marks typically associated with promoters (Figure 5A).

We asked whether the cluster of somatic variants in TPP1 are coding variants in TPP1-L or promoter variants in TPP1-S. To differentiate between these two possibilities, we cloned four different TPP1 plasmid constructs and incorporated a C-terminal FLAG-tag: 1) a TPP1 genomic plasmid construct that contains the entire genomic region of TPP1 (3.5 kilobases including 895 base pairs upstream of the TPP1-S translational start site, 637 base pairs upstream of the TPP1-L translation start site, and all exons and introns of both isoforms), 2) TPP1-S cDNA plasmid expressing only TPP1-S, 3) TPP1-L cDNA plasmid expressing TPP1-L and TPP1-S, and 4) TPP1-L (M87A) cDNA plasmid expressing only TPP1-L (Sherilyn Grill et al., 2019). We used TPP1-S, TPP1-L, and TPP1-L (M87A) as controls. Immunofluorescent staining confirmed that the C-terminally tagged TPP1 colocalized with TRF2 at telomeres (Figure 5B). Western blots 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 melanoma cell lines LOX and MEL624 (Figure 5C). Taken together, these results support the conclusion that the cluster of TPP1 variants we identified are localized to the promoter region of TPP1-S.

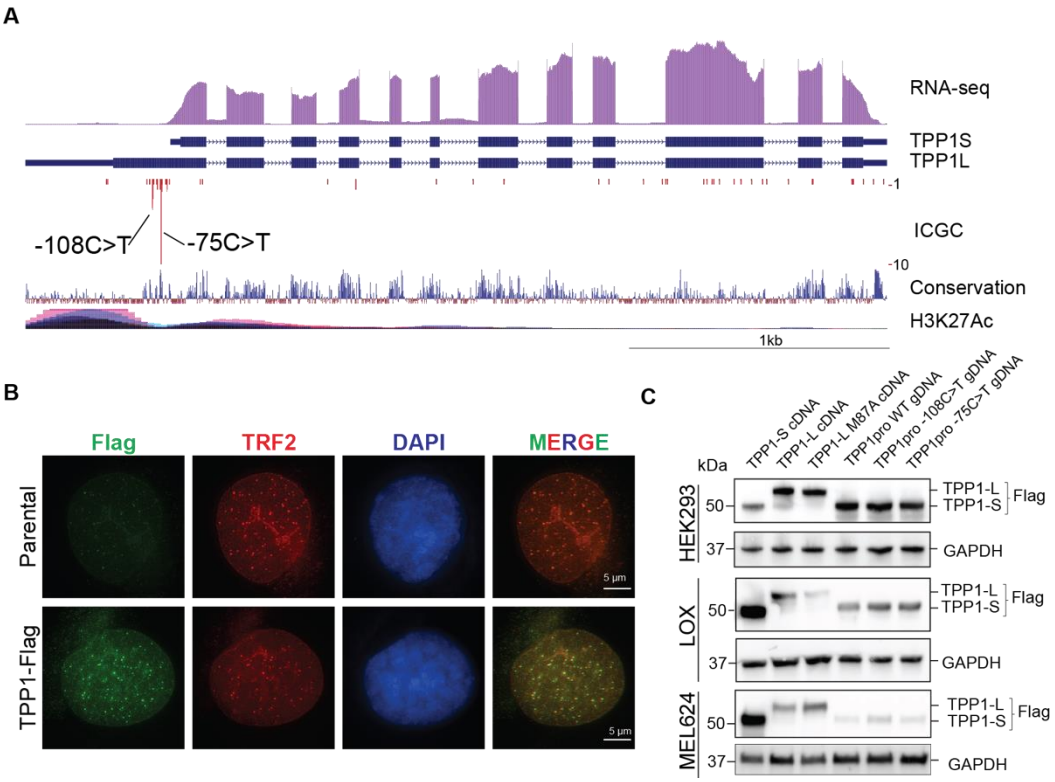


Figure 5. Identification of a Cluster of Somatic Promoter Variants in *TPP1*.
(A) The genomic locus of the ACD gene is depicted with dark blue rectangles indicating the exons for TPP1-S and TPP1-L respectively (UCSC genome browser). The red bars below the gene tract show the location of the somatic variants (ICGC database). The size of the bars corresponds 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 cell lines stably 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 blots of HEK293, LOX, and MEL624 cells transfected with plasmids encoding the cDNAs for TPP1-S and TPP1-L, and TPP1-L-M87A (incapable of expressing TPP1-S), and plasmids expressing the entire genomic locus of TPP1 with and without the most common promoter variants.

To determine which isoform of TPP1 exists in melanoma cells, we analyzed RNA-seq data from 12 melanoma cell lines and 61 micro-dissected nevi and melanoma samples (RNA-seq data have been deposited in the NCBI Gene Expression Omnibus under GEO: GSE153592 and GSE112509) (Manfred Kunz et al., 2018; Motwani et al., 2021). We investigated the historical annotations of the *TPP1* gene from RefSeq and GENCODE along with RNA Annotation and Mapping of Promoters for the Analysis of Gene Expression (RAMPAGE) and Cap Analysis Gene

Expression (CAGE) data from GENCODE. We found that TPP1 was annotated as both the long isoform and the short isoform. In a 2019 analysis, only the short TPP1 isoform was annotated (light blue and dark blue panels in Figure 6). Taken together, TPP1-S is the only isoform expressed in melanomas (top panel in Figure 6), and the cluster of somatic variants is located in the highly conserved regions of the *TPP1* promoter (bottom panels in Figure 6).

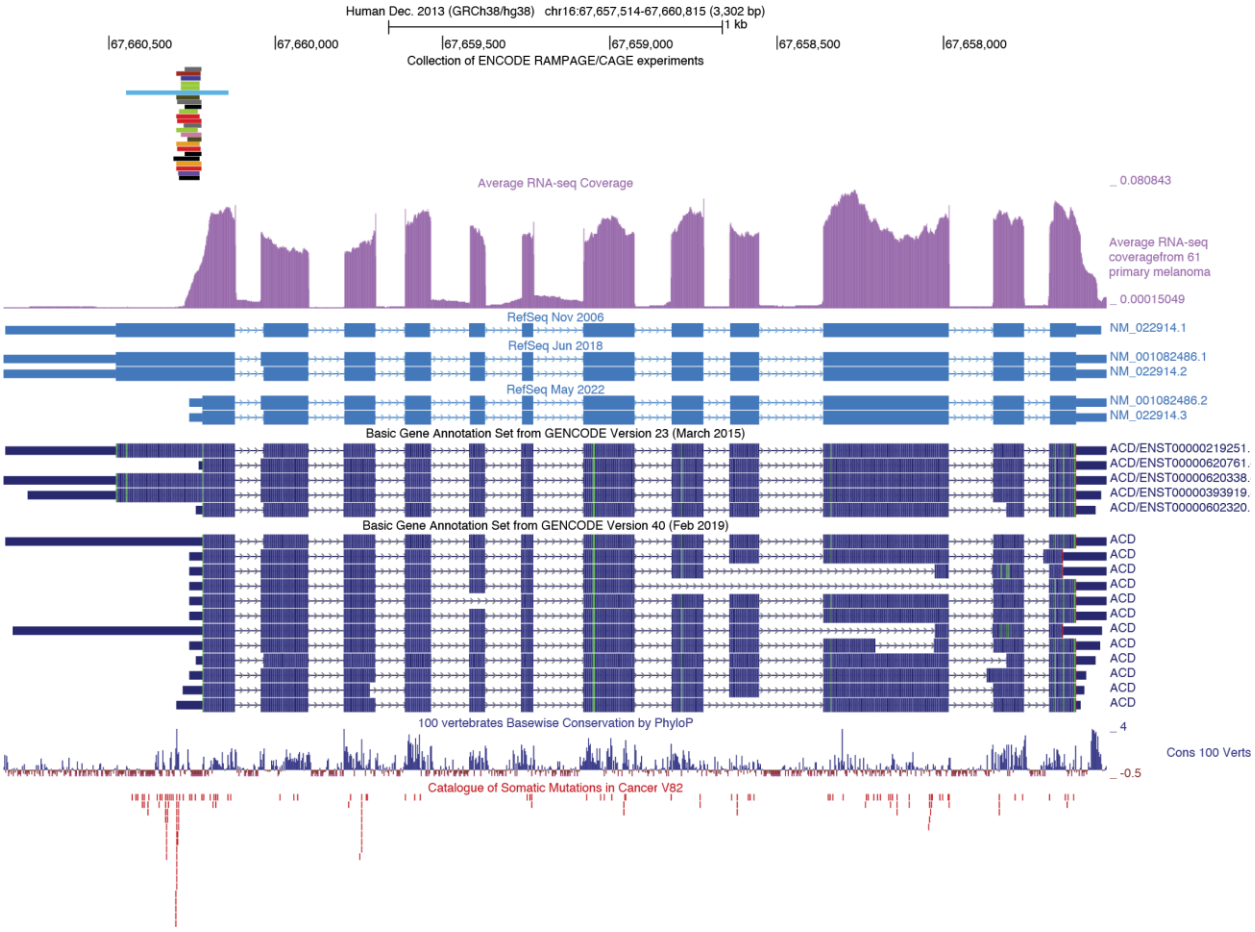


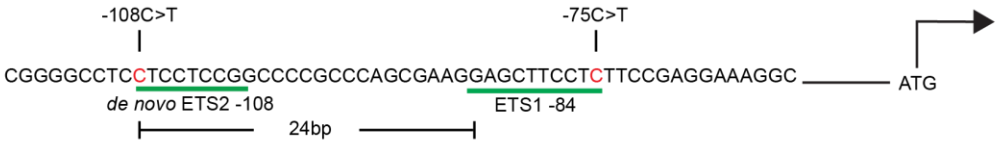
Figure 6. Historical Annotations of the *TPP1* Gene.
Screen capture from the UCSC genome browser showing the historic annotation of TPP1 from RefSeq and GENCODE. RNA-seq coverage from 61 primary nevi and melanoma samples demonstrates the short isoform is expressed in melanoma. RAMPAGE/CAGE data from 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 are shown in the bottom panels.

3.2 *De Novo* Transcription Factor Binding Sites Created by *TPP1* Promoter Variants

To begin to understand the potential consequences of the cluster of variants we identified, we analyzed the sequences of the wild type and variant promoters using the TRANSFAC database. TRANSFAC contains eukaryotic DNA sequence motifs and the transcription factors that bind to and act through these motifs (Wingender, Dietze, Karas, & Knüppel, 1996). We performed the analyses as a screening tool for transcription factor binding sites created by the *TPP1* promoter variants. We used the PROMO-ALGGEN software (version 8.3 of TRANSFAC) (Farré et al., 2003; Messeguer et al., 2002) to identify the predicted transcription factor binding motifs (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) in the TPP1 promoter and the two most common variants: -75 C>T and -108 C>T. The novel variants created *de novo* ETS transcription factor binding motifs (Figure 7) that are similar to those created by the *TERT* promoter mutations, where mutations also created *de novo* ETS transcription factors binding motifs (Horn et al., 2013). The precise sequences created by the variants are distinct. The -108 C>T variant creates *de novo* ETS1, ETS2, and ELK1 binding sites (Figure 7A-B), which harbor 5′TTCC3′, the core binding sequence for ETS transcription factors. The -75 C>T variant creates a *de novo* TFIID binding site (Figure 7C), which is adjacent to an existing ETS site in the context of a sequence that is enriched for mutations in melanoma (Fredriksson et al., 2017) and co-localizes with the annotated transcriptional start site for TPP1-S mRNA (Figure 6).

