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Decoding the mechanisms of cancer and stem cell immortality

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Abstract

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Telomeres are the repetitive sequences at the ends of linear chromosomes. The key functions of telomeres are to protect the cells from losing genomic information and to prevent chromosome ends from being repaired by the double strand break repair machinery. To counteract loss of telomeric DNA, cells can express a reverse transcriptase, telomerase, that synthesizes telomeric repeats *de novo*. In humans, telomerase activity is mostly restricted to germ and stem cells, so the telomeres of most somatic cells progressively shorten with each cell division. Once telomeres become critically short, they are recognized as sites of DNA damage and cells cease to proliferate. By this mechanism, telomere shortening functions as a tumor suppression mechanism. TERT, the protein component of telomerase, becomes silenced once stem cells differentiate. However, in 90% of cancer cells, TERT is transcriptionally re-activated. Thus, telomerase regulation is crucial for our understanding of telomere length regulation in stem cell maintenance and tumorigenesis. To understand how telomerase acts on telomeres, I attempted to endogenously tag telomerase. To do this I inserted epitope tags at the endogenous TERT locus in hESCs using genome editing. However, I found that all the tested tags cause defects in telomere maintenance, which was previously not appreciated in experiments using exogenous overexpression.

Recently, point mutations in the TERT promoter were identified as the most frequent non-coding mutations in cancer. To elucidate the role of TERT promoter mutations (TPMs) in tumorigenesis, I genetically engineered these TPMs into human embryonic stem cells (hESCs) using genome editing. Using the resulting isogenic hESC lines, I demonstrated that TPMs lead to a failure of TERT silencing upon differentiation from stem into somatic cells. To understand role of TPMs in tumorigenesis, I monitored long-term telomere maintenance and proliferation in human fibroblasts engineered to carry TPMs. I found that TPMs immortalize cells but do not prevent telomere shortening and telomere fusions. *In vitro*, around the time when telomere fusions occurred, TERT expression was gradually increased. Thus, TPMs are required, but not sufficient, for cancer cell immortality and contribute to tumorigenesis in two steps. First, TPMs expand proliferation capacity of a cell by elongating only the shortest telomeres but do not prevent overall telomere shortening. In the second step, TPMs fuel tumorigenesis by not fully suppressing genomic instability. In order for cells to immortalize they need to upregulate TERT during this second step.

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CHAPTER ONE

Importance of the ends of linear chromosomes and its maintenance

Problems at the ends of linear chromosomes

In multicellular organisms fertilized from a single cell, maternal and paternal genomic information has to be handed down and protected throughout development. During the process of cell division, genomic DNA is faithfully replicated, yet the ends of linear chromosome are not fully synthesized due to the limits of the mechanism of DNA replication(Olovnikov, 1973; Watson, 1972). Briefly, DNA polymerase uses RNA primers to initiate DNA synthesis but can only extend in the 5'-3' direction by adding a complementary deoxynucleotide to 3'-OH(Steitz, 1999). Normally, RNA primers are removed from newly synthesized Okazaki fragments and lagging strands are patched by DNA ligase, however, at the 3'-end, there is no downstream template DNA to place the RNA primers(Stillman, 1994). Therefore, upon each round of DNA replication, genomic information of 3'end will be lost — this known as the "end replication problem".

Another problem at the ends of linear chromosomes is that the ends can be recognized as a DNA double strand break (DSB) site by the cell's internal DNA repair machinery(Blackburn, 2001; de Lange, 2002). Typically, breakages of genome should be repaired to maintain integrity of genome, otherwise it results in activation of DSB signaling cascades finishing with apoptosis or senescence of the cell(Ciccia and Elledge, 2010). Additionally, failure of repairing double strand breaks can cause genomic instability leading to tumorigenesis(Jackson and Bartek, 2009). Thus, cells have DSB repair machinery that surveys and repairs damage of the genome via non-homologous end joining (NHEJ), a rapid, error-prone repair machinery or homologous recombination (HR), a faithful repair machinery, depending on cell cycle stage(Branzei and Foiani, 2008). A cell has to distinguish between DSB sites and natural ends of chromosomes, otherwise chromosome ends will be unintentionally repaired, resulting in re-arrangement of the genome landscape (Maciejowski and de Lange, 2017). This can lead to genomic instability and increase tumorigenic potential.

Furthermore, free DNA ends are also subject to degradation by nucleases, leading to loss of genomic information(Mimitou and Symington, 2009). Overall, Linear chromosomes provide diversity and complexity of genome, yet cells have to resolve problems of the ends to maintain genomic stability.

Telomeres

To prevent loss of genomic information at chromosome ends, there is a buffer sequence called telomeres(Blackburn and Gall, 1978; Szostak and Blackburn, 1982). In mammals, telomeres are tandem repeats of six nucleotides (nt), 5'-TTAGGG-3', capping the end of chromosomes(Moyzis et al., 1988). The length of telomeres differs from species to species as well as on cell type(Cooke and Smith, 1986; Zakian, 1995). In human embryonic stem cells, the length of telomeres is roughly 13kb. The 5' region of telomeres consists of non-canonical TTAGGG telomeric repeats called subtelomeres while the 3' of telomere ends with 100-200 nt of G-rich single-stranded DNA overhang(Makarov et al., 1997; McElligott and Wellinger, 1997; Wu et al., 2012). This single-stranded telomeric DNA forms a loop structure, called the T-loop, by invading

double-stranded regions of telomeres to displace duplex repeats(Griffith et al., 1999). Although the functions and mechanisms of T-loops are not fully understood, it at least caps the telomere ends and prevents telomere end-end fusions.

In addition to repetitive DNA, there is a telomere-specific six protein complex called shelterin (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1) that has essential roles in how telomeres function with regards to the end replication problem(Smogorzewska and de Lange, 2004). The shelterin complex suppresses DNA damage responses mediated by the ATM and ATR signaling pathway, as well as inappropriate HR at telomeres (Palm and de Lange, 2008). As an example, deletion of TRF2 leads to p53 dependent cell cycle arrest by activation of ATM kinase pathway and results in apoptosis or senescence of the cell (more details are described in the following ***Telomere shortening as a tumor suppression mechanism*** section). Thus, telomeres and their associated shleterin complex are equipped to provide solutions for the problems that linear chromosomes endure.

Telomere maintenance by telomerase

Although telomeres are a solution to counteract loss of genomic information by the end replication problem, upon each replication cycle telomere length is continuously shortened(Harley et al., 1990). In many organisms, telomeres are maintained or even elongated during development and self-replication, suggesting that a mechanism to counteract loss of telomeres exists(Bernards et al., 1983; Szostak and Blackburn, 1982). Initially, a few models were hypothesized including homologus recombination(Heumann, 1976), however the evidence points to the existence of an enzyme capable of synthesizing telomeric repeats de novo(Shampay et al., 1984). The enzyme was finally discovered in a ciliate organism, Tetrahymena thermophile and later in human (Greider and Blackburn, 1985; Morin, 1989). This specialized enzyme, named telomerase, is a reverse transcriptase that can add telomeric repeats to the 3' single strand overhang(Greider and Blackburn, 1987).

Telomerase is a ribonucleoprotein composed of the catalytic protein component TERT and the RNA template TR(Greider and Blackburn, 1989). TERT is composed of the TERT N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the RT domain and the C-terminal extension (CTE) domain(Wu et al., 2017). The TEN domain has a crucial role in the recruitment of telomerase to telomeres mediated by the interaction between the TEN domain and a member of the shelterin complex, TPP1(Nandakumar et al., 2012; Schmidt et al., 2014; Sexton et al., 2014). Additionally, the TEN domain as well as the CTE-domain include regions called dissociates activities of telomerase (DAT) regions (Armbruster et al., 2001; Banik et al., 2002). Telomerase with DAT mutations still possess its catalytic activity, yet fail to immortalize primary fibroblasts(Counter et al., 1998), supporting requirement of these regions for localization of telomerase at telomeres. Another important feature is that telomerase is a processive enzyme meaning that telomerase can add multiple repeats without dissociating from its substrate — repeat addition processivity (RAP) (Greider, 1991). Mutations in the TEN domain result in reduced RAP in vitro(Nandakumar et al., 2012; Robart and Collins, 2011), thus the N-terminal of telomerase is not only important for telomerase localization but also its activity. The TRBD interacts with CR4/5 motif of TR in human(Bley et al., 2011). The structure of TR is very diverse between different organisms, but it always contains the template region complementary to the telomeric sequence(Wu et al., 2017).

TR is a non-coding RNA transcribed by RNA polymerase II (Pol II) and processed for maturation(Wu et al., 2017). The 3'-end of TR forms the H/ACA motif and TR precursor is exonucleolytically processed by PolyA-specific ribonuclease (PARN)(Moon et al., 2015; Tseng et al., 2015). The H/ACA hairpin loop associates with dyskerin, NOP10, NHP2 and NAF1 which are critical for TR biogenesis(Meier, 2005; Mitchell et al., 1999). Another factor associated with active telomerase is telomerase Cajal body protein 1 (TCAB1). TCAB1 co-localizes with TR at Cajal body where snRNP maturation and processing occurs. TCAB1 is suggested to have a role in active telomerase assembly and trafficking of telomerase to telomeres(Venteicher et al., 2009). However, a cell with TCAB1 knock out or a cell carrying TR with deficiencies in the TCAB1 binding region can still maintain its telomeres for long-term proliferation (Vogan et al., 2016), thus further characterization of the role of TCAB1 in telomere maintenance is required.

Mutations in any of the telomerase holoenzyme component, TERT, TR, dyskerin and TCAB1 as well as PARN and the sheltrin component TIN2 can cause rare genetic disease dyskeratosis cngenita (DC). DC patients develop premature aging phenotype associates with a bone marrow failure syndrome that is characterized by white patches in the mouth, nail dystrophy and abnormal skin pigmentation as well as risks for malignant tumors, pulmonary fibrosis and liver cirrhosis. Severe symptoms of DC are associated with highly regenerative tissues including failure of differentiation in hematopeietic stem cells. Two independent studies using DC patient derived iPSCs resulted in opposite telomere maintenance phenotype(Agarwal et al., 2010; Batista et al., 2011). This highlights the importance of understanding link between telomere homeostasis by telomerase and replicative capacity of different cell types.

Telomerase regulations in human stem cells and somatic cells

Complexity of a given tissue is achieved by many different types of cells derived from a progenitor or stem cell. For example, embryonic stem cells (ESCs) are derived from inner cell mass of a blastocyst and have a potential to become any cell type in any of the three germ layers, known as pluripotency (Thomson et al., 1998). An alternate example of a stem cell is intestinal stem cells. Intestinal stem cells locate at a base of crypt, proliferating and differentiating into cell types that either support the stem cell niche or secretion cells to form the intestinal crypt structure(Clevers, 2006). This type of adult stem cell is multipotent, which can differentiate into a specific lineage of cells. Stem cells and germ cells are immortal to maintain their self-renewing and differentiation capacity into progeny cells. Thus, these cells are commanded to have high replicative capacity and it is important to maintain genomic stability to prevent inaccurate genomic information from being inherited by its offspring cells. Therefore, in most higher eukaryotes, telomerase activity is exclusively detectable in stem cells and germ cells (Wright et al., 1996). The key of limiting telomerase activity in stem cells is achieved by expression level of TERT but not TR(Meyerson et al., 1997; Nakamura et al., 1997). TR is ubiquitously expressed among different cell types regardless of telomerase activity and the half-life of TR RNA is very stable(Yi et al., 1999). In human immortal cell lines, about 1000 molecules of TR exist in a cell(Xi and Cech, 2014). On the other hand, expression of TERT is very dynamic. In most of somatic cells TERT is transcriptionally silenced and it is expressed only in telomerase positive stem cells at a low level(Kolquist et al., 1998; Martín-Rivera et al., 1998). It is estimated that even in immortal cell lines,

expression of TERT is less than 10 - 100 molecules per cell(Xi and Cech, 2014). This indicates that the number of active telomerase holoenzymes is less than the number of chromosome ends, thus not all of telomeres are elongated upon each cell cycle. Supporting this estimation, in yeast, shorter telomeres are preferentially elongated by telomerase (Hemann et al., 2001; Teixeira et al., 2004). TERT transcript contains various splicing isoforms including some mRNAs that skip exons coding the TEN-domain or the RT catalytic domain(Kilian et al., 1997). It is hypothesized that these "junk" TERT mRNA might contribute to limit the telomerase activity level(Wong et al., 2014), yet due to its low abundance and large variability in different cell lines, roles of TERT splicing in telomerase regulation are not clear.

As described above, the primary determinant of the telomerase enzymatic activity is repression of TERT upon somatic differentiation. For example, in human fibroblasts, there are no TERT mRNA molecules detectable and chromatin immunoprecipitation (ChIP) for Pol II at the TERT promoter shows absence of signal, supporting shut down of TERT transcription upon differentiation. Interestingly this silencing process is reversible during the process of reprograming from somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This mode of gene regulation is similar to that of OCT4, SOX2 or KLF4, known as the Yamanaka factors — transcription factors required for reprograming(Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In the case of these genes, cis- and trans- regulatory elements as well as epigenetic regulation have been extensively investigated(Loh et al., 2006; Rodda et al., 2005). However, the expression of the Yamanaka factors is on or off between pluripotent stem cells and somatic cells, while levels of TERT silencing is more tissue/cell type dependent. Currently, mechanisms underlying tissue-specific TERT repression have not been understood.

Another challenge of understanding TERT regulation is a critical difference between human and mouse telomere biology. Mice are a strong model to study in vivo regulations that a human tissue culture system fails to recapitulate, including tissue specific gene regulations. However, mice have longer telomeres, 50-100kb, compared to human and upon somatic differentiation and TERT expression is not silenced(Cheng et al., 2017; Greenberg et al., 1998; Horikawa et al., 2005). One approach to compensate for the absence of a mouse model can be the use of human pluripotent stem cells (hPSCs) (Hockemeyer and Jaenisch, 2016). Protocols for differentiation of hPSCs into various cell types have been extensively investigated and constantly improved including protocols for telomerase positive adult stem cells such as intestinal stem cells or neuronal precursor cells as well as telomerase negative cell types such as fibroblasts or neurons(Chambers et al., 2009; Forster et al., 2014). Together with induction of TERT during the re-programming process, hPSCs offer a dynamic observation of the TERT regulation cycle in vivo.

Telomere shortening as a tumor suppression mechanism

In 1961, Hayflick discovered that proliferation of normal human cells ceases and cause senescence after limited number of cell divisions(HAYFLICK and MOORHEAD, 1961). In addition, co-culture of younger cells with later passaged cells did not changed the replicative capacity of a cell(HAYFLICK, 1965). These observations indicated the existence of a cellular mechanism like an intercellular biological clock. Shortly after

discovery of telomeres and telomerase, it was shown that in fibroblasts, the length of telomeres shorten over the lifetime of a cell due to lack of telomerase activity(Harley et al., 1990). Involvement of DNA checkpoints such as p53 or RB in replicative senescence was suggested by recovery from senescence using mutated SV40 T antigen lacking p53/RB binding(Shay et al., 1991). Breakage of chromosomes can induce p53 and ATM dependent apoptosis, while this response is suppressed by TRF2 at telomeres(Fabrizio d'Adda di Fagagna et al., 2003; Karlseder et al., 1999; Takai et al., 2003). Once telomeres becomes shortened or dysfunctional, DNA damage foci such as γ-H2AX, 53BP1, Mre11 and Rad 17 (telomere dysfunction-induced foci (TIF)) becomes detectable at telomeres(Fabrizio d'Adda di Fagagna et al., 2003; Takai et al., 2003). In vitro, TRF2 directly interacts with ATM and inhibits autophosphorylation of ATM(Karlseder et al., 2004). However, a cell with inhibition of TRF2 and lacking ATM can still cause telomere induced senescence indicating that the ATM signaling pathway is not sufficient to explain the chromosome end protection at telomeres(Denchi and de Lange, 2007). Independent from the ATM signaling pathway, the ATR pathway is suppressed at telomeres by single strand telomeric binding protein Pot1 by excluding RPA from the 3' overhang of telomeres(Denchi and de Lange, 2007; Gong and de Lange, 2010).

Using the mechanisms described above, telomere shortening functions as a tumor suppression mechanism(Greenberg et al., 1999a). However, it still remains elusive how the length of telomere is recognized by the shelterin complex and how that information is conveyed to the suppression of DNA damage responses or how NHEJ and HR are suppressed between individual telomeres.

Roles of telomerase in cancer

As functions of telomere deteriorate, replicative senescence is induced to prevent genomic instability in a cell. Replicative senescence can be overwritten by inactivation of the cell cycle checkpoints to enable further proliferation with critically short telomeres(Chin et al., 1999). In human cells transformed with SV40, critically short telomeres are associated with genomic instability including dicentric chromosomes by telomere-end fusions(Counter et al., 1992). In all mortal cells, dicentric chromosomes derived from telomere end fusions are associated with critically short telomeres and no telomerase activity was detected in the population. On the other hand, in immortal cells, short telomeres were maintained for further proliferation after telomere crisis and frequency of chromosome fusions was stabilized. These immortal cells showed telomerase activity so that attenuated telomeres were healed. In vivo experiments using mouse lacking telomerase and INK4a showed that telomere shortening impairs tumorigenesis and restoration of telomerase regained tumorigenic capacity(Greenberg et al., 1999a). These results highlight the importance of telomerase during tumorigenesis especially acquisition of TERT expression during crisis(Counter et al., 1992; Meyerson et al., 1997; Nakayama et al., 1998). Indeed, telomerase activity as a result of TERT expression is detected in 80-90% of human tumors (Kim et al., 1994).

Since TERT expression is silenced in normal human somatic cells, key regulatory steps to understand TERT expression were mainly focused on two aspects: (i) how TERT expression is silenced in normal somatic cells (ii) how TERT expression is activated in cancer cells. Previous extensive studies reported on *trans*- and *cis*- regulatory elements of the TERT promoter such as c-Myc and/or Sp1 that strongly activated TERT transcription

(Kyo et al., 2000; Wang et al., 1998; Wu et al., 1999). Other reports linked hypoxia, estrogens and EGF signaling to increased TERT expression through the transcription factors HIF-1(Anderson et al., 2005; Lou et al., 2007; Nishi et al., 2004; Yatabe et al., 2004), ERα(Kimura et al., 2004; Kyo et al., 1999; Misiti et al., 2000) or Ets(Maida et al., 2002) respectively, while Mad1 and Menin have been reported to function as repressors of TERT expression (Lin and Elledge, 2003). In mice, involvement of Klf4 working with the Wnt pathway in ES cells or NF-κB in mouse liver cells was suggested(Hoffmeyer et al., 2012; Yin, 2000). In addition to these transcription factors and their respective binding sites in the TERT promoter, *cis*-regulatory elements have been described for the TERT promoter. CTCF, known as a repressor or insulator, can be recruited to the TERT promoter in CpG methylation dependent manner(Eldholm et al., 2013; Renaud, 2005; Renaud et al., 2007), and histone deacetylation can also contribute to TERT repression(Zhu et al., 2010). In addition to epigenetic regulations, bioinformatic analysis identified that 5kb upstream of the TERT transcription start site is conserved among primates and may be a potential enhancer(Tran et al., 2010). Another chromatin landscape that can have influence on TERT regulation is telomere length-dependent loops. This regulation, known as telomere position effect, is a concept that heterochromatic status of telomeres influence expression of genes near chromosome ends(Baur et al., 2001; Gottschling et al., 1990). The TERT gene is located at the chromosome 5p end, and it is suggested that TERT expression can be regulated by telomere length(Kim et al., 2016). In some cancer, rearrangement of the chromosomal region proximal to TERT or gene amplification of TERT contributes to increasing TERT expression(Cao et al., 2008; Nagel et al., 2010; Peifer et al., 2015; Valentijn et al., 2015).

Despite many studies on TERT expression, molecular and genetic mechanisms governing TERT expression are not fully understood. This is partly due to very low TERT expression, as analyses are relying on transient transfection of TERT reporter plasmids which do not reflect physiological regulations. Single cell derived clonal isolation from cancer cell lines showed heterogeneity of telomerase activity and TERT expression as well as disconnection between the telomerase activity level and telomere length(Bryan et al., 1998). Lack of defined genetic information in cancer cell lines makes it difficult to study telomerase regulations including TERT expression.