These analyses suggest that mutations 75 bp and 108 bp upstream of the ATG translational start site of TPP1-S could promote transcription via the creation of binding motifs for ETS and TFIID (Figure 7D).

A



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

Native and *de novo* transcription binding motifs

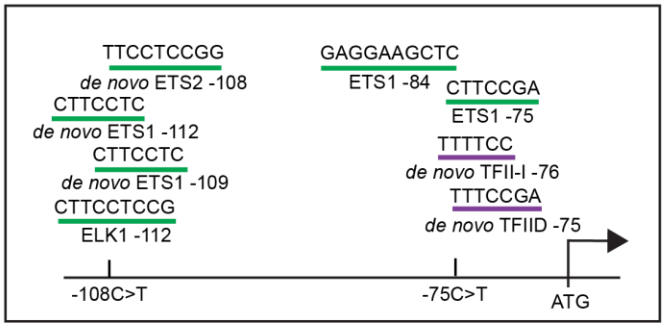


Figure 7. Recurrent Variants in the TPP1 Promoter Create ETS Transcription Factor Binding Sites.
(A) Schematic of the *TPP1* promoter showing the sequence context and location of the most common variants and their positions relative to the TPP1-S translational start site. (B-D) Promoter analysis using PROMO shows that the -108 variant creates a core ETS binding site while the -75 variant modifies an existing ETS transcription factor binding site. The -75 variant falls in the region of the annotated transcriptional start site for TPP1-S and creates several TFIID binding motifs (purple).

3.3 *TPP1* Promoter Variants Require ETS Transcription Factors for Transcriptional Activation

We sought to determine the functional consequences of the somatic variants in the promoter of *TPP1*, specifically, whether *de novo* ETS binding motifs could activate *TPP1* transcriptional activity. We analyzed *TPP1* gene expression from two large cancer databases, including those cancers with a high frequency of *TERT* promoter mutations, and found significantly increased TPP1 expression in skin cancer, bladder cancer, and liver cancer compared to their normal cells (Figure 8A-B).

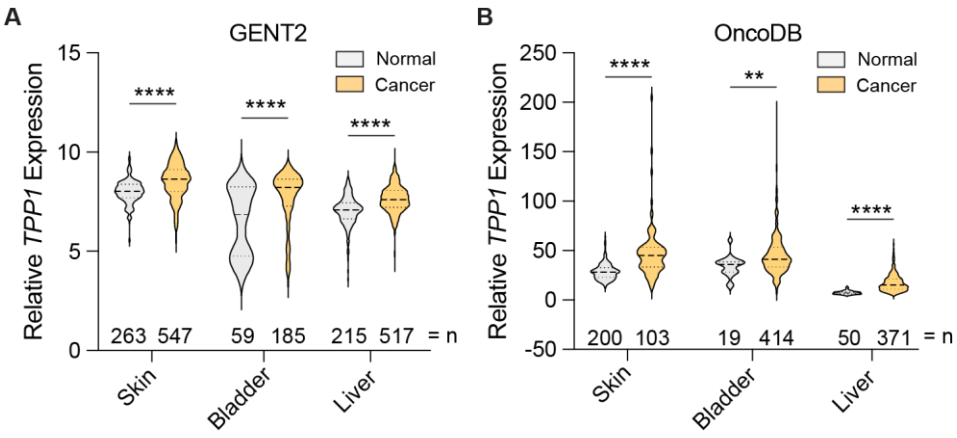


Figure 8. *TPP1* Expression is Elevated in Several Cancers.
(A) Gene expression data from the Gene Expression database of Normal and Tumor Tissues 2 (GENT2). (B) Normal and tumor gene expression data from OncoDB. Numbers below each of the violin plots indicate the number of samples included. These two database were selected because their differing methodologies decreased the probability of including overlapping datasets. GENT2 uses exclusively microarray data whereas OncoDB uses RNA-seq data. Mann-Whitney test was used to compare each of the groups of samples. **P < 0.01 and ****P < 0.0001.

To further investigate *TPP1* promoter variants, we tested progressively smaller fragments of the *TPP1* proximal promoter in luciferase assays to determine the minimum fragment required to recapitulate wild-type *TPP1* transcriptional activation. We found a 285 bp fragment is sufficient

for full basal transcriptional activity (Figure 9A). We next tested the *TERT* promoter mutations in luciferase reporter assays as positive controls for the *TPP1* promoter variants experiments. The *TERT* promoter -124 C>T mutation significantly increases luciferase activity compared to the wild-type *TERT* promoter as previously reported (Figure 9B) (R. J. Bell et al., 2015; Huang et al., 2013). Next, we introduced the -75 C>T or -108 C>T variants into the minimal 285 bp *TPP1* promoter fragment. We found little effect on luciferase expression in HEK293 cells but saw a small but significant increase in luciferase expression in the melanoma cells lines LOX and MEL624 (Figure 10A).

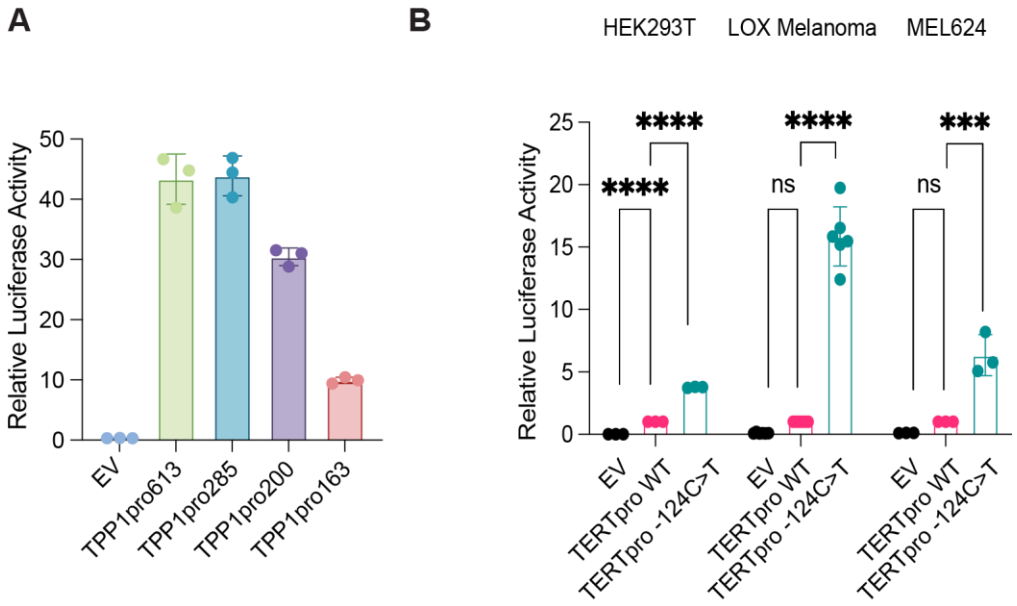


Figure 9. Luciferase Activity of *TPP1* Promoter Mutations and *TERT* Promoter Mutations.
(A) Luciferase reporter constructs with specific fragments of the *TPP1* proximal promoter containing 613, 285, 200, and 163 base pairs of the proximal promoter relative to the TPP1-S translational start site. The 285 base pair fragment was the minimal sequence sufficient for full basal activity of the reporter. The mean activity and standard deviation from three independent experiments are shown. (B) Luciferase assays were performed with a 163 base pair fragment of the *TERT* proximal promoter in melanoma and non-melanoma cell lines. The *TERT* promoter mutations have significant effects on the transcriptional activity in both non-melanoma cells lines (HEK293) and two melanoma derived lines (LOX and MEL624). Abbreviations: EV, empty vector; *TERT*pro, *TERT* promoter; WT, wild-type.

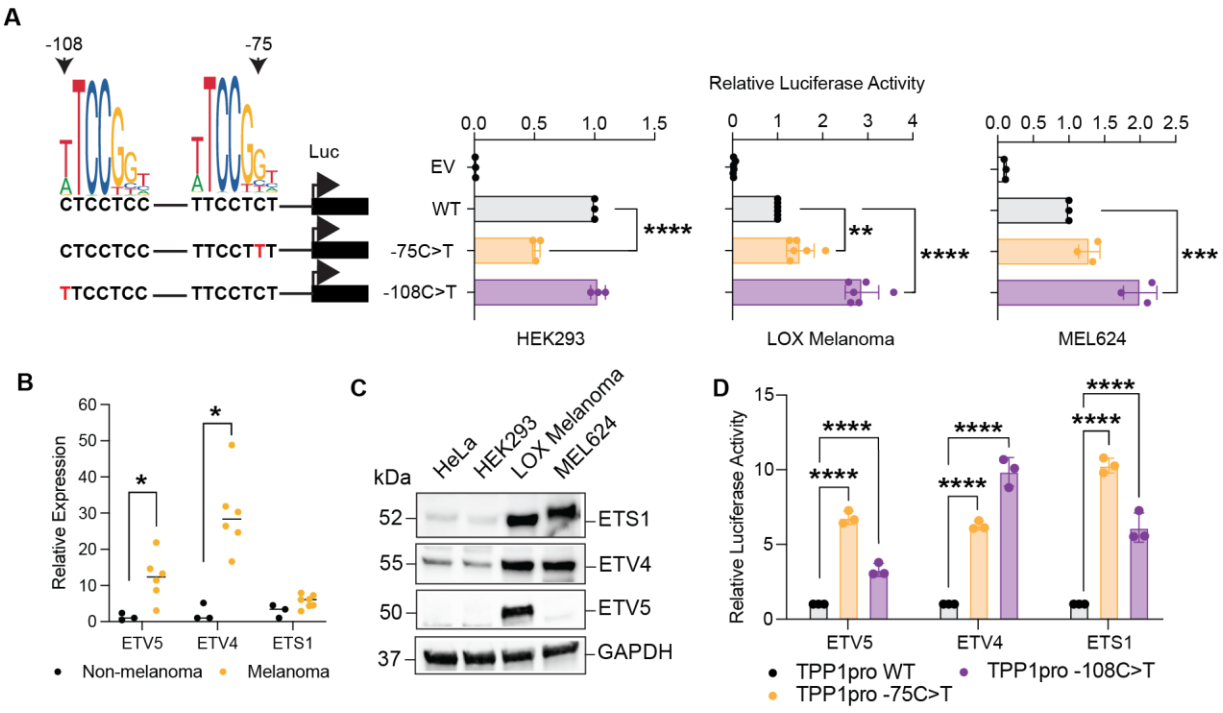


Figure 10. ETS Transcription Factors Activate the Variant *TPP1* Promoters.
(A) Luciferase assays were performed with a 285 base pair fragment of the *TPP1* proximal promoter in melanoma and non-melanoma cell lines. The *TPP1* promoter variants had little effect on the transcriptional activity in a non-melanoma cells line (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). Median values are shown and groups were compared using Mann-Whitney test. (C) Western blot shows high expression of ETS transcription factors in the melanoma cell lines LOX and MEL624. (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 expressing one of the three ETS transcription factors indicated. 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.

We asked what transcription factors in melanoma cells are differentially expressed versus non-melanoma cells and are required for *TPP1* transcriptional activity. Analysis of the gene expression of 27 ETS family members in 426 melanoma samples from the ICGC revealed the abundant expression of ETS1, ETV4, and ETV5 (Figure 11). To validate the ETS gene expression in melanomas, we performed the quantitative PCR (qPCR) and Western blotting on the non-melanoma cell lines (HeLa, BJ fibroblasts, and HEK293) and several melanoma cell lines derived by short-term primary culture. We found relatively higher gene expression of ETV5, ETV4, and

ETV1 transcripts and greater expression of ETS proteins in melanoma cells compared to non-melanoma cells (Figure 9B-C).