Cancer-associated TERT promoter mutations

In 2013, genome wide sequencing studies on human melanomas identified single nucleotide mutations at the promoter region of TERT(Horn et al., 2013; Huang et al., 2013). These TERT promoter mutations (TPMs) occur at high frequency in other types of tumors as well, especially those derived from low rates of self-renewing cells such as gliomas, myxoid liposarcoma, bladder cancer, urothelial cancer, liver cancer and medulloblastoma(Killela et al., 2013). Within all types of cancer, TPMs are the most frequent non-coding mutation(Heidenreich et al., 2014). Interestingly, TPMs are seldom observed in tumors in high self-renewing tissues such as intestine or blood(Heidenreich et al., 2014). These observations indicate TPMs are selected for specific tissue and cell types.

TPMs were discovered at three major positions in the TERT promoter, -57A/C, -124C/T and -146C/T (each number indicating the position from the translation start site). -57 was discovered in a familial form of melanoma and 124 and -146 were sporadic

mutations(Horn et al., 2013; Huang et al., 2013). All of three mutations form de novo ETS transcription factor binding sites (TTCCGG) in the TERT promoter. There are 27 transcription factors in the ETS family and gene regulatory networks by the ETS factors are important in mammalian development from embryos as well as in cancer(Sharrocks, 2001). One of the ETS factors, GABPa, is suggested to upregulate TERT expression associated with TPMs(Bell et al., 2015). GABPa is the only ETS factor functioning as a heterodimer (with GABPb) and bioinformatics analysis has proposed that the distance between the endogenous ETS binding sites in the TERT promoter and TPMs fits for dimerization of GABPa/b. Another study showed that 146 TPM but not 124 cooperate with the non-NFkb pathway for TERT expression(Li et al., 2015). On top of these conflicting results, there is an indication of involvement of telomere position effect in TERT expression through TPMs(Akıncılar et al., 2016), however further validation is required in genetic and mechanistic manner. TPMs occur as a heterozygous mutation and monoallelic expression associates with epigenetic changes were observed in hepatocellular carcinoma cell lines (Stern et al., 2015).

Patients with TPM tumors have poor survival rate(Borah et al., 2015) and studies in glioma and melanoma revealed tumors with TPMs have shorter telomere length compared to those without TPMs(Ceccarelli et al., 2016; Hayward et al., 2017). As described above, short telomere is protective against tumorigenesis in mouse model, thus this seemingly contradicted observations highlight importance of understanding role of TPMs in telomere maintenance during tumorigenesis. TPMs and mutations of ATRX, a gene involved in telomerase independent telomere maintenance known as ALT (alternative lengthening of telomere)(Bryan et al., 1997; Heaphy et al., 2011), are mutually exclusive(Ceccarelli et al., 2016; Hayward et al., 2017). This means TPMs are required for telomere maintenance in tumors yet whether it is sufficient is not clear. Other cancer associating mutations in the Ras signaling pathway, cell cycle regulation or chromatin remodelers are recurrently acquired with TPMs(Ceccarelli et al., 2016; Hayward et al., 2017). It is important to understand the sequential order of these mutations and how each mutation is genetically interacting during tumorigenesis. Most importantly, dissecting the role of TPMs in cellular proliferation via telomere length regulation is key for understanding of cancer cell immortality. This requires analyzing TPMs in a genetically defined setting.

Genome engineering in human embryonic stem cells

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Thomson et al., 1998), collectively referred to as pluripotent stem cells (hPSCs), are currently used in disease modeling to address questions specific to humans and to complement our insight gained from other model organisms(Soldner and Jaenisch, 2012; Soldner et al., 2011). Recently, genetic engineering using site-specific nucleases has been established in hPSCs (Dekelver et al., 2010; Hockemeyer et al., 2009; Hockemeyer et al., 2011; Yusa et al., 2011; Zou et al., 2009), allowing a level of genetic control previously limited to model systems. Thus, we can now perform targeted gene knock-outs, generate tissue-specific cell lineage reporters, overexpress genes from a defined locus, and introduce and repair single point mutations in hPSCs(Dekelver et al., 2010; Hockemeyer et al., 2009; Hockemeyer et al., 2011; Yusa et al., 2011; Zou et al.,

2009). This ability to genetically engineer pluripotent stem cells will significantly facilitate the study of human disease in a genetically defined setting.

Genetic manipulations of hPSCs by conventional homologous recombination have proven to be too inefficient for routine applications. This poses a challenge in gene targeting of hPSCs, which is attributable to a lower frequency of homology recombination (HR) events. Pioneering experiments using endonucleases, such as SCE-I(Bibikova et al., 2003; Porteus and Baltimore, 2003), have shown homology-directed repair (HDR) events to be stimulated by a DNA double-strand break (DSB) made in close proximity to the recombination site(Jasin, 1996). Since SCE-1 has a fixed recognition site, it has limited use in gene targeting across the genome. These early experiments, however, have led to the development of engineered site-specific DNA nucleases that introduce a DSB at a defined genomic position, thus facilitating gene editing at that site. The first site-specific DNA nuclease to be engineered was the zinc-finger nuclease (ZFN), which is a chimeric protein composed of zinc-finger DNA-binding domains and a FOK1 cleavage domain originally found in *Flavobacterium okeanokoites* (Hockemeyer et al., 2009; Urnov et al., 2005). The zinc finger DNA binding motif establishes specificity to about 3 bases and can be arrayed to recognize longer targeting sequences. ZFNs are designed as pairs with appropriately spaced DNA binding sites that permit the dimerization of the FOK1-nuclease domains. As this dimerization is required for nuclease activity, ZFNs can be engineered to form obligatory heterodimers, which restricts catalytic activity and prevents non-specific DNA cleavage. The most advanced ZFN libraries currently use an array of up to 6 zinc fingers in a ZFN, allowing for an 18 base pair recognition site. An alternative gene-editing tool, known as transcription activator-like effector nucleases (TALENs) has been used to genetically modify hPSCs (Hockemeyer et al., 2011). Like ZFNs, TALENs consist of a DNA-binding domain and a FOK1 nuclease domain(Boch et al., 2009; Moscou and Bogdanove, 2009). However, unlike ZFNs, the DNA-binding domain of TALENs is adapted from transcription activator-like effectors (TALEs), which were originally discovered in the plant pathogen Xanthomonas spp. bacteria and found to alter transcription in plant hosts. TALE DNA-binding is accomplished via repeat domains that correlate recognition of one repeat to one base. The base preference of each repeat domain is determined by two adjacent amino acids called a repeat variable di-residue (RVD)(Miller et al., 2010). By combining different RVDs, TALENs can be designed to target nearly any sequence. In addition to the aforementioned gene-editing tools, RNA-guided nuclease adapted from Cas9 is an emerging genome engineering method with wide ranging applications. Cas9 derived from clustered regularly interspaced short palindromic repeats (CRISPR), is involved in the "adaptive immune system" of microbes(Deveau et al., 2010). The CRISPR system is composed of the Cas nuclease and non-coding RNA elements called guide RNAs. The guide RNAs possess distinct repeat elements derived from exogenous DNA targets known as protospacers and protospacer adjacent motifs (PAMs)(Jinek et al., 2012). The most commonly used CRISPR/Cas9 system is derived from *Sterptococcus pyogenes* and bears the PAM sequence 5'-NGG-3'. The 20 base pairs of sequence immediately preceding PAM sequence is fused with the other trans activating RNA components, and the resulting chimeric RNA is used as a single-guide RNA (sgRNA)(Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Due to its simplicity of preparation, the CRISPR/Cas9 system have been commonly used for genome editing compared to ZFNs

and TALENs, yet each approach has its pros and cons, which may dictate the choice for a given experiment(Fu et al., 2013; Hsu et al., 2013; Ran et al., 2013).

Several genes have now been successfully targeted in human cells using site-specific nucleases. The easiest approach involves disruption of gene function by non-homologous end-joining (NHEJ)(Perez et al., 2008; Reyon et al., 2012), while more complex modification of the genome are generally achieved by creating a site-specific DSB with a nuclease and providing a donor DNA template to promote resolution by HDR(Hockemeyer et al., 2009; Hockemeyer et al., 2011). Several proof-of-concept experiments illustrate the utility of this approach. For example, the AAVS1 locus as a "safe harbor" locus to enable the ectopic expression of genes in hPSCs(Smith et al., 2008). This locus is dispensable in hPSCs, does not show strong variegation and silencing effects, and can be used for the robust expression of genes in hPSCs and differentiated cell types. In addition, endogenous genes, such as Oct4, could be modified using site-specific nucleases with a repair donor plasmid that integrates a GFP reporter gene(Hockemeyer et al., 2009; Hockemeyer et al., 2011). Further, to introduce a so-called "scarless" point mutation with a single base pair resolution, homology-directed repair is used with single strand oligonucleotide coupled with FAC-sorting(Soldner et al., 2011). Additionally, site-specific nucleases could be used for a targeted chromosomal deletion in hPSCs(Lee et al., 2010; Orlando et al., 2010; Yang et al., 2013). As shown in prior studies, there are innumerable applications for gene targeting using site-specific nucleases. Using genome editing in hPSCs, it is possible to study regulation of the endogenous telomerase or introducing de novo TPMs in genetically defined manner.

CHAPTER TWO

Endogenous TERT N-terminal tagging affects human telomerase function at telomeres *in vivo*

Based on Chiba et al., *Molecular & Cellular Biology* 2017

ABSTRACT

Telomerase action at telomeres is essential for the immortal phenotype of stem cells and the aberrant proliferative potential of cancer cells. Insufficient telomere maintenance can cause stem cell and tissue failure syndromes, while increased telomerase levels are associated with tumorigenesis. Both pathologies can arise from only small perturbation of telomerase function. To analyze telomerase at its low endogenous expression level, we genetically engineered human pluripotent stem cells (hPSCs) to express various N-terminal fusion proteins of the telomerase reverse transcriptase from its endogenous locus. Using this approach, we uncovered that these modifications can perturb telomerase function in hPSCs and cancer cells, resulting in telomere length defects. Biochemical analysis suggests that this defect is multi-leveled including changes in expression and activity. These findings highlight the unknown complexity of telomerase structural requirements for expression and function *in vivo*.