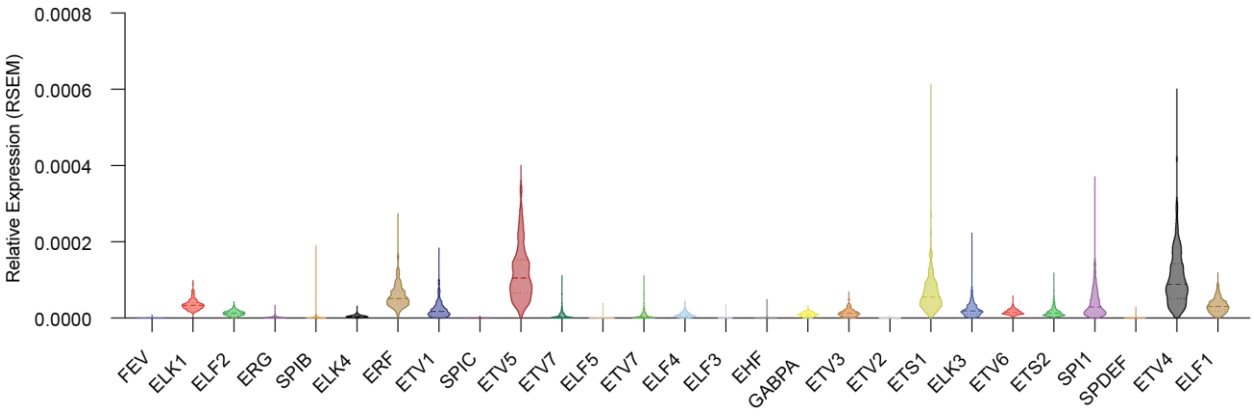


Figure 11. Expression of ETS Transcription Factors in Melanomas.
RNA-seq data was obtained from 426 melanoma samples from the ICGC data portal. Relative expression of 27 ETS family members is shown.

To determine the role of the *de novo* ETS transcription factors binding motifs generated by *TPP1* promoter variants, we conducted luciferase reporter assays using the *TPP1* promoter in HEK293 with the expression of three ETS factor candidates: ETV5, ETV4, and ETS1. Expression of the ETS factors significantly increased luciferase expression of the *TPP1* promoter variants in HEK293, similar to the luciferase expression seen in LOX and MEL624 cells (Figure 10D). Taken together, these data suggest that the *TPP1* promoter variants are activated by ETS transcription factors, which are abundantly expressed in melanomas.

3.4 TPP1-S Overexpression is Sufficient to Cause Telomere Elongation

TPP1 regulates telomere lengthening by recruiting telomerase to chromosomal ends and increasing telomerase processivity, which is facilitated by the OB-fold domain of TPP1 via its

interaction with POT1 (Jayakrishnan Nandakumar et al., 2012; F. Wang et al., 2007; Franklin L. Zhong et al., 2012). Both isoforms of TPP1 localize to the telomeres. They are able to recruit telomerase to telomeres. However, only the overexpression of TPP1-S induces hyper-elongation of telomeres (Sherilyn Grill et al., 2019). To examine the effect of TPP1 overexpression on telomere length, we generated HeLa stable cell lines overexpressing TPP1-S, TPP1-L WT, and variants using the Flp-In system.

First, we examined if coding variants in the N-terminus of TPP1-L alter its ability to regulate telomere length. We engineered stable cell lines to express either C-terminal FLAG-TPP1-L (59 kD, aa 1-544) or C-terminal FLAG-TPP1-S (50 kD, aa 87-544) and compared expression of the transgenes in the two sets of cell lines. Individual clones were propagated for 90 days. Immunoblots from lysates prepared 35 and 63 days post-transfection showed consistent and stable transgene expression over the selected time points. TPP1-S appeared to be more abundant in cells than TPP1-L.

Immunoblots showed weaker TPP1-L signals than TPP1-S signals (Figure 12A). To dissect this issue, we asked whether TPP1-L is unstable due to ubiquitin mediated proteasome degradation, decreased expression, or protein mis-folding. The TPP1 protein is degraded in a proteasome-dependent manner (Zemp & Lingner, 2014). To verify that TPP1 is degraded via the proteasome, we determined the protein expression upon proteasome inhibition. HeLa cells stably expressing TPP1-S or TPP-L were treated with the proteasome inhibitor MG132 (20 μM) for 6 hours, which had no effects on protein levels (Figure 12 B-C).

Typically, cycloheximide (CHX) treatment blocks protein translation, so we are able to measure protein half-life without interference from new protein synthesis. To determine whether TPP1-L expression is regulated by proteasome-dependent degradation we performed a time course

experiment in HeLa cells stably expressing TPP1-S or TPP-L. We treated cells with DMSO (negative control) or MG132 (5 μM) followed by the addition of CHX (100 μg/ml). Analysis of TPP1-S- and TPP1-L-2xFlag protein levels over 24 hours revealed that TPP1-L cannot be rescued by proteasome inhibitor treatment (Figure 12D-I) suggesting that unstable TPP1-L is caused by a complex mechanism. It may be destabilized relative to TPP1-S, translated less efficiently or degraded by proteasome-independent degradation.

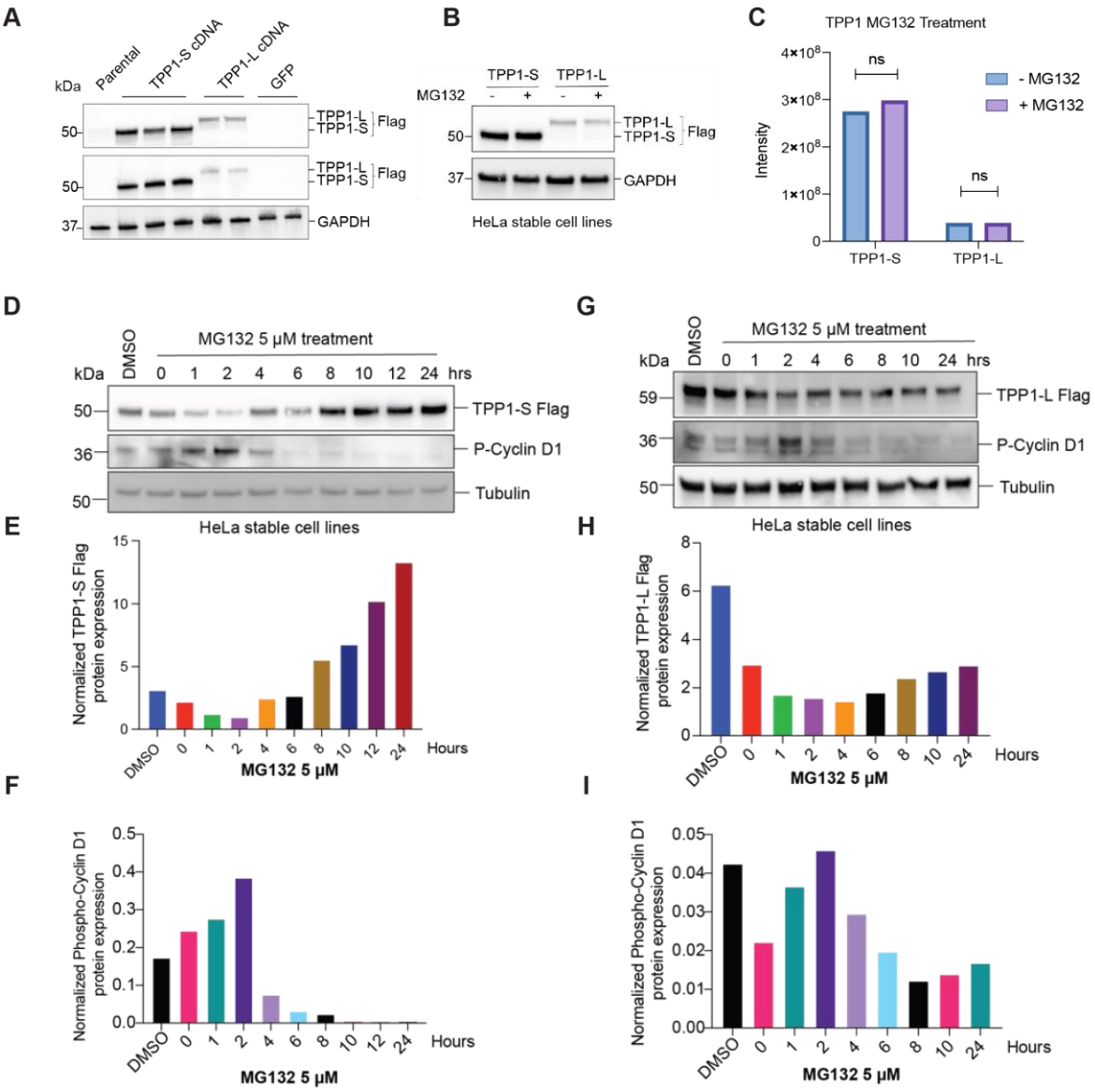


Figure 12. The TPP1-L Protein is Unstable and Cannot be Rescued by Treatment with a Proteasome Inhibitor.

(A) Western blotting of FLAG and GAPDH from lysates extracted from HeLa cell lines stably expressing TPP1-S and TPP1-L. (B) Western blotting for TPP1-S and TPP1-L in cells treated with MG132 (20 μM) for 6 hours. (C) The graph shows the protein expression levels pictured in Fig.11B. (D) and (G) Time course analysis of HeLa cells stably expressing TPP-S- or TPP1-L 2xFlag treated with cycloheximide (CHX 100 μg/ml) and MG132 (5 μM) or solvent (DMSO). TPP1 protein levels were analyzed by Western blotting at the indicated time points. (E) and (H) Western blot analysis and quantification of TPP1 from separate experiments are shown. (F) and (I) Western blot analysis and quantification of Phosphocyclin-D1 (positive control for MG132 treatment) from separate experiments are shown.

I next examined the effects of the stable expression of TPP1-S and TPP1-L on telomere lengthening. I collected cell lines at day 90 of propagation and performed telomere restriction

fragment (TRF) Southern blot analysis. Overexpression of TPP1-S elongated telomeres but overexpression of TPP1-L significantly shortened telomeres, consistent with a previous report (Sherilyn Grill et al., 2019) (Figure 13A, 13D). Interestingly, overexpression of TPP1-L wild-type or the variants in long-term culture had no impact on telomere length (Figure 13B, 13C, 13F). These data confirm that increased expression of TPP1-S can lead to telomere lengthening in telomerase-expressing cells.