INTRODUCTION

Telomerase is a reverse transcriptase that synthesizes *de novo* telomeric repeats, which have the sequence 5'-TTAGGG-3' in vertebrates, and thereby maintains telomeres (Blackburn et al., 2006; Greider and Blackburn, 1985). Its enzymatic activity counteracts terminal sequence loss from linear chromosomes and ensures the long-term proliferative capacity of human stem cells (Armanios and Blackburn, 2012). When aberrantly active, telomerase expression can grant the immortal phenotype of human cancer cells (Kim et al., 1994). The minimal components for catalytic activity reconstitution are the protein component telomerase reverse transcriptase (TERT) and the non-coding telomerase RNA (TR), which bears the template region to synthesize telomeric repeats. Telomerase is expressed at low levels in stem cells and cancer cells. Estimates range from 50 to a few hundred functional RNPs generated from 2-20 copies of TERT mRNA per cell (Cohen et al., 2007; Ducrest et al., 2002; Xi and Cech, 2014; Yi et al., 2001). Under these conditions, telomerase can be in substoichiometric abundance in relation to the number of telomeres that are present after DNA replication. Telomerase is not generally present at telomeres, but is actively recruited during S-phase to a subset of telomeres through protein-protein interactions that occur between telomerase and the telomere. This interaction is mediated by the N-terminal domain of TERT called the TEN-domain (Telomerase Essential N-terminal domain) (Podlevsky and Chen, 2012) and the telomere by the shelterin complex, a six-member protein complex (Doksani and de Lange, 2014). Specifically, a small region in the shelterin protein TPP1 called the TEL-patch interacts with the telomerase TEN-domain (Abreu et al., 2010; Nandakumar et al., 2012; Sexton et al., 2014; Tejera et al., 2010; Wang and Lei, 2011; Wang et al., 2007; Xin et al., 2007; Zhong et al., 2012). This interaction is essential for telomere maintenance as cells genetically engineered to lack an acidic loop within the TEL-patch phenocopy telomerase knockout cells (Sexton et al., 2014). Furthermore, residue swap experiments that exchange critical amino acids in the TEN-domain and the TEL-patch indicate a direct interaction between TERT and TPP1 (Schmidt et al., 2014). Beyond telomerase recruitment, TPP1, together with its shelterin interacting partner POT1, can have additional activating and inhibitory roles on telomerase action at telomeres as reviewed in (Hockemeyer and Collins, 2015) and (Schmidt and Cech, 2015).

TPP1 binds to the telomere through its interaction with TIN2, which itself binds to the double stranded telomeric binding proteins TRF1 and TRF2 (Ye et al., 2004a). In addition, TPP1 recruits the single stranded binding protein POT1 to telomeres (Liu et al., 2004; Ye et al., 2004b). Perturbation of the shelterin protein-interaction network by overexpression or loss-of-function results in telomere length changes in human cells (Hockemeyer et al., 2005; Kim et al., 1999; Loayza and De Lange, 2003; Smogorzewska et al., 2000; van Steensel and de Lange, 1997; Ye et al., 2004b). However, how these proteins function in cells in which telomere length is at homeostasis is not well understood. At telomere homeostasis, telomere shortening caused by nucleolytic degradation and the end replication problem is at equilibrium with telomere elongation. Yet, telomeres at different chromosomes ends within one cell or telomeres of the same chromosome within a cell population can differ in length. Previous experiments suggest that overall telomere length homeostasis is established by a process that stochastically elongates shorter telomeres preferentially over long telomeres (reviewed in (Hockemeyer

and Collins, 2015). The underlying counting mechanism that distinguishes telomeres of different length and communicates this to telomerase is currently not well understood.

Several lines of evidence indicate that telomerase, and particularly the process of telomerase recruitment to individual telomeres, must be studied in the context of the physiological expression levels regulated within the endogenous genetic context. Importantly, overexpression of telomerase in human cells leads to rapid telomere elongation that has been suggested to be unrestrained and not subject to the regulatory mechanisms that establish telomere homeostasis(Cristofari and Lingner, 2006). This excessive action of telomerase at telomeres suggests that overexpression of telomerase can bypass the transient nature of telomerase localization to telomeres; when overexpressed, several TERT molecules constitutively localize to most telomeres within a cell, which is not observed in naturally telomerase-positive cells (Zhong et al., 2012).

Until recently, direct observation of telomerase action at telomeres without overexpression has been considerably hampered by the lack of a reliable antibody detecting endogenous levels of TERT. In fixed cells, fluorescence *in situ* hybridization (FISH) for the telomerase RNA or the localization of Cajal bodies to telomeres have been used as proxies for the localization of TERT to telomeres (Cristofari and Lingner, 2006; Stern et al., 2012). However, recent genetic data suggest that these associations might not be directly reporting on telomerase action at telomeres (Chen et al., 2015; Tomlinson et al., 2010; Vogan et al., 2016). With the advent of genome editing in human pluripotent stem cells, an experimental system became available that can overcome these challenges. Robust protocols to genetically modify human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) (Hockemeyer and Jaenisch, 2016; Hockemeyer et al., 2009; Hockemeyer et al., 2011; Soldner et al., 2011), collectively referred to as hPSCs, have recently become available. With these technical developments, epitope tags or fluorescent reporter genes can now be inserted into the hPSC genome to endogenously mark cells for imaging or biochemical purification.

hPSCs are an ideal model system to study telomerase regulation, as they are telomerase-positive (Takahashi et al., 2007; Thomson et al., 1998), but can be rapidly differentiated into telomerase-negative cells. Moreover, the reverse process of telomerase reactivation can be studied during the reprogramming of somatic cells into iPSCs(Agarwal et al., 2010; Batista et al., 2011). Due to their constitutive telomerase expression, hPSCs are immortal. However, in contrast to tumor cells, they have functional DNA surveillance and cell cycle checkpoints and therefore are well suited for studying the effects of telomere length regulation and dysfunction on these pathways.

Here, we report on the analysis of genetically engineered hPSCs that express TERT with a variety of N-terminal fusions from the endogenous locus. We chose to modify the N-terminus instead of the C-terminus of TERT because the addition of a hemaglutinin-tag (HA-tag) at the C-terminus of TERT results in a striking defect in telomere maintenance despite almost wild-type enzymatic activity *in vivo*(Counter et al., 1998). Interestingly, separation-of-function mutants in TERT termed DAT mutants that retain catalytic activity but fail to immortalize human primary fibroblasts have been identified at both the C-terminus and in the N- terminal TEN-domain(Armbruster et al., 2001; Armbruster et al., 2004; Banik et al., 2002; Counter et al., 1998). The N-terminal DAT mutants alter residues subsequently identified as part of the interaction interface

with TPP1 required for localization and activity of telomerase at telomeres(Schmidt et al., 2014; Sexton et al., 2014; Stern et al., 2012). Mutations near the N-terminus can also have pronounced defects on TERT's ability to sequentially add multiple telomeric repeats without dissociating from the telomere, a specialized feature of telomerase called repeat addition processivity (RAP)(Greider, 1991).

We employed a genome editing strategy to tag TERT endogenously at its N-terminus based on a protocol that we previously established to genetically engineer hPSCs to carry mutations in the TERT promoter (Chiba et al., 2015). This protocol was developed to engineer hPSCs that carry mutations in the TERT promoter, representing the most frequent noncoding mutations in human cancer as reviewed in (Heidenreich et al., 2014). Here, we have extended this targeting strategy to test several N-terminal modifications of TERT with the goal of directly tagging TERT in hPSCs to increase the ease of immunofluorescence and biochemical experiments. Unexpectedly, during these experiments we observed significant changes in telomerase biology as a result of N-terminal tagging. We demonstrate that most N-terminal fusion proteins generated by inserting sequences at the translational start site of TERT show a pronounced defect in telomerase action at telomeres and result in reduced telomere length. Biochemical and cellular assays suggest that this defect is multi-factorial, indicating that much remains to be learned about the cellular mechanisms for regulation of telomerase action at telomeres.

RESULTS

Endogenous epitope tagging of TERT using genome editing

To understand the regulation of TERT in living cells, we engineered the endogenous TERT locus in hESCs to carry a Halo-tag (Los et al., 2008) at the N-terminus (Halo-TERT hESCs). A Halo-tag covalently binds to its synthetic ligands conjugated with fluorescent dyes such as Tetramethylrhodamine (TMR). We chose this strategy over conventional GFP tagging as it is expected to result in more photostable and brighter fluorescently-marked proteins and thereby facilitates single molecule imaging. To establish hESC lines with TERT tagged at the N-terminus, we modified the two-step, scar-less genome editing of the TERT locus that we previously reported (Chiba et al., 2015) (Figure 2.1A). First, the region between -1462 and +67 base pairs (bp) relative to the translational start site (ATG) of the TERT gene was deleted using two Cas9s. Cells homozygous for this deletion ($TERT^{\Delta/\Delta}$) lacked TERT expression, and consequently, telomerase activity. As a result, these cells showed progressive telomere shortening and died about 150 days after the deletion event (Sexton et al., 2014). However, in cells that were subjected to a second targeting step that reintroduced the deleted element, telomerase activity and cellular viability was restored and telomeres elongated back to a wild-type length over time. This second editing step was conducted using a specific sgRNA against the new junction (-1462 to +67) to reinsert into the deleted region either the wild-type sequence or sequences that insert a tag after the first ATG (Figure 2.1A). After the second targeting, targeted cells restore TERT expression and are gradually enriched while untargeted parental $TERT^{\Delta/\Delta}$ hESCs die at around 150 days (Figure 2.1B).

Initially, we focused on generating cells that expressed an N-terminal Halo-tag-targeted TERT from the endogenous locus. We successfully generated Halo-TERT expressing cells that proliferated past the proliferative capacity of $TERT^{\Delta/\Delta}$ cells (data not shown). However, direct comparison of Halo-TERT and isogenic wild-type cells (wt), in which we introduced the wild-type sequence without the tag, revealed a striking proliferation defect of Halo-TERT hESCs. Halo-TERT cells had reduced colony size and showed cell morphology associated with dysfunctional telomerase in hESCs (18). Proliferation and viability of these cultures were impacted too severely to permit further characterization.

To test the hypothesis that these proliferation defects were due to the endogenous N-terminal tagging of TERT, we decided to systematically test the effects of the smaller epitope tags 3xFlag (abbreviated below as Flag), HA or YbbR-TEV-3xFlag (YbbR; a label-accepting tag derived from the ybbR open reading frame of the *Bacillus subtilis* genome (Yin et al., 2005)) inserted into the endogenous TERT locus. (Table 2.1). In addition, we tested whether the addition of a flexible polypeptide linker between the Flag tag and TERT would restore TERT function. To this end we directly compared Flag-TERT and 3xFlag-GS10 (Flag-GS10) and 3xFlag-GS20 (Flag-GS20), where GS10 and GS20 indicate linkers consisting of 10 or 20 repeats of glycine and serine, respectively (Table 2.1).