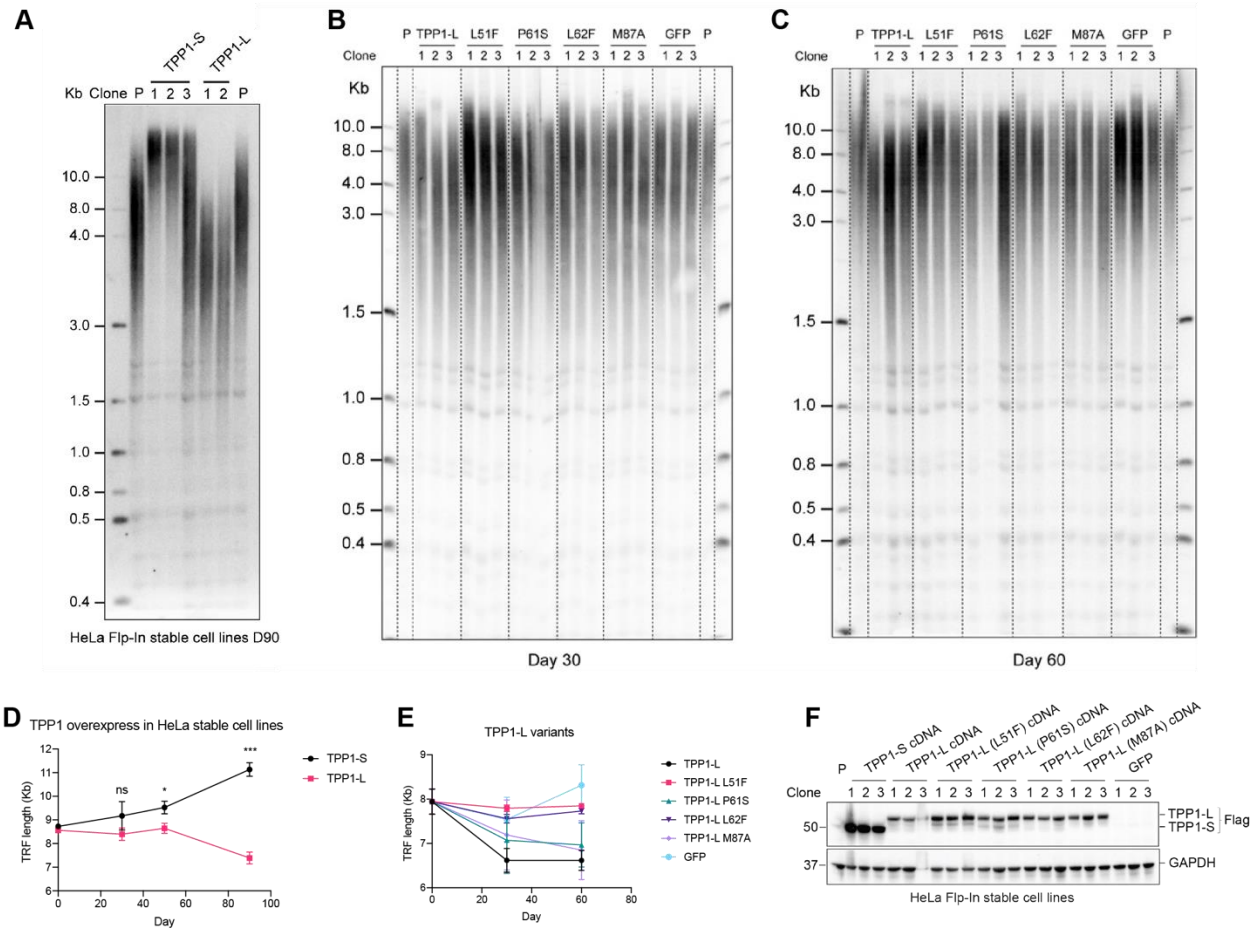


Figure 13. Overexpression of TPP1-S, but not TPP1-L, Causes Telomere Lengthening.
(A) Southern blot of telomeres in HeLa cell lines stably expressing TPP1-S or TPP1-L for 90 days. Two independent clones of each are shown. P – parental cell line used to establish each of the modified clones. (B) and (C) Southern blots of telomeres in HeLa cell lines stably expressing TPP1-L and coding variants for 30 days and 60 days, respectively. (D) and (E) Plots of the mean TRF length of parental, TPP1-S, and TPP1-L cell lines from (B) and (C) respectively. (F) Western blotting for FLAG and GAPDH of lysates from TPP1-S, TPP1-L wild-type and variants extracted from HeLa stable cell lines.

3.5 TERT and TPP1 Act Synergistically to Lengthen Telomeres

Human fibroblasts in culture have been used extensively as a model of cellular senescence due to their lack of telomerase activity. Human fibroblasts gradually lose telomere length over time until they hit a critical threshold that triggers senescence (Harley, Futcher, & Greider, 1990). To determine the role of TPP1 in cell proliferation, we asked whether the overexpression of TPP1-S could extend the proliferative capacity of cells that express limiting amounts of telomerase (undetectable telomerase activity in fibroblasts (Kim et al., 1994)). Using lentiviral transduction, we introduced TPP1, TERT, or both into primary BJ fibroblasts to generate stable clones. We monitored their proliferative capacity for 90 days. Western blotting of the lysates from three independent clones of transduced BJ fibroblasts detected expression of TPP1 and TERT proteins (Figure 14B). In proliferation assays, control (untransduced) fibroblasts and fibroblasts transduced with TPP1 underwent replicative senescence after 40 days. Clones expressing TERT or TERT and TPP1 overcame replicative senescence and proliferated longer than the untransduced cells (Figure 14A). Our findings are consistent with a previous report that TERT overexpression is sufficient to immortalize primary fibroblasts (Bodnar et al., 1998).

Next, we examined the impact of TPP1 overexpression on telomere length in BJ fibroblasts. In control and TPP1-transduced fibroblasts, telomere length was heterogenous with a median length of about 6 kb after 15 passages (Figure 14C). Introduction of TERT alone led to telomere lengthening, which is also consistent with a previous report (Bodnar et al., 1998). However, Chiba *et al.* presented conflicting results in melanoma samples and a fibroblast model. They demonstrated *TERT* promoter mutations promote cellular immortalization in two-steps: an initial phase in which *TERT* promoter mutations cannot prevent bulk telomere shortening, but extend the cellular life span with healing of the shortest telomeres, and a second phase in which

an unknown factor up-regulates telomerase activity and melanoma cells become fully immortal (Chiba et al., 2017a). We found that co-expression of TERT together with TPP1 caused a synergistic effect on telomere lengthening (Figure 14C). Our results suggest TPP1 is one of the missing factors that co-operates with TERT to drive telomere elongation and allows cells to become fully immortalized.

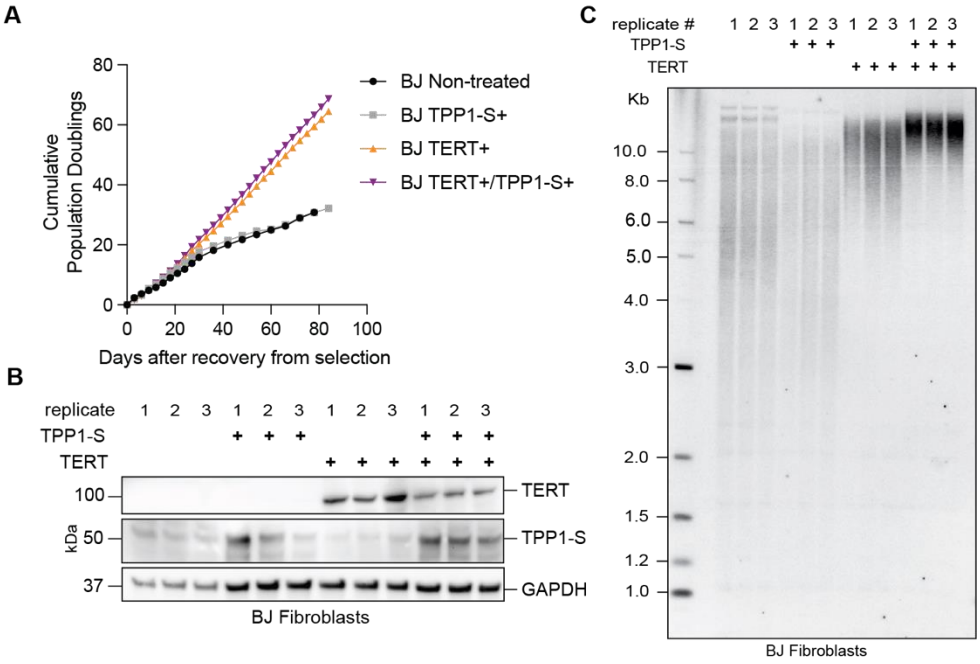


Figure 14. TPP1-S Overexpression is Synergistic with TERT Overexpression to Cause Telomere Lengthening.
(A) Growth curves of cumulative population doublings of BJ fibroblasts expressing TPP1-S or TERT (average of three independent transductions for each group). (B) Western blots showing expression of each of the transgenes in cells collected from (A). (C) Southern blot of telomere lengths of BJ fibroblasts in (B) 15 passages after transduction showing synergistic telomere lengthening in cells with exogenous TPP1 and TERT overexpression. *TPP1* promoter variants synergize with hTERT to increase telomere repeats.

3.6 *TPP1* Promoter Mutations Synergize with *TERT* to Increase the Addition of Telomere Repeats in Melanoma Cells

We showed that TPP1 and TERT overexpression co-operate to elongate telomeres and immortalize BJ fibroblasts beyond TERT overexpression alone. We next asked whether TPP1 and TERT overexpression are sufficient to increase *de novo* telomeric sequences. We introduced the two most common variants (-75 C>T and -108 C>T) into MEL624 and LOX cells (Figure 15A) via CRISPR/Cas-9 mediated genome editing. Six clones of LOX and MEL624 were positive for EarI (uncleaved in -75 C>T) and BseRI (uncleaved in -108 C>T) restriction enzyme screening (Figure 15B). Sanger sequencing was used to verify the presence of the mutations (Figure 15C). We examined *TPP1* gene expression after modifying the endogenous promoter with the -75 or -108 variants. Both *TPP1* promoter variants significantly increase expression of *TPP1* in LOX and MEL264 cells (Figure 16). The modified endogenous promoter (Figure 17A) showed greater increases in *TPP1* expression compared to the increases seen in luciferase assays (Figure 10A), which suggests that additional factors may contribute to *TPP1* expression in the context of melanoma.

We asked whether TPP1 expression is able to induce changes in telomere length. We were unable to detect any changes in telomere length via telomere restriction fragments (TRF) on a Southern blot due to the extremely long telomeres in MEL624 and LOX cells (>20 kb).

Instead, we expressed a telomerase RNA encoding a variant telomeric repeat sequence: 5′TTAGGT3′ (a wild-type telomeric repeat sequence is 5′TTAGGG3′), which can be incorporated into telomeres and localized with a peptide nucleic acid fluorescent probe. The percentage of telomeres with variant repeats was detected by FISH (Figure 16B). Melanoma cells with a modified *TPP1* promoter incorporated significantly more 5′TTAGGT3′ variant repeat sequences

on telomeres (Figure 16C-16E). These findings confirm that *TPP1* promoter variants synergize with *hTERT* to increase the addition of telomeric repeats in melanoma cells.