Each of these editing approaches in $TERT^{\Delta/\Delta}$ were successful in restoring cellular viability past the proliferative capacity of $TERT^{\Delta/\Delta}$. Next, single-cell derived targeted cells were isolated and genotyped by PCR and Southern blotting (Figure 2.1C and D;

Figure 2.3B and C). As expected from our previous report (Chiba et al., 2015), in each case (wild-type and all of the N-terminal TERT tagging), only one of the deleted TERT loci was restored resulting in hemizygous cell lines TERT $^{tag/\Delta}$ (Figure 2.1D).

Short telomeres associated with endogenous tagging of TERT

We analyzed TERT mRNA expression in the genetically engineered hESCs (Figure 2.2A). RT-qPCR analysis of the targeted cells established that TERT mRNA expression in all tagged lines, with the exception of YbbR-TERT, was similar to cells edited to restore the wild-type untagged TERT. In YbbR-TERT cells, TERT expression was reduced to about 40% compared to wild-type cells and these cells showed a slight proliferative defect that was less severe than the one seen in Halo-TERT cells. Despite this mild proliferation defect, YbbR-TERT cells could be maintained in culture more than 100 days and were used for further analysis. All other cell lines proliferated at a rate indistinguishable from the wild-type cells. All tagged hESCs, including YbbR-TERT cells, remained pluripotent based on the expression of the pluripotency marker OCT4 (Figure 2.2A). Next, we analyzed telomerase activity in these cells. YbbR-TERT cells had a slight reduction in telomerase activity when measured using the TRAP assay (Figure 2.2B). We previously established that the telomerase RNA hTR, rather than TERT, is limiting for telomerase activity in wild-type hESCs and that TERT+/- cells do not have a 50% reduction in telomerase activity (Chiba et al., 2015). This argues that the reduced TERT mRNA level in YbbR-TERT cells cannot fully account for their reduced telomerase activity. A similar reduction in activity was also evident in HA-TERT cells, despite the wild-type TERT expression in these cells. Cells expressing Flag-TERT, regardless of the presence or size of a GS linker, had overall levels of telomerase activity equivalent to wild-type TERT cells (Figure 2.2B).

To further characterize telomerase function in these endogenous-locus tagged TERT hESCs, we measured telomere length at several time points after the second editing step. Before the second editing event, which restores TERT function, telomeres were short but elongated in cells complemented with the wild-type sequence (without tag) over time back to the telomere length set-point of the original hESC wild-type cells. Surprisingly, all tagged versions of TERT except Flag-GS10-TERT were unable to elongate telomeres to a length similar to isogenic wild-type TERT cells (Figure2. 2C). The median telomere hybridization signal intensity in wild-type TERT cells increased from 6.3 kilobases (kb) to 7.8 kb over the term of 64 days. In contrast, telomeres remained stable at 4.5 kb in HA-TERT cells and at 3.5 kb in YbbR-TERT cells over the same period of time (Figure 2.2D). These defects in restoring telomere length could be explained by the reduced TERT expression in YbbR-TERT cells and the reduced telomerase activity in YbbR-TERT and HA-TERT cells. However, Flag-TERT and Flag-GS20-TERT also showed a deficit in telomere elongation (Figure 2.2C). Their telomeres elongated from 6.1 kb to 6.8 kb and 5.6 kb to 6.4 kb respectively (Figure 2.2D). Shorter telomeres in Flag-TERT and Flag-GS20-TERT cells cannot be simply explained by reduction of expression levels of TERT or telomerase activity as these did not differ significantly from isogenic wild-type edited cells (Figure 2.2A).

To exclude the possibility that these shorter telomeres were artifacts of this particular genome editing experiment, three independent experiments were performed, and they confirmed the reproducibility of shorter telomeres associated with endogenous tagging of TERT (Figure 2.2E). Telomeres in Flag-GS10-TERT cells elongated to a similar extent as cells restored to wild-type in these independent experiments (Figure 2.2E).

Next, we analyzed the telomere length distribution of individual clones isolated after the second targeting step (Figure 2.3). In wild-type TERT cells, telomeres showed some telomere length heterogeneity between clones, but where on average longer than . telomeres in Flag- and YbbR-TERT cells (Figure 2.3A). Flag-TERT clones #1, #5, #7, #11, #12, #13 and #15 possessed significantly shorter telomeres (Figure 2.3A). Based on Southern blot data, these clones were aberrantly targeted at the 5' end of TERT. Additional Southern blot genotyping using three different probes revealed that these clones had an aberrant integration of the donor plasmid backbone 5' upstream of the right homology arm (Figure 2.3B and C). Similar events have been described previously (Hockemeyer et al., 2009; Rouet et al., 1994) and are possibly the result of heterologous targeting where one side (here the 3' homology arm) of the double stranded break is repaired by homology mediated repair and the other side by non-homologous end joining. Still, when analyzing only correctly targeted cells, telomeres in Flag-TERT clones were collectively shorter than telomeres compared to wild-type TERT cell lines (Figure 2.3A and D). This analysis highlights that the defect in telomere elongation is highly penetrant, and is not the result of the aberrant behavior of a subset of cells.

Telomere length comparison between Flag and Flag-GS10 cells suggested that including a GS10 linker between TERT and the Flag tag could alleviate the telomere length defect. To test if this is a general feature of N-terminal tagging of TERT, we evaluated the impact of the GS10 linker in combination with the HA tag. Again, HA-TERT cells showed significant defects in telomere length maintenance, despite wild-type levels of TERT expression and telomerase activity (Figure 2.2A, B; 2.4A and B). In contrast, HA-GS10-TERT cells had almost wild-type telomere lengths (Figure 2.4C). These results indicate that the insertion of a GS10 linker can reduce defects associated with genetically tagging the TERT N-terminus.

Slower telomere elongation kinetics by overexpression of Flag-TERT relative to untagged TERT

N-terminal tagging of TERT is often used as a modification to mark overexpressed TERT. Therefore, we wanted to determine whether the effects that we detected for the N-terminal tagging of TERT at its endogenous locus in hESCs are specific to the stem cell system compared to the field-standard transgene-mediated TERT overexpression in cancer cell lines. To this end, we knocked out TERT ($TERT^{-/-}$) in HCT116 cells (Vogan et al., 2016), a colon carcinoma cell line, using the same strategy as previously described in hESCs (Sexton et al., 2014). In these $TERT^{-/-}$ cells, telomeres shorten progressively, and cells eventually die due to critically short telomeres (Vogan et al., 2016). If TERT is ectopically expressed in these cells, viability and telomere maintenance are restored and telomeres elongate. We used this assay to measure the

ability of variously tagged forms of TERT to elongate telomeres. We overexpressed either wild-type or 3xFlag-GS-TERT (see Table 2.1 for AAVS1 transgene tag sequence) driven by a CAGGS promoter from the AAVS1 locus (Figure 2.5A). After insertion of these TERT overexpression constructs into the AAVS1 locus of $TERT^{-/-}$ cells, telomere length gradually recovered in TERT and 3xFlag-GS-TERT cells. Yet, 3xFlag-GS-TERT overexpressing cells showed slower telomere elongation rates compared to wild-type TERT overexpression, despite 3xFlag-GS-TERT cells having greater telomerase activity and protein expression levels (Figure 2.5B, C and D).

Next, we addressed the effects of TERT overexpression in hESCs that were either Flag-TERT, YbbR-TERT, or wild-type ($TERT^{wt/\Delta}$) at the endogenous locus. In these cells, we overexpressed either TERT, 3xFlag-TERT, TR or GFP from the AAVS1 locus following integration of these by genome editing (Figure 2.6A). In agreement with our previous reports (Chiba et al., 2015), TERT overexpression was almost neutral in hESCs , resulting in only modest telomere elongation (Figure 2.6B). In contrast, TR overexpression led to dramatic telomere elongation in wild-type cells (Figure 2.6B). Similarly, in endogenous-locus Flag-TERT cells, overexpression of TERT or Flag-TERT from the AAVS1 locus was largely neutral while TR overexpression led to telomere elongation (Figure 2.6B). However, a strikingly inverse outcome was observed in YbbR cells. In these cells, TERT or 3xFlag-TERT overexpression resulted in pronounced telomere elongation while telomere elongation due to TR overexpression was blunted (Figure 2.6B). These results highlight that N-terminal tagging of TERT can lead to distinct defects. In YbbR-TERT cells, TERT expression is limiting for telomerase levels. In contrast, TERT in Flag-TERT cells is not limiting and the reduced telomere elongation in these cells appears to results from a post-assembly defect of telomerase RNP containing Flag-TERT. These data demonstrate that N-terminal modifications of TERT can impair telomerase function in both stem cells at a physiologically endogenous expression and also upon TERT overexpression in cancer cells.

N-terminally tagging of TERT results in reduced telomerase repeat addition processivity

Previous studies showed that the TEN-domain of TERT plays important roles for repeat addition processivity (RAP). We hypothesized that N-terminal tagging of TERT might disrupt RAP. To specifically test this hypothesis, we measured the processivity of endogenously tagged telomerase. We used an oligonucleotide-purification method (Wu et al., 2015) for the purification of human telomerase from genetically engineered hESCs. This purification enables enrichment of endogenous telomerase, independent of an epitope tag. Lysates from genetically engineered hESCs were incubated with the telomeric template affinity oligonucleotide beads for binding. Following serial washes, enriched telomerase was eluted from the beads using the displacement oligonucleotide, which cannot be extended by telomerase due to addition of 2'-3'-dideoxycytidine at the 3'-end. Then, the eluted fraction was assayed for telomerase activity using a direct extension assay in the presence of excess telomeric-repeat primer, which allows to assay RAP (Figure 2.7A). All N-terminally tagged telomerases tested showed reduced RAP compared to telomerase isolated from isogenic cells edited to express wild-type TERT (Figure 2.7B). This result was confirmed in several independent experiments (Figure

2.7C). These data suggest that shorter telomeres in the tagged cells could in part result from reduced RAP of N-terminally modified telomerases.

DISCUSSION

Key regulatory steps of telomerase involve its assembly, recruitment to, and activity at telomeres. Here, we report that N-terminal amino-acid additions to TERT can impair telomerase function in multiple ways: TERT mRNA expression levels can be changed, and telomerase's overall activity, as well as its RAP, can be reduced (summarized in Table 2.2). We do not fully understand the molecular basis for all of these defects, yet our findings allow us to draw the key conclusion that N-terminal fusions to TERT can adversely alter TERT expression and protein function, resulting in telomere maintenance at a reduced telomere length set-point. These findings are important for future attempts to investigate steps of telomerase regulation and telomere length control at physiological telomerase expression levels.

Based on our experiments, we predict that the successful visualization of TERT in naturally telomerase-positive primary cells will be more challenging than has been previously demonstrated for Hela and HEK 293 cells (Schmidt et al., 2016; Xi et al., 2015). At least in HEK293 cells, TERT tagging resulted in an approximately 8-fold higher total TERT mRNA expression. This could suggest that the targeting strategy used can result in an epigenetic lesion aberrantly increasing TERT expression after editing. For both cell types, Hela and HEK 293 cells, only a subset of TERT gene loci has been modified, so that the tagged-TERT is expressed in the presence of wild-type protein (Schmidt et al., 2016; Xi et al., 2015). It is therefore possible that the residual wild-type TERT maintained telomeres in these cells, while the tagged TERT might have been predominantly TERT alone (free of hTR) or assembled into functionally compromised enzyme. It is important for future studies of active RNP localization to use an editing strategy that ensures that the visualized TERT protein is at an endogenous expression level and has endogenous TERT function in the absence of any wild-type TERT. Our findings suggest that pluripotent stem cells provide a sensitive system that can detect physiologically perturbed cellular regulation of telomere length maintenance. Due to this sensitivity, we expect that using the stem cell system will uncover novel nuances of telomerase regulation and telomere maintenance.

Our findings raise questions of how to most usefully epitope tag TERT at its endogenous locus in future studies. From our comparison between HA-TERT and HA-GS10-TERT, we conclude that the addition of a flexible spacer can reduce the negative effects of TERT N-terminal tagging on telomere maintenance. However, cells edited to express the Flag-GS20-TERT allele showed a reduced telomere elongation rate compared to cells restored to wild-type, suggesting that the linker length impacts tagged TERT function. Our experiments identified Flag-GS10 or HA-GS10 as N-terminal TERT tagging modifications not deleterious for telomerase function. These tags will aid in the biochemical characterization of TERT in stem cells as well as visualization of TERT in fixed samples but do not provide a strategy for single-molecule live-cell imaging of TERT.

Previous experiments established that the linker region between the TEN-domain and the remainder of TERT is dispensable for catalytic activity of telomerase (57). Based on this, we hypothesized that an insertion of Halo in this linker region could allow for the functional tagging of TERT. We tested this by inserting a Halo, YbbR and Flag tag at a position between Y325 and A326 of TERT. Unfortunately, these experiments have been

so far unsuccessful in generating a TERT with retained biological function (data not shown). Similarly, adding a GS10 linker between TERT and an N-terminal Halo tag (Halo-GS10 TERT hESCs) did not fully restore TERT function to wild-type level (data not shown).

The defects in telomere elongation in Flag-TERT cells in comparison to wild-type TERT cells could be caused by recruitment defects of tagged telomerase, similar to previously described TERT DAT mutations(Armbruster et al., 2001; Armbruster et al., 2004; Banik et al., 2002; Counter et al., 1998), in addition to the RAP impairment. Understanding the molecular basis for telomerase recruitment to and RAP at telomeres is highly relevant to telomere biology, as a subset of disease-associated mutations in TERT are specifically defective in RAP or impair recruitment (Gramatges et al., 2013; Robart and Collins, 2010; Zaug et al., 2013). Currently, studies of these TERT mutants are limited by the need for overexpression of TERT to characterize their biochemical defects. The assay used here to characterize the RAP defects associated with tagging the N-terminus of TERT is highly sensitive and can relate changes in RAP to changes in telomere length. This approach provides a system to characterize the impact of RAP defects on telomere length in an endogenous telomerase subunit expression setting.

Figure 2.1

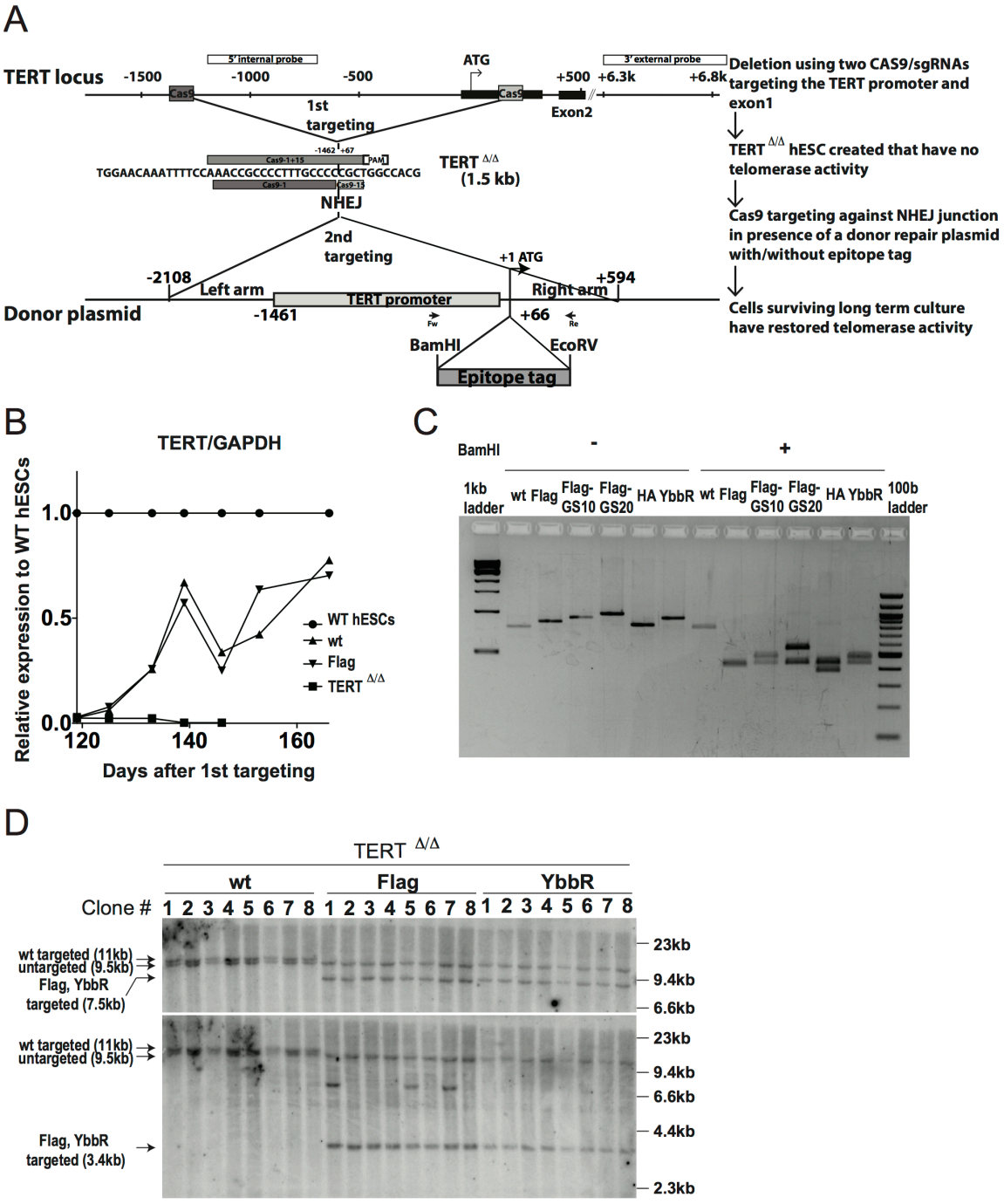

A
TERT locus
-1500
5' internal probe
-1000
-500
ATG
+500
Exon2
+6.3k
3' external probe
+6.8k
1st targeting
TERT Δ/Δ (1.5 kb)
TGGAACAAATTTTCCAAACCGCCCCTTTGCCCCCGCTGGCCACG
NHEJ
2nd targeting
+1 ATG
-2108
Left arm
TERT promoter
Right arm
+594
Donor plasmid
-1461
+66
BamHI
EcoRV
Epitope tag
Deletion using two CAS9/sgRNAs targeting the TERT promoter and exon1
TERT Δ/Δ hESC created that have no telomerase activity
Cas9 targeting against NHEJ junction in presence of a donor repair plasmid with/without epitope tag
Cells surviving long term culture have restored telomerase activity
B
TERT/GAPDH
Relative expression to WT hESCs
1.0
0.5
0.0
120
140
160
Days after 1st targeting
WT hESCs
wt
Flag
TERT Δ/Δ
C
BamHI
-
+
1kb ladder
wt Flag
Flag-GS10
Flag-GS20
HA YbbR
wt Flag
Flag-GS10
Flag-GS20
HA YbbR
100b ladder
D
TERT Δ/Δ
wt
Flag
YbbR
Clone # 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
wt targeted (11kb)
untargeted (9.5kb)
Flag, YbbR targeted (7.5kb)
wt targeted (11kb)
untargeted (9.5kb)
Flag, YbbR targeted (3.4kb)
23kb
9.4kb
6.6kb
23kb
9.4kb
6.6kb
4.4kb
2.3kb

Figure 2.1
Generation of endogenously N-terminally tagged hESCs using scarless two-step genome-editing
(A) Schematic overview of the two-step genome-editing approach used to insert epitope tags into the N-terminus of endogenous TERT. First, a TERT knock-out cell line ($TERT^{\Delta/\Delta}$) that lacks the region between 1.5 kb upstream and 66 bp downstream of the 1st ATG was established using two Cas9/sgRNAs (sg-1 and sg-15). Second, an sgRNA against the newly synthesized NHEJ-derived junction (-1462 and +67: sg1+15) was co-electroporated with donor plasmids containing the deleted regions with, or without, epitope tags after the 1st ATG of TERT. After the second targeting, the cells were passaged until all of the parental $TERT^{\Delta/\Delta}$ hESCs died due to telomere shortening.
(B) Relative expression levels of TERT mRNA measured by quantitative RT-PCR over a time course after genome-editing (day 0: first editing; day 86: second editing). Expression of targeted wild-type (wt) and Flag-TERT hESCs are relative to non-targeted original WT hESCs. $TERT^{\Delta/\Delta}$ cells are plotted until the last time point before $TERT^{\Delta/\Delta}$ cultures died. Expression of TERT was normalized to GAPDH. wt and $TERT^{\Delta/\Delta}$ cell data are identical to the data in (Chiba et al., 2015).
(C) PCR genotyping of the targeted cells after selection. Bulk DNA was used for PCR over the first ATG and the products were digested with BamHI to confirm the insertion of epitope tags.