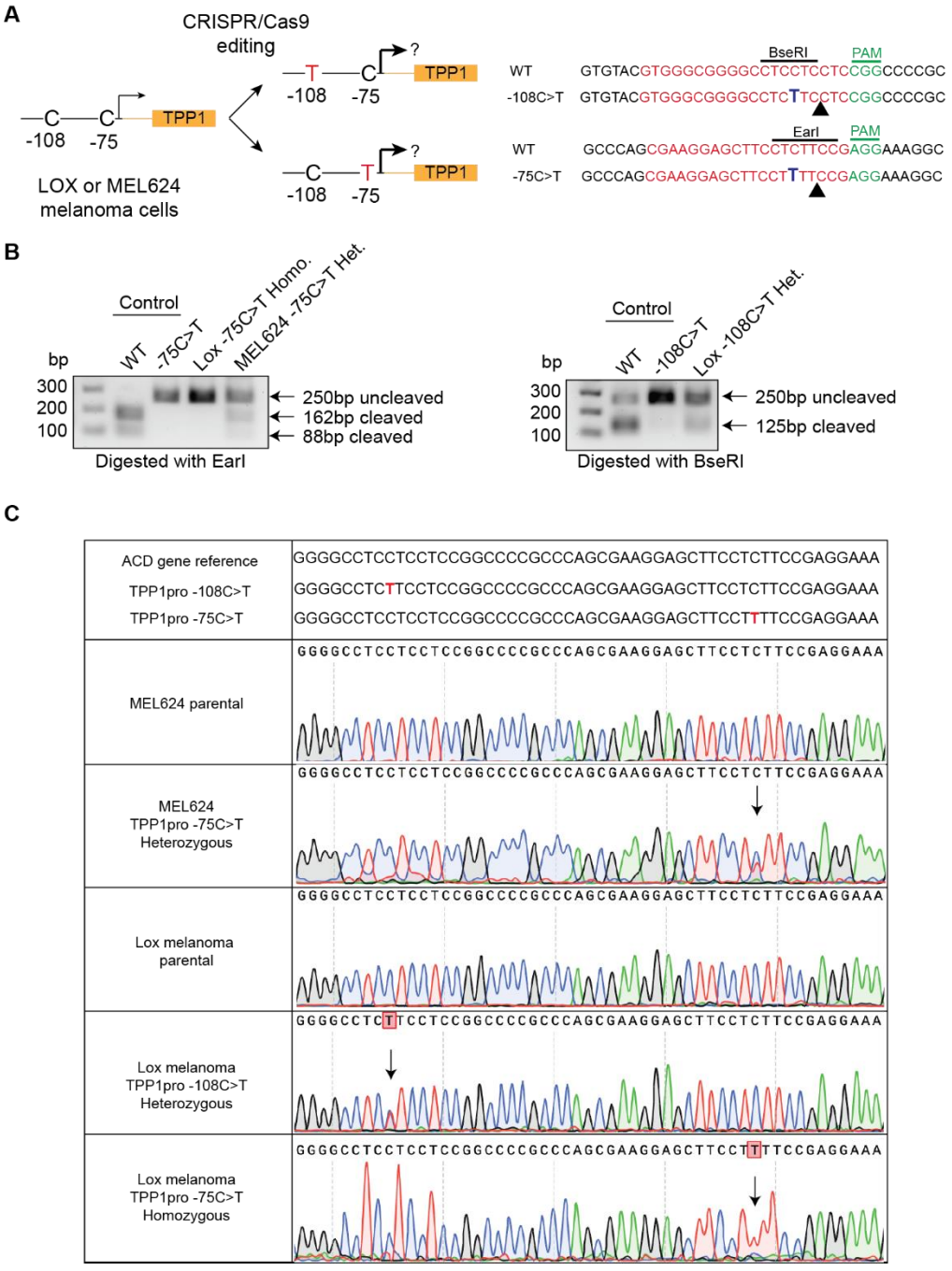


Figure 15. Verification of Genome Editing of Melanoma Cell Lines.
(A) Schematic of the experimental approach for CRISPR/Cas9 genome editing in LOX and MEL624 cells. Sequences used for targeting (crRNAs, sense strand) are shown (red) in the context of the targeted nucleotides (blue) and the protospacer adjacent motif (PAM, green). (B) Representative restriction digests of screening PCRs performed on

successfully edited clones. (C) Representative chromatograms showing sequence verification of the indicated modified melanoma cell lines. Cas9, CRISPR-Associated Protein 9.

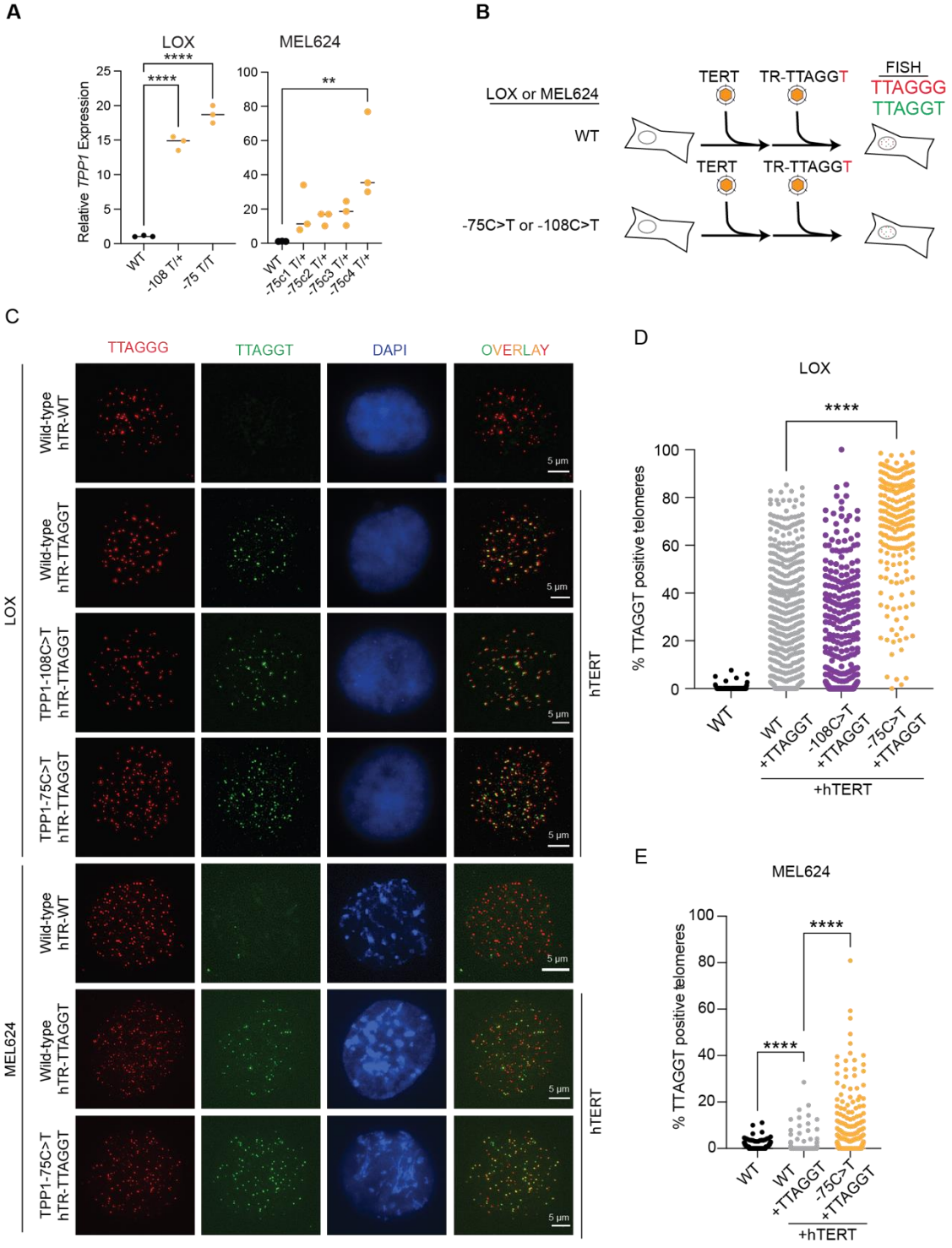


Figure 16. *TPP1* Promoter Mutations Increase Expression of the Endogenous Transcript.

(A) Quantitative PCR of *TPP1* expression following the introduction of promoter mutations in LOX and MEL624 cells. Labels below the graph indicate the presumed zygosity based on sequencing. The median of three independent measurements from each clone is shown 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 5′TTAGGT3′), cells are passaged and the canonical and variant telomeres are quantitated. (C) Fluorescent in situ hybridization for the WT (5′TTAGGG3′; red) and variant (5′TTAGGT3′; green) in parental or genome edited LOX and MEL624 cells. Images were taken 7 days after transduction with lentiviruses. (D) and (E) Quantitation of the fraction of telomeres that had both 5′TTAGGG3′ and 5′TTAGGT3′ signals from a single clone in LOX and MEL624, respectively. Groups were compared using ANOVA with Dunnett's correction for multiple comparison. **P<0.01 and ****P < 0.0001.

3.7 Co-occurrence of Somatic *TERT* and *TPP1* Promoter Mutations in Cancer

The mutually exclusive nature of *TERT* promoter mutations was identified in melanomas and other cancers (Huang et al., 2013; Patrick J. Killela et al., 2013; X. Liu et al., 2013; João Vinagre et al., 2013). These *TERT* promoter mutations are the most common mutations in melanoma (about 75%) and were first described in familial and sporadic melanomas (Horn et al., 2013). These mutations were reported to increase the expression of *TERT* and enable telomere maintenance in somatic cells. We sought to determine the frequency of the *TPP1* promoter variants and the *TERT* promoter mutations in melanomas and whether they are mutually exclusive or co-occur. First, we sequenced the promoters of *TERT* and *TPP1* from 19 available lines. Seventeen melanoma cell lines harbored *TERT* promoter mutations: seven cell lines carried -124 C>T (36.84%), eight cell lines carried -146 C>T (42.10%) mutations, and two cell lines carried other *TERT* promoter mutations (10.53%). Two cell lines harbored a wild-type *TERT* promoter (10.53%) (Figure 17A).

We next analyzed the TPP1 promoter. Only one cell line harbored the -106 C>T *TPP1* promoter (5.26%) and eighteen cell lines carried a wild-type *TPP1* promoter (94.74%) (Figure 17B). Interestingly, we found the co-occurrence of *TERT* and *TPP1* promoter mutations in one

melanoma cell line from the total of 19 melanoma cell lines (Figure 17C). The high frequency of *TERT* promoter mutations suggests that focusing on cell lines may bias our results toward *TERT* promoter mutations.

Therefore, we focused on clinical samples derived from a large collection of primary and metastatic tumors. Pathologists identified the tumor regions from paraffin-embedded blocks. We micro-dissected the tumors from slides, isolated genomic DNA, amplified the *TERT* and *TPP1* promoter regions and verified the *TERT*- and *TPP1*- promoter sequences by Sanger sequencing. Unfortunately, tissue fixation with formalin adversely impacted the quality of the genomic DNA. We assayed the gDNA using nested PCR and all samples contained the same mutation position. We hypothesized that the high number of running cycles (45x) may have contributed to the unusual results. We were unable to interpret the data (data not shown here).

Due to the impact of the co-occurrence of the *TERT* and *TPP1* mutations in melanomas, we were curious to understand their frequency in other cancers. We next investigated the co-occurrence of somatic *TERT* and *TPP1* promoter mutations in cancer from the COSMIC somatic variants. *TPP1* promoter mutations have been primarily found in melanomas but are seen with lower frequency in other tumor types (Figure 18). The rationale for the disproportional number of variants in melanomas is unknown but may be related to the high mutation burden and reliance on telomerase activation in this cancer.

We analyzed WGS in 139 cutaneous melanoma samples with high coverage depth 85× (Hayward et al., 2017) for the frequency of *TERT* promoter mutations and *TPP1* promoter variants. As previously reported, we found *TERT* promoter mutations in 83% of the samples and *TPP1* promoter variants in eight samples (6%) (Horn et al., 2013; Huang et al., 2013). Interestingly, seven of eight samples carried both a *TERT* promoter mutation and a *TPP1* promoter variants and

the remaining sample that carried only a *TPP1* promoter variant without *TERT* promoter mutations (Figure 19). Due to the low depth of coverage in current WGS data sets, we suspect that they may have underestimated the *TERT* promoter mutations. Low coverage data may impact both *TERT* and *TPP1* promoter sequencing data from those samples. Additional studies using targeted resequencing will be required to determine the frequency of *TPP1*- and *TERT*- promoter mutations in cancers other than melanoma in the future.

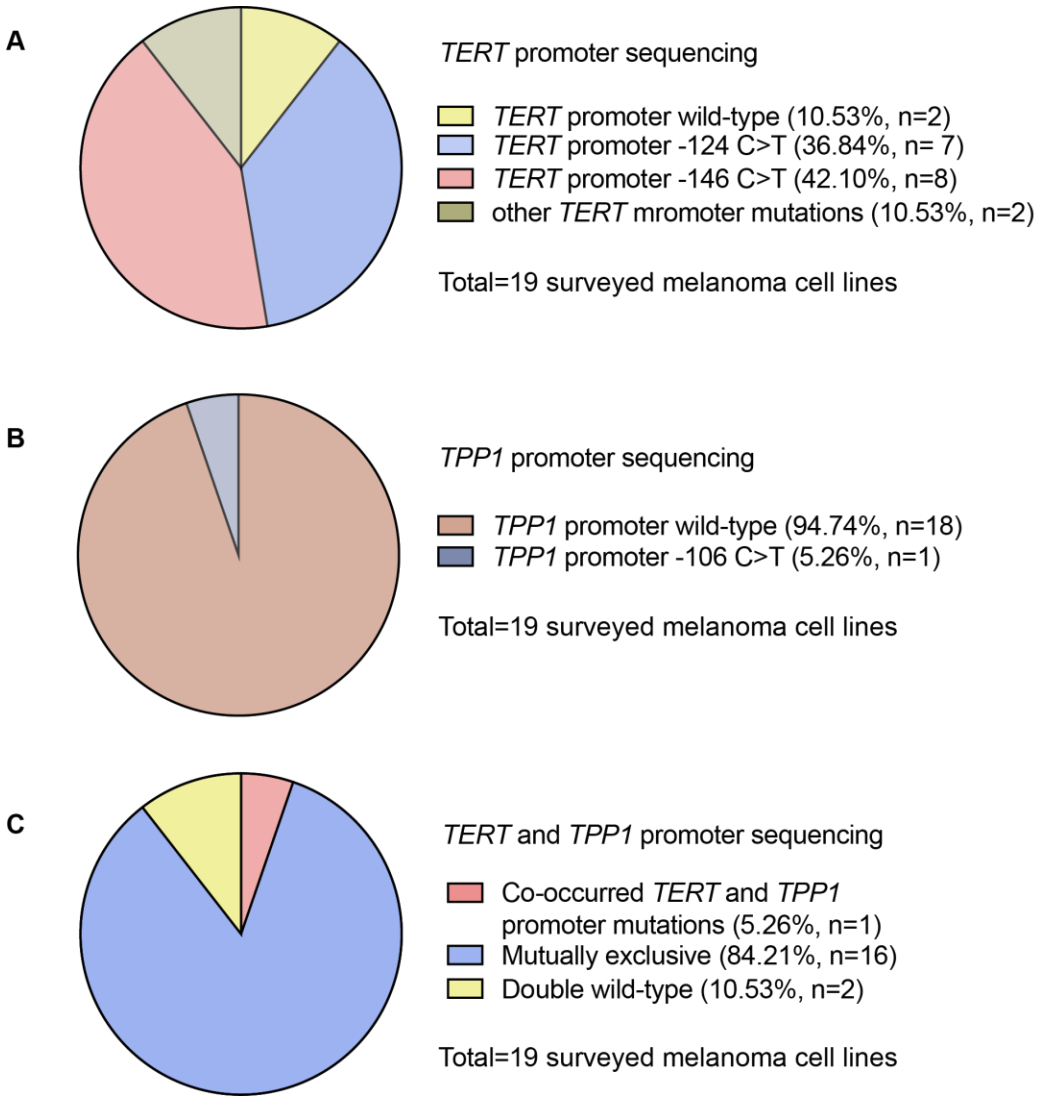


Figure 17. Frequency of *TERT* and *TPP1* Promoter Mutations in Melanoma Cell Lines.

(A) Pie chart of *TERT* promoter mutational status in 19 surveyed melanoma cell lines. (B) Pie chart of *TPP1* promoter mutational status in 19 surveyed melanoma cell lines. (C) Pie chart of *TERT* and *TPP1* promoter mutational status in 19 surveyed melanoma cell lines. *TERT* and *TPP1* promoter regions were amplified by PCR and Sanger sequencing was used to verify the mutational status.

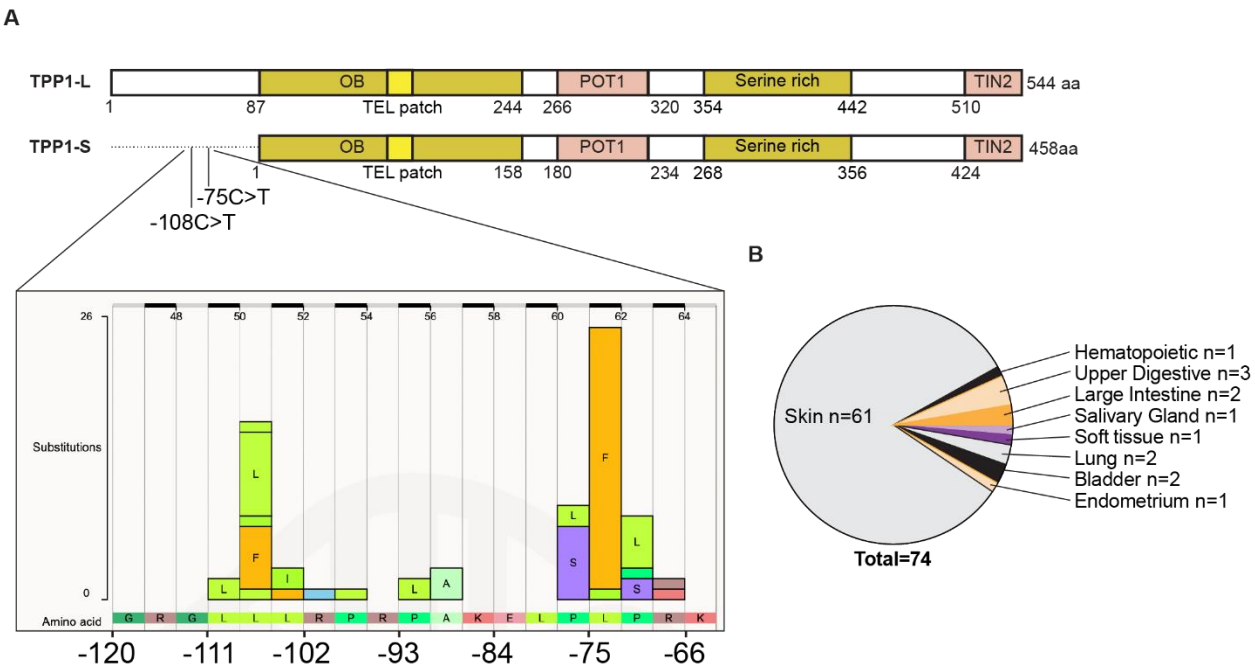


Figure 18. Spectrum of Cancers Harboring *TPP1* Promoter Mutations.
(A) Schematic of the TPP1-L and TPP1-S isoforms with screen capture of the cluster of mutations in the *TPP1* promoter from the COSMIC database. The COSMIC database uses GENCODE basic annotations which prioritizes longer transcripts 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 the hypothesis that these variants are impacting TPP1-L. (B) Distribution of reported *TPP1* promoter variants across major cancer types in the COSMIC database (v96).

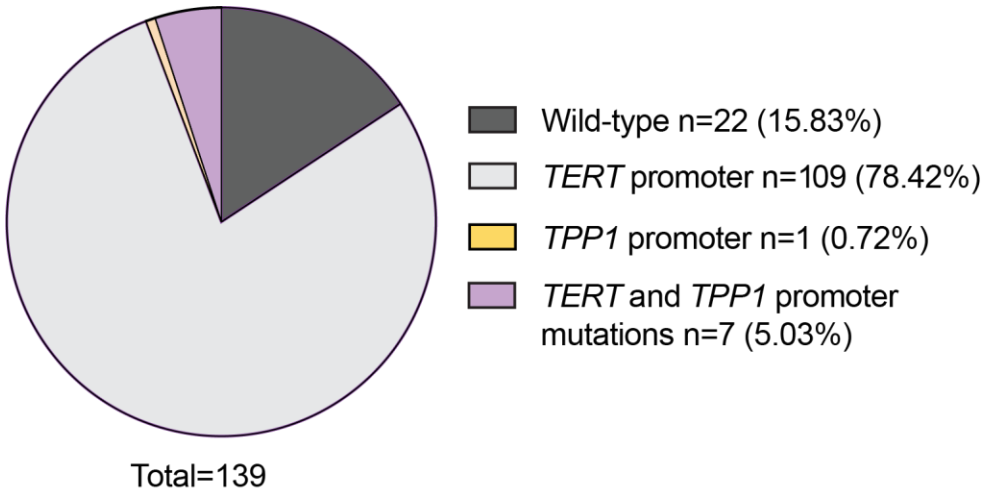


Figure 19. Co-occurence of *TERT*- and *TPP1*- Promoter Mutations in Cutaneous Melanomas.
Proportion of cutaneous melanomas that carried *TERT* promoter mutations, *TPP1* promoter variants, both *TERT* promoter mutations and *TPP1* promoter variants, and double wild-type from Hayward et al. (Hayward et al., 2017).

3.8 A Model of Telomere Length Dynamics in Melanoma Progression

The acquisition of a *TERT* promoter mutation in melanoma cells did not immediately stop telomere shortening. Telomeres continue gradually shortening until telomerase activity increases to a level sufficient to maintain the short length of all telomeres. However, melanoma cells with short telomeres may cease proliferating once telomeres have become critically shortened or they need another mechanism to overcome this barrier to immortalization. We propose that telomere maintenance in melanomas happens in two steps. The first hit occurs when *TERT* promoter mutations arise early in tumorigenesis to upregulate telomerase. This is insufficient to prevent bulk telomere shortening. Telomeres gradually shorten until reaching a critical length, which is maintained. The second hit to lengthen telomeres is then required. *TPP1* promoter mutations provide the second hit in melanoma cells to help them escape crisis and lengthen the telomeres to become immortal (Figure 20).

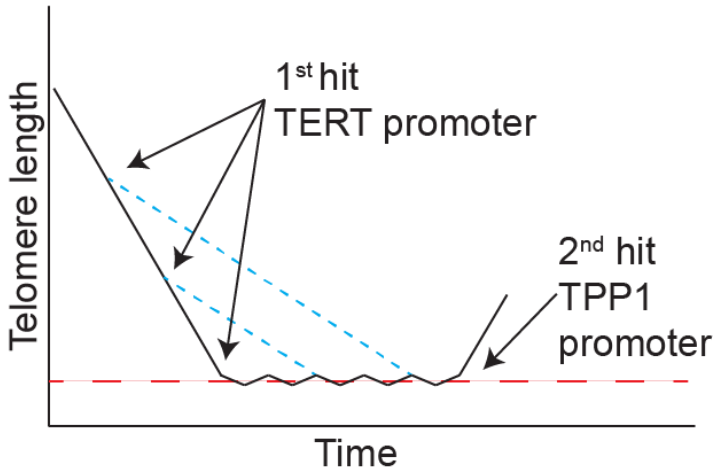


Figure 20. Model of Telomere Length Dynamics in Melanoma Progression.
Model of telomere length dynamics in melanoma progression. *TERT* promoter variants occur early to slow telomere attrition, but are not sufficient to prevent telomere shortening (blue dashed lines in model). Telomere shortening continues until cells enter crisis (red dashed line). Additional mutations, like the *TPP1* promoter, are likely required to fully maintain telomeres and escape crisis (2nd hit).