(D) Southern blot genotyping of targeted hESCs. After all $TERT^{\Delta/\Delta}$ hESCs died, single cell-derived colonies were isolated from bulk populations. Genomic DNA was digested with BamHI and blotted using either 3'-external (top) or 5'-internal (bottom) probes. Untargeted allele appears as a 9.5 kb band. The size of the correctly targeted allele is 11 kb (wild type) or 7.5 kb (Flag, YbbR), respectively, for the external probe and 11 kb (wild type) or 3.4 kb (Flag, YbbR), respectively, for the internal probe.

Figure 2.2

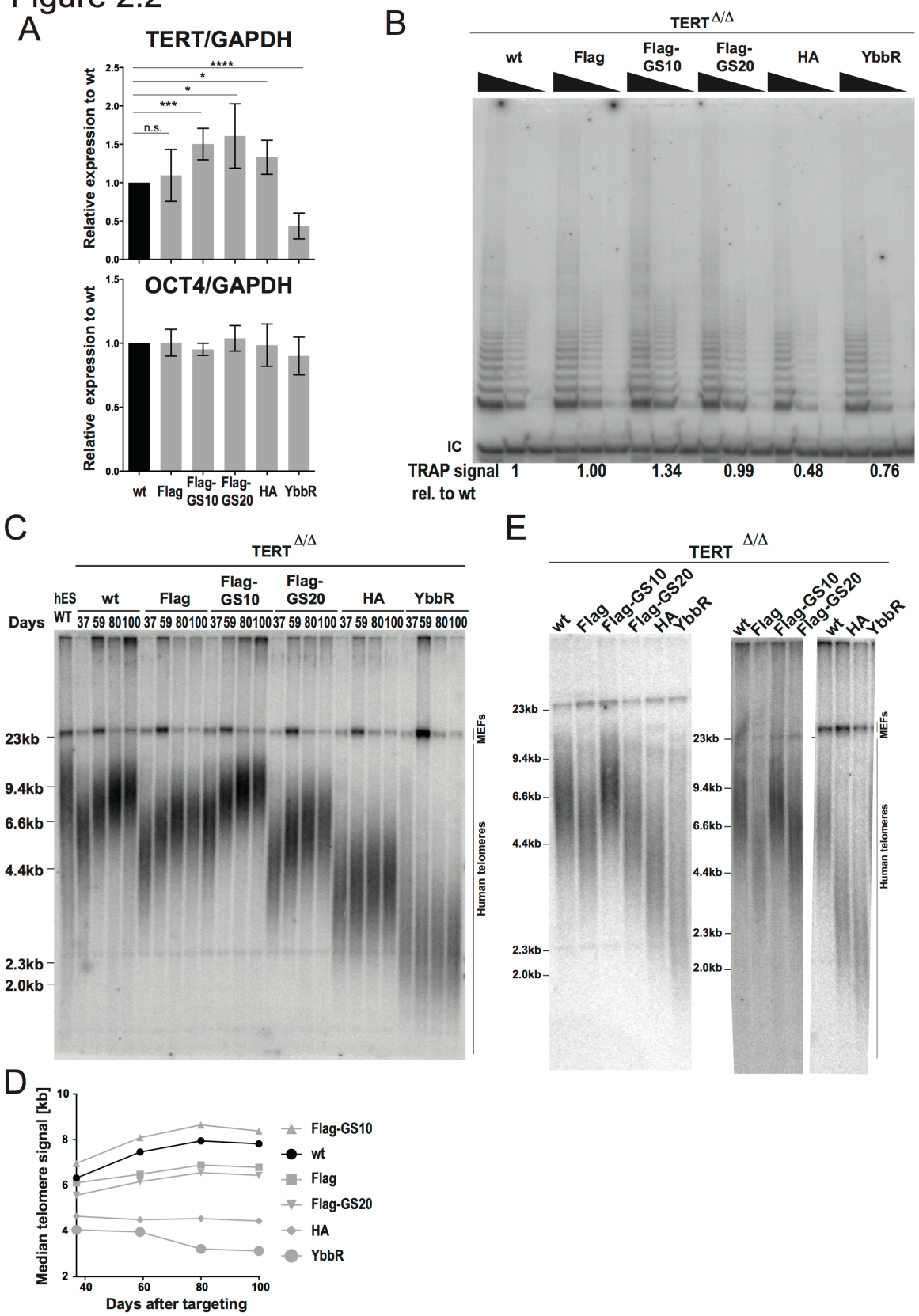

A
TERT/GAPDH
Relative expression to wt
n.s.
***
*
*
****
OCT4/GAPDH
Relative expression to wt
wt
Flag
Flag-GS10
Flag-GS20
HA
YbbR
B
TERT Δ/Δ
wt
Flag
Flag-GS10
Flag-GS20
HA
YbbR
IC
TRAP signal rel. to wt
1
1.00
1.34
0.99
0.48
0.76
C
TERT Δ/Δ
hES WT
wt
Flag
Flag-GS10
Flag-GS20
HA
YbbR
Days
37 59 80 100
23kb
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
MEFs
Human telomeres
D
Median telomere signal [kb]
Days after targeting
Flag-GS10
wt
Flag
Flag-GS20
HA
YbbR
E
TERT Δ/Δ
wt
Flag
Flag-GS10
Flag-GS20
HA
YbbR
23kb
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
MEFs
Human telomeres

Figure 2.2
Endogenous tagging of TERT disrupts telomere maintenance and results in shorter telomere length compared to wild-type hESCs
(A) Relative expression level of TERT and OCT4 mRNA in the endogenously epitope tagged hESCs compared to isogenic wt cells measured by quantitative RT-PCR. Expression of TERT and OCT4 were normalized to GAPDH. n.s. $p>0.05$, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ (two-tailed Student's t-test).
(B) Telomeric repeat amplification protocol (TRAP) assay of whole cell extracts from non-clonal wt, Flag, Flag-GS10, Flag-GS20, HA and YbbR hESCs using a decreasing amount of protein (200, 40, and 8 ng). TRAP signals relative to wt hESCs were quantified and are shown at the bottom of the lanes (40 ng).
(C) Telomere restriction fragment assay of the targeted bulk population hESCs over a time course after targeting (day 37, 59, 80, and 100 after the second targeting). Restoration of telomerase after the second targeting resulted in substantial telomere elongation and an overall increase in telomere signal intensity. 2 µg of genomic DNA after digestion with MboI and AluI were loaded in each lane and hybridized with a TTAGGG radioactive probe.
(D) Quantification of median telomere length shown in (C).
(E) Telomere restriction fragment assay of the indicated cell lines that independently targeted from (C). Samples were collected at day 51 (left gel), 53 (middle) and 37 (right) respectively after the second targeting.