4.0 Discussion

Melanoma tumorigenesis is a complex progression from normal melanocyte to malignant melanoma. Melanoma is initiated by the acquisition of point mutations from UV light induced DNA damage. Frequent *TERT* promoter mutations in melanoma suggest that telomerase activation is fundamental in tumorigenesis. While *TERT* promoter mutations activate enough telomerase activity to maintain short telomere length after cells enter crisis and to extend the proliferative capacity of cells, it is not enough to immortalize melanoma cells. Although the role of telomerase in tumorigenesis driven by *TERT* promoter mutations is well established, details regarding how melanomas maintain their long telomeres and become immortal remain incompletely understood.

Here I report a second non-coding mutation in the promoter of *TPP1* co-operates with *TERT* promoter mutations to fully immortalize melanomas.

Defining the precise boundaries between regulatory elements including promoters, enhancers, and other regulatory elements is impossible because these elements can overlap or be located at the great distances from the transcription initiation start site. For our analysis, we used histone 3 lysine 27 acetylation epigenetic marks that indicate the active chromatin in the core promoter region. We found the cluster of *TPP1* variants falls in the region in between the two peaks of histone marks that typically represent the transcriptional start site (Figure 5). In 2019, the TPP1 annotations changed from identifying both TPP1-S and TPP1-L to only identifying TPP1-S in human cells as well as in other species. This change effectively re-annotated the cluster of TPP1 variants located in this region, which recur primarily in cutaneous malignant melanomas, from coding variants to a cluster of variants in the proximal promoter of *TPP1*. Our functional studies

further showed that this region is indeed a regulatory region and that the cluster of variants functions to increase expression of TPP1.

The mechanism by which *TERT* promoter mutations drive *TERT* reactivation has been well described. The identical 11 bp DNA sequence motif (5′-CCCCTTCCGGG-3′) containing a consensus binding site for ETS transcription factors created by the *TERT* promoter mutations (Huang et al., 2013) suggests ETS family transcription factors are necessary for promoter activation. Surprisingly, among 27 ETS factors, GABPA is the member responsible for activating the mutant *TERT* promoter (R. J. A. Bell et al., 2015). GABPA, which selectively binds to and regulates the mutant *TERT* promoter across multiple cancer types including melanoma, can only function as a heterodimer or heterotetramer with GABPB (Barger et al., 2022; LaMarco, Thompson, Byers, Walton, & McKnight, 1991; Oikawa & Yamada, 2003; Thompson, Brown, & McKnight, 1991). However, further investigation the effects of GABP in the context of *TPP1* promoter variants in melanoma cells might be interesting.

For the current study, *in silico* analysis suggested *TPP1* promoter variants: -108 C>T and -75 C>T create *de novo* ETS binding motifs (Figure 7). The *TPP1* promoter -108 C>T variant can generate *de novo* ETS transcription binding motifs, while the *TPP1* promoter -75 C>T variant generates a *de novo* ETS site adjacent to the native ETS binding motifs. Both *TPP1* promoter variants significantly increase *TPP1* transcriptional activity (Figure 10). Our hypothesis for this phenomenon is the *de novo* ETS factor binding motif (created by variant -108C>T) co-operates with the native ETS factor binding motif to activate TPP1. This is a mechanism similar to that of the *TERT* promoter mutations (R. J. Bell et al., 2015).

I found no evidence of the high expression of GABP from the RNA-seq data of 426 melanomas. I was unable to determine whether GABP knock-down was sufficient to decrease

expression of TPP1 due to technical difficulties (low transfection efficiency in tested cell lines). Thus, we identified the ETS factor as the critical factor activating TPP1 transcription activity in melanoma. Our results when we overexpress ETS factors and *TPP1* promoter variants in HEK293 cells support a role for ETS factors in the activation of the *TPP1* promoter mutants. Non-melanoma cells such as HeLa cells, HEK293, and RPE cells express limited amounts of ETS factors, reflecting the specific characteristics of melanoma cells that are not seen in other cell types. The combination of high levels of ETS and *TPP1* promoter mutations in melanocytes may allow the cells to become immortal.

Based on the findings of this study, mutations in the TPP1 promoter create *de novo* ETS binding motifs that act in concert with highly expressed ETS transcription factors to upregulate TPP1 in melanomas. The recurrence of *TERT* and *TPP1* promoter mutations in melanomas supports the proposed mechanism that somatic hotspot mutations in regulatory regions play an important role in tumorigenesis.

The potential mechanism of telomere lengthening in cells with altered TPP1 expression is unclear. Conflicting data show that decreasing TPP1 (via knock-down) (O'Connor, Safari, Xin, Liu, & Songyang, 2006) or increasing TPP1 (via overexpression) (Sherilyn Grill et al., 2019) expression can drive the telomere elongation, but loss of TPP1-S (TPP1 knock-out) (Bisht, Smith, Tesmer, & Nandakumar, 2016; Sherilyn Grill et al., 2021) leads to telomere shortening and deprotection. One potential explanation is that a defect in the POT1-TPP1 balance might affect the stoichiometries of POT-TPP1 heterodimers, which unexpectedly disturb the telomere length feedback regulation that drives telomere elongation. Along with the previous study, the tighter-binding POT1–TPP1 heterodimer may not slide along the DNA and thus would be unable to accommodate additional POT1–TPP1 dimers at increasing stoichiometries (Taylor, Podell,

Taatjes, & Cech, 2011). Our hypothesis here is the imbalance of TPP1 and POT1 may decrease the affinity of POT1–TPP1 for DNA and help the POT1–TPP1 heterodimer slide along the DNA more easily, allowing the complex to recruit and facilitate more telomerase enzyme to elongate the telomere.

The progression from melanocyte to melanoma is initiated by mutations known to activate the MAPK pathway—such as *BRAF* and *NRAS* mutations—in benign lesions, followed by *TERT* promoter mutations that activate telomerase, and finally the disruption of the G1–S checkpoint (e.g., *CDKN2A* mutations) in the late stage of melanoma (Shain et al., 2015). This model provides us with interesting questions: 1) Do *TERT* promoter mutations happen prior to *TPP1* promoter mutations? 2) What is the advantage of *TPP1* promoter mutations in tumors with *TERT* promoter mutations? Mutations in *TPP1* promoter alone are seen at a lower frequency than mutations in both *TERT* and *TPP1* promoters. It is possible this is due to pre-malignant cells containing TERT promoter mutations and longer telomeres allow more cell divisions and clonal expansion that, in turn, allows for the second, transformation-driving mutations seen in the *TPP1* promoter.

Driver mutations, which push the cell along the tumorigenesis path, are a hot topic in cancer evolutionary biology (Rheinbay et al., 2020; Weinhold, Jacobsen, Schultz, Sander, & Lee, 2014). WES analysis, which revealed only driver mutations found in coding regions, has progressed to WGS analysis that enables scientists to identify potential driver mutations in non-coding regions. Previous studies that had examined promoter regions for clusters of mutations had likely missed those in TPP1 because the studies used genome annotations that included TPP1-L, which is not expressed in human cells, and would have annotated these variants as coding variants.

We also analyzed RNA-seq data from 61 primary nevi and melanoma samples [available from the NCBI Gene Expression Omnibus under GEO: GSE112509 (Manfred Kunz et al., 2018)]

and found that TPP1-S is the only isoform expressed in primary melanoma tumors. This data confirmed our findings that TPP1 somatic mutations are in non-coding regions.

We demonstrated that the co-expression of TPP1 and TERT induced significantly more telomere elongation than overexpression of TERT alone in primary BJ fibroblasts (Figure 13C).

Our hypothesis could be further supported by a genome editing experiment in which we insert the promoter mutations into normal melanocytes and determine whether they can transform the cells. We could also introduce the two most common variants of *TPP1* (-75 C>T or -108 C>T) together with two most common *TERT* promoter mutations (-124 C>T or -146 C>T) into primary BJ fibroblasts and observe whether they bypass Hayflick's limit and show enhanced replicative capacity and telomere elongation. Another experiment to determine whether the *TPP1* promoter variants are sufficient to lengthen telomeres would be to perform the gene editing with either one of the two most common TPP1 promoter variants in melanoma cell lines with short telomeres. This experiment would allow easier analysis of bulk telomere changes than LOX or MEL624, which already have lengthy telomeres. These experiments may support our findings that TPP1 promoter mutations in melanoma drive increasing TPP1-S expression to regulate telomerase and lengthen their telomeres.

In a previous study of the genetic evolution of melanomas from precursor lesions (Shain et al., 2015) 77% of intermediate lesions and melanomas *in situ* harbored *TERT* promoter mutations indicating these mutations are selected at the early stage of melanoma progression. These findings raise the following questions: 1) When are *TPP1* promoter variants selected during melanoma progression? 2) If *TERT* promoter mutations co-occur with *TPP1* promoter variants, which mutation happens first? 3) Is there a relationship between these mutations, for example, if one occurs, does it drive the other mutation? It is unclear from our data when the variants in the *TPP1*

promoter arise relative to cancer progression and the acquisition of *TERT* promoter mutations. Answering these questions will require the analysis the samples from different stages of melanoma progression. It would be interesting and challenging to define the order of genetic alterations and evolutionary trajectories in the progression of melanomas which can help researchers to develop the new therapeutic drugs to stop melanomas or improve clinical outcomes.

There are several limitations in this study. One limitation is the questionable accuracy and reliability of defining non-coding mutations as somatic drivers in cancer using annotations from GENCODE v.19 (Rheinbay et al., 2020). TPP1 mutations located at the N-terminus region were identified as coding mutations but actually act as TPP1-S promoter mutations. Our results demonstrate that annotations must be verified experimentally.

Moreover, cancer heterogeneity—meaning they contain a mixture of subpopulations, including tumor cells and non-tumor cells, that express distinct genotypes and phenotypes—makes analyzing the gDNA from these populations complicated. To overcome the problem of tumor heterogeneity, we used cell lines, which are more homogeneous.

Another challenging problem is trying to use gDNA from FFPE sample sections to sequence the *TPP1*- and *TERT*- promoters. FFPE samples yielded poor quality DNA to use as templates for *TPP1*- and *TERT*- promoter PCR. This obstacle cannot be solved by nested PCR or increasing the PCR cycles due to the contamination may happen during these processes.

To overcome these hurdles, we analyzed deep coverage WGS from a previous study of melanoma samples that included primary tumors, metastases, and cell lines (Hayward et al., 2017). Notably, Hayward *et al.* identified eight genes in addition to *TERT* that showed the potential to be driver mutations. The *TPP1* gene was not identified because of mis-annotation.

In summary, the present work uncovered one missing piece of the puzzle in the telomere maintenance mechanism that drives the immortalization of melanoma cells. We identified *TPP1* promoter variants that co-occurred with *TERT* promoter mutations in about 5% of cutaneous melanomas. Acquisition of a *TERT* promoter mutation as the "first-hit" enables melanomas to reactivate telomerase and bypass senescence with short telomeres. Insufficient levels of telomerase cannot immortalize melanocytes. At the very least, a second hit must happen in order to reactivate telomerase. We propose that *TPP1* promoter mutations are the "second-hit" required for melanoma cells to lengthen their telomeres and become immortal.

The development of cancer therapies targeting telomerase has been challenging due to poor efficacy and unfavorable side effects. Novel findings from this study open up potentially new targets for melanoma treatments to improve clinical outcomes.

5.0 Conclusions

Tumor cell proliferation and enabling replicative immortality are interdependent hallmarks of cancer. Reactivation of telomerase is essential to maintain telomeres and enable replicative immortality in cancer. UV radiation is the major environmental risk factor inducing the somatic mutations that contribute to tumorigenesis. Melanomas harbor the highest somatic mutation burden among all cancer types (Martincorena & Campbell, 2015). Spontaneous somatic mutations accumulate throughout an individual's lifetime due to unrepaired errors induced by UV damage. The landscape of genomic alterations in cutaneous melanomas is wide-ranging due to the variability in high mutation burden attributed by mainly UV exposure or some unknown carcinogen.

Melanomas are usually characterized by the recurrent mutations in proto-oncogenes including *BRAF* (Davies et al., 2002), *NRAS* (Albino, Le Strange, Oliff, Furth, & Old, 1984), *NF1* (Cirenajwis et al., 2017; Kiuru & Busam, 2017; Thielmann et al., 2021), and *KIT* (Curtin, Busam, Pinkel, & Bastian, 2006) and tumor suppressor gene such as *CDKN2A*, *CDK4*, and *PTEN* (Curtin et al., 2005). Mutations detected by whole exome sequencing studies create a framework for targeted treatments and immune therapies. However, WES approaches, which focus on protein coding regions, neglect most non-coding regions of DNA. With WGS approaches, two independent studies identified *TERT* promoter mutations in about two-third of sporadic and familial cutaneous melanomas (Horn et al., 2013; Huang et al., 2013). UV signature mutations always drive C>T or CC>TT substitutions in the *TERT* promoter region to create *de novo* ETS binding motifs that increase gene expression (Brash, 2015; Horn et al., 2013; Huang et al., 2013). In the absence of a known mechanism responsible for telomere maintenance in the remaining one-

third of melanomas, this study investigated a novel telomere maintenance mechanism that contributed to replicative immortality. This dissertation describes:

1) A novel mechanism of telomere maintenance in cancer, represented by a cluster of variants in the *TPP1* promoter region that we identified in melanoma samples. The *TPP1* promoter variants created *de novo* ETS transcription factors binding sites that increase TPP1-S expression and promote telomerase activity and processivity.

2) A novel method to classify tumors. Previous studies have demonstrated that two *TERT* promoter mutations (-124 C>T and -146 C>T) are mutually exclusive, meaning if one of these *TERT* promoter mutations occurs in a melanoma tumor, the other *TERT* promoter mutation will not occur. This study shows that *TERT* promoter mutations co-occurred with *TPP1* promoter mutations in about 5% of cutaneous melanomas suggesting that *TPP1* promoter mutations cooperate with the *TERT* promoter mutations to increase telomere repeats addition.

5.1 A Two-step Model for Promoting the Immortalization of Melanomas

In this study, we provide evidence that there is a special link between melanomas and long telomeres. However, the acquisition of mutations in the *TERT* promoter during transformation from normal melanocyte to malignant melanoma does not support telomere length maintenance (Chiba et al., 2017b). *TERT* promoter mutations do not prevent bulk telomere shortening but they maintain short telomeres and expand cellular life span. This is not sufficient to immortalize cells, suggesting that *TERT* promoter mutations could only partially explain the telomere length in melanoma. In this study, we examined DNA sequencing data from melanomas looking for mutations in telomere-related genes (Figure 4). We identified variants in TPP1 that act as promoter

variants (Figure 5). These variants increase *TPP1* transcription activity (Figure 10) via *de novo* ETS binding motifs (Figure 7).

More than a decade ago, researchers showed that TPP1 can enhance telomerase processivity (Latrick & Cech, 2010; Jayakrishnan Nandakumar et al., 2012). The current study shows that having excess TPP1 and telomerase can immortalize cells by driving significant telomere lengthening (Figure 14). To confirm this hypothesis, we introduced *TPP1* mutations into melanoma cells using CRISPR-Cas9 genome editing (Figure 15). We observed increased telomerase activity and an increased amount of TPP1 protein (Figure 16).

The co-occurrence of *TERT* promoter mutations and *TPP1* promoter mutations in about 5% of cutaneous melanomas (Figure 19) can partially fill the gap in understanding the mechanism of telomere maintenance. In the genetic evolution of melanomas, it is unclear exactly where in tumorigenesis the TPP1 promoter variants arise. We hypothesized that *TPP1* promoter variants arise in the later stage after the acquisition of *TERT* promoter mutations. This order would allow telomerase activity to be boosted by an increase in TPP1 protein. However, some missing pieces in melanoma tumorigenesis need to be uncovered to understand the whole mechanism. This pathway could provide the opportunity to develop therapies to these new targets.

We propose that TERT and TPP1 act synergistically to drive cell immortalization in a two-step model (Figure 20). As the first-hit, *TERT* promoter mutations arise in early tumorigenesis in cells with critically short telomeres. The TERT mutations contribute to delay replicative senescence but lack sufficient telomerase activity to drive telomere elongation. *TPP1* promoter mutations serve as the second-hit at a later stage of tumor progression. These mutations promote telomerase activity and enable telomere elongation and immortalization.

TPP1 is one of the missing factors required to understand the immortalization process. Historically, variants or mutations in regulatory regions have been difficult to identify. But, as we have demonstrated in our research, variants within non-coding genomic regions can greatly affect disease. Identifying regulatory variants associated with disease is complex and involves both computational analyses and laboratory-based investigation. Thanks to the advances in sequencing technologies, it is now cheaper and easier than ever to sequence patient genomes in order to identify variants associated with diseases. Our work demonstrates the crucial importance of validating these mutations experimentally.

Finally, this present work highlights the significance of alterations in telomere maintenance during the tumorigenesis of cutaneous melanomas and provides potential diagnostic markers and future therapeutic targets for cancer.

5.2 Future Directions

Previous studies have shown the genetic evolution of melanoma progression from precursor lesions to malignant tumors (Chiba et al., 2017b; Shain & Bastian, 2016; Shain et al., 2015). Interestingly, 77% of intermediate melanomas and melanomas *in situ* harbored *TERT* promoter mutations (Shain et al., 2015). This study shows that *TPP1* promoter variants were found in roughly 5% of cutaneous melanomas and almost always co-occurred with *TERT* promoter mutations. Our model predicts that *TERT* promoter mutations occur in an early stage of melanoma tumorigenesis. These mutations are required for telomerase reactivation to bypass telomere crisis and maintain telomere length to allow cellular proliferation until *TPP1* promoter mutations are acquired. To better understand the genetic evolution of melanomas, TPP1 is a candidate gene to

investigate further. Future studies are required to address when *TPP1* promoter mutations arise during melanoma progression. However, we need the appropriate approaches and systems to further investigate.

The introduction of TERT and TPP1 into human primary fibroblasts demonstrated a synergistic effect in telomere lengthening but it remains unclear how melanocytes transform into malignant melanomas. Using a genome editing approach to introduce the *TERT* and *TPP1* promoter mutations into a normal melanocyte could be the ideal experiment to test whether TERT and/or TPP1 are sufficient to immortalize cells. However, we need more time and resources to investigate this model. Possible approaches include using a genome editing approach to introduce *TERT* and/or *TPP1* promoter mutations into embryonic stem cells to determine whether the mutants can drive ESCs differentiate into fibroblasts.

Mutated promoters -75 C>T and -108 C>T create *de novo* ETS transcription binding motifs, which are the primary mechanism for promoting TPP1 transcription. The mutated *TPP1* promoter -75C>T can also create a *de novo* TCT binding motif, which is located at the +2 position relative to the +1 transcription start site. The TCT motif is a key component of the RNA polymerase II system that is required for the transcription of ribosomal protein (RP) gene promoters (Parry et al., 2010). The TCT motif appears to be present in the core promoter of the RP genes from *Drosophila* to humans but not in yeasts (Parry et al., 2010). However, the impacts of TCT motifs in human is unknown. The specialized TCT-based transcription system has been studied in *Drosophila* but never have been done in humans. We need more resources, facilities, and collaborations to further investigate this system, which could apply in humans. However, the analysis of TCT-based transcription system could provide new insights into a broader understanding of transcriptional regulation.

TPP1 promoter variants were identified in cutaneous melanomas and other cancer types. The ICGC data reported TPP1 variants in the N-terminal region upstream to Methionine 87, where they are TPP1-S promoter variants, in multiple cancers including lung, bladder, ovarian, gastric, pediatric brain, liver, thyroid, esophageal, endometrial, breast, and renal cancers. A subsequent experiment is to test whether *TPP1* promoter variants in other cancer cell types can promote the transcriptional activation of *TPP1* and lengthen telomeres similar to our results in melanoma cells. One challenging issue is that different cell types typically express ETS transcription factors differentially. We cannot expect to see the same increase in TPP1 transcriptional activity in TPP1 promoter variants from different cell types due to differential ETS expression. Melanomas show a high rate of *TERT* promoter mutations not seen in other cancers. The higher percentage of *TPP1* promoter variants found in melanomas supports the specific link between melanomas and long telomeres. However, some cancers such as thyroid, liver, and glioblastoma multiforme have been reported with a high percentage (>50%) of *TERT* promoter mutations (Bell et al., 2016). These tumor types could be candidates to investigate further the role of *TPP1* promoter variants. Expanding the mechanistic understanding of mutations in the TPP1 promoter in wide variety of cancers could help inform newer strategies to develop new targeted cancer therapies.

Appendix A Melanoma Cell Lines

The *TERT* promoter and *TPP1* promoter of nineteen melanoma cell lines were sequenced in collaboration with the Melanoma Center Biospecimen Repository at the University of Pittsburgh Hillman Cancer Center. The analysis is detailed below.

Appendix A.1 Sequencing of the *TERT* Promoter and *TPP1* Promoter in Melanoma Cell Lines

Appendix Table1 lists the TERT and TPP1 promoters in 19 melanoma cell lines from a collaboration with the Melanoma Center Biospecimen Repository at the University of Pittsburgh Hillman Cancer Center. Promoter regions were identified by Sanger sequencing.

Appendix Table 1. Melanoma Cell Lines with *TERT* and *TPP1* Promoter Sequencing Used in This Study

Cell line	*TERT* promoter	*TPP1* promoter
LOX	homozygous -124 C>T	wild-type
MEL624	heterozygous -124 C>T	wild-type
A375	heterozygous -146 C>T	wild-type
M308	homozygous -124 C>T	wild-type
SkMEL37	homozygous -146 C>T	wild-type
FEMX	heterozygous -146 C>T	wild-type
526MEL	homozygous -146 C>T	wild-type

Appendix Table 1. Melanoma Cell Lines with TERT and TPP1 Promoter Sequencing Used in This Study

(continued)

Cell line	*TERT* promoter	*TPP1* promoter
983MEL	homozygous -146 C>T	wild-type
M255	double heterozygous -138/39 CC>TT	wild-type
TPF-11-743	wild-type	wild-type
Colo829	heterozygous -124 C>T	wild-type
TPF-14-346	double heterozygous -124/25 CC>TT	wild-type
2020-MEL-42	heterozygous -146 C>T	wild-type
2020-MEL-52	heterozygous -124 C>T	Heterozygous -106 C>T
96-099-MEL-2020-31	heterozygous -124 C>T	wild-type
96-099-TPF-15-230	heterozygous -146 C>T	wild-type
96-099-TPF-19-219	wild-type	wild-type
96-099-TPF-19-235	homozygous -124 C>T	wild-type

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