Figure 2.3

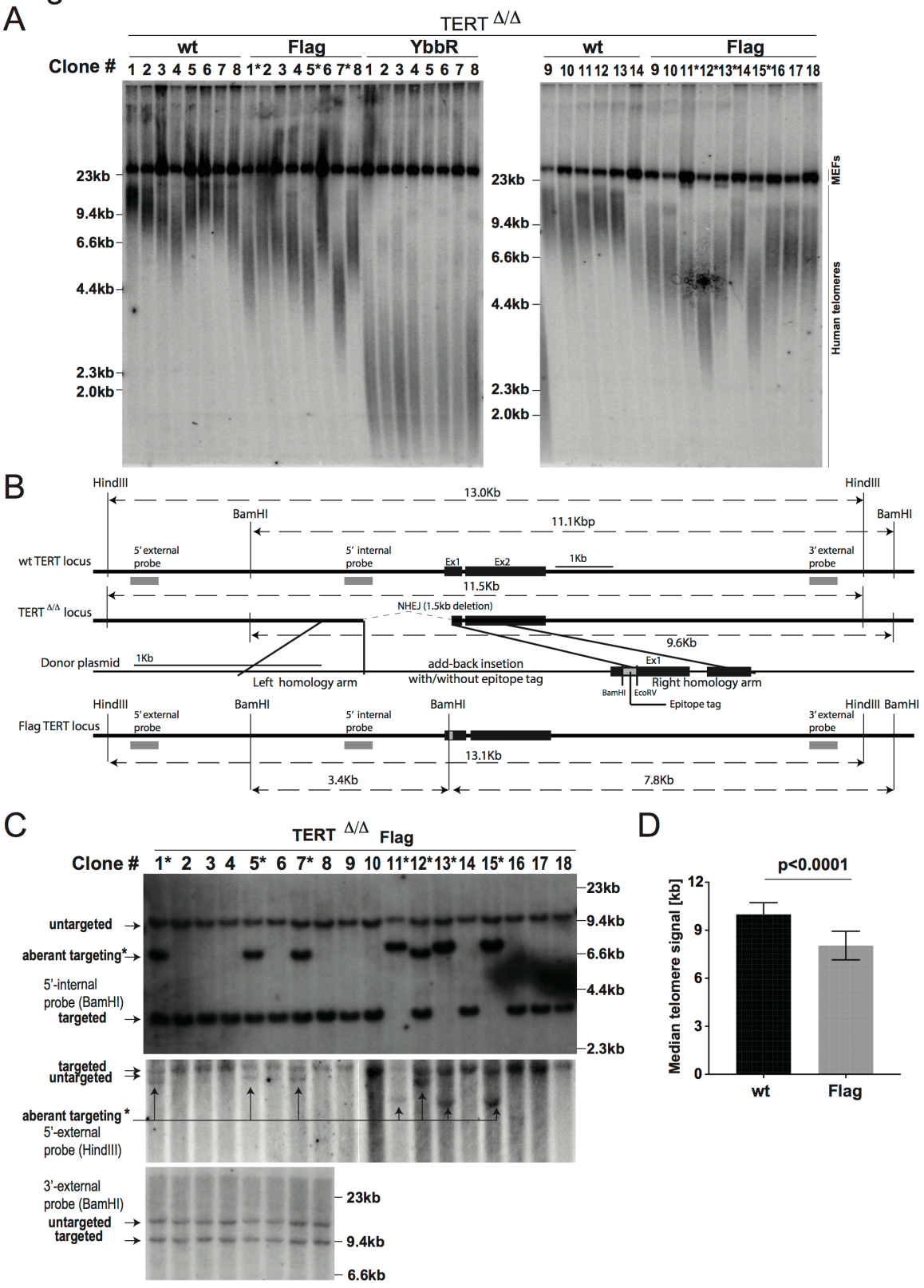

A
TERT Δ/Δ
wt
Flag
YbbR
wt
Flag
Clone # 1 2 3 4 5 6 7 8 1*2 3 4 5*6 7*8 1 2 3 4 5 6 7 8
9 10 11 12 13 14 9 10 11*12*13*14 15*16 17 18
23kb
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
MEFs
Human telomeres
B
HindIII
BamHI
13.0Kb
11.1Kbp
wt TERT locus
5' external probe
5' internal probe
Ex1
Ex2
1Kb
3' external probe
11.5Kb
TERT Δ/Δ locus
NHEJ (1.5kb deletion)
9.6Kb
Donor plasmid
1Kb
Left homology arm
add-back insetion with/without epitope tag
Ex1
Right homology arm
BamHI
EcoRV
Epitope tag
Flag TERT locus
13.1Kb
3.4Kb
7.8Kb
C
TERT Δ/Δ Flag
Clone # 1* 2 3 4 5* 6 7* 8 9 10 11*12*13*14 15* 16 17 18
untargeted
aberant targeting*
5'-internal probe (BamHI)
targeted
23kb
9.4kb
6.6kb
4.4kb
2.3kb
targeted untargeted
aberant targeting *
5'-external probe (HindIII)
3'-external probe (BamHI)
untargeted
targeted
23kb
9.4kb
6.6kb
D
p<0.0001
Median telomere signal [kb]
12
9
6
3
0
wt
Flag

Figure 2.3
Southern blot genotyping of targeted hESC clones and clonal variability of telomere length
(A) Telomere restriction fragment assay of the targeted hESCs over a time course after targeting. 2 μg of genomic DNA, after digestion with MboI and AluI, was loaded into each lane and hybridized with a TTAGGG radioactive probe. Aberrantly targeted clones are marked with an asterisk.
(B) Schematic overview of genotyping for N-terminal tagging at the endogenous TERT locus. Genomic DNA from single cell-derived hESC clones was isolated and digested with either BamHI or HindIII. 5'-internal, 5'-external and 3'-external probes were used for genotyping.
(C) Southern blot analysis for clonal Flag-TERT hESCs using three different probes. Clone #1, #5, #7, #11, #12, #13 and #15 were aberrantly targeted 5' upstream of the right homology arm, as indicated with an asterisk.
(D) Quantification of median telomere length from correctly genome edited individual clones (wt: n=14, Flag; n=11). $p<0.0001$ (two-tailed Student's t-test).

Figure 2.4

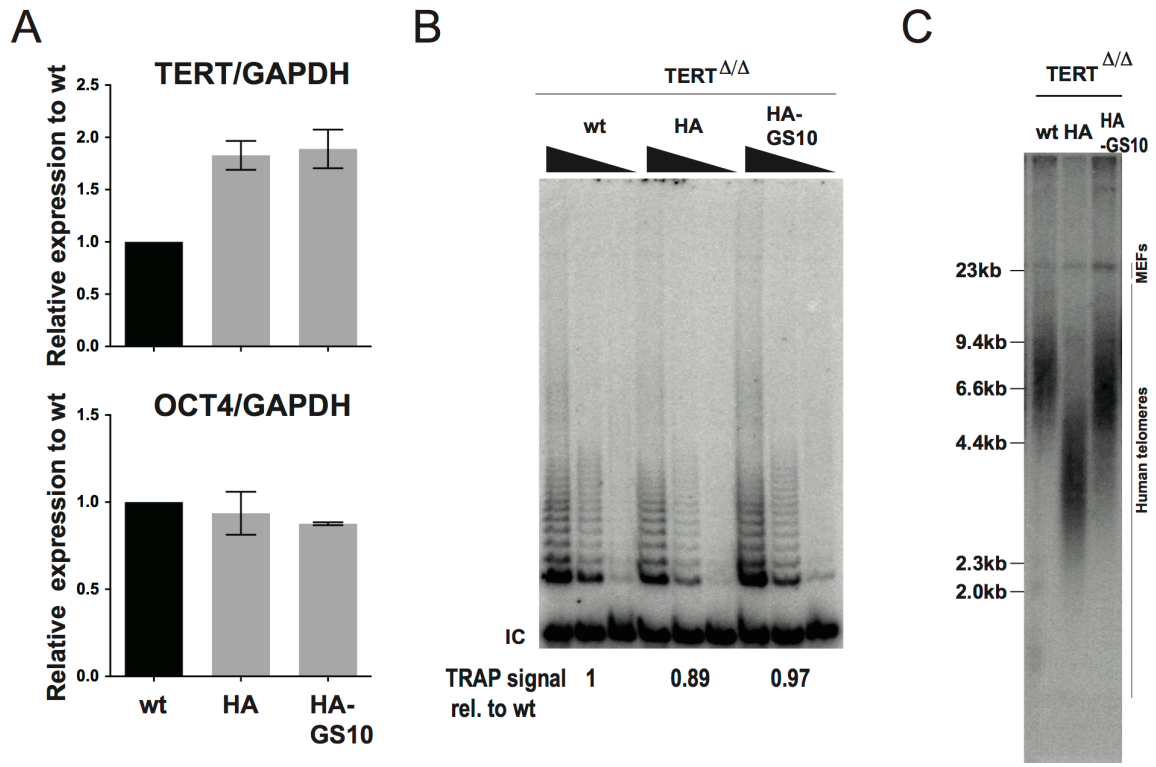


Figure 2.4.
A GS-10 linker can alleviate defects in telomere maintenance in endogenously HA-tagged hESCs
(A) Relative expression level of TERT and OCT4 mRNA in the endogenously epitope tagged hESCs compared to isogenic wt cells measured by quantitative RT-PCR. Expression of TERT and OCT4 was normalized to GAPDH. HA and HA-GS10 cells were targeted independently from Figure 2.
(B) Telomeric repeat amplification protocol (TRAP) assay of whole cell extracts from non-clonal wt, HA and HA-GS10 hESCs using a decreasing amount of protein (200, 40, and 8 ng). TRAP signals relative to wt hESCs were quantified and are shown at the bottom of the lanes.
(C) Telomere restriction fragment assay of the targeted hESCs 42 days after the second targeting.

Figure 2.5

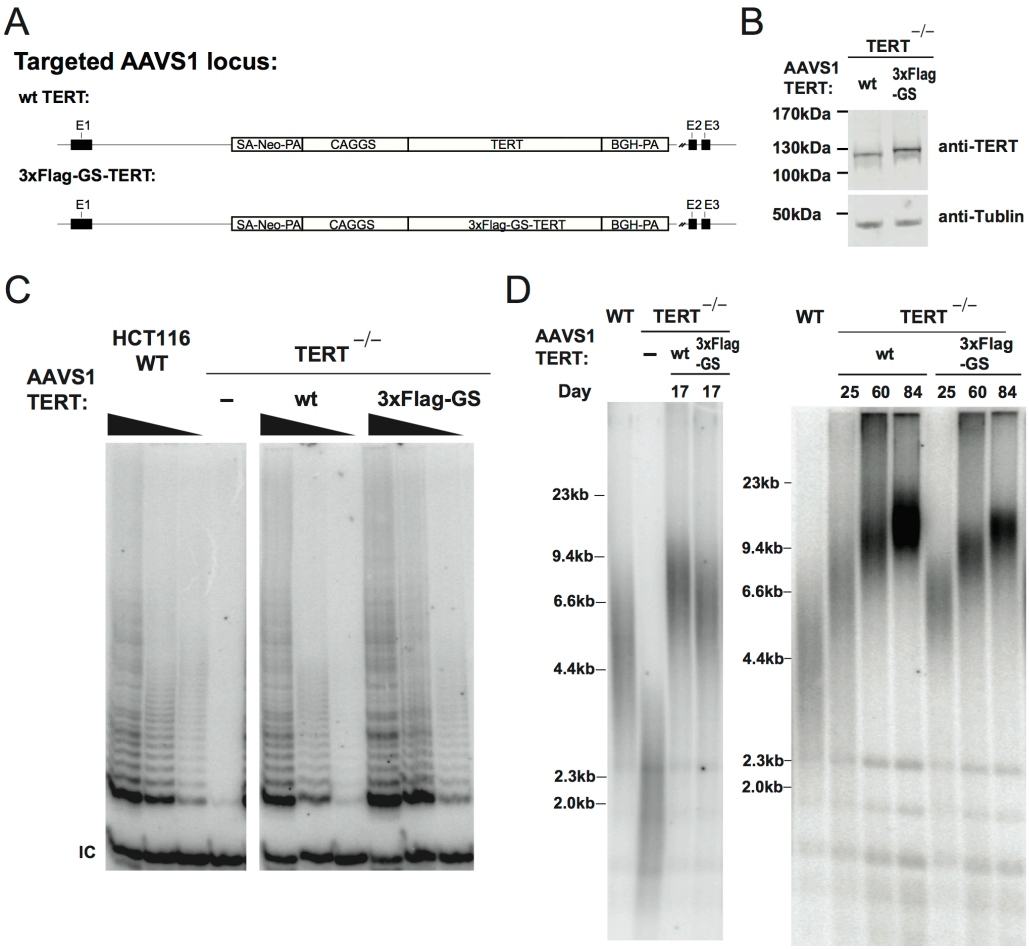


Figure 2.5
Overexpression of Flag-TERT in TERT$^{-/-}$ cells reveals slower telomere elongation compared to wt TERT
(A) Targeting schematic of wt or 3xFlag-GS-TERT overexpression from the AAVS1 locus in the TERT knock-out (KO) HCT116 cell line (TERT$^{-/-}$). TERT$^{-/-}$ HCT116 cell line was established by insertion of a stop codon into exon 1 of TERT using targeting vectors described previously (Sexton et al., 2014).
(B) Immunoblot of total TERT and tubulin protein in wt and 3xFlag-GS-TERT overexpressing TERT$^{-/-}$ cells from whole cell extracts.
(C) TRAP assay of whole cell extracts from parental HCT116, TERT$^{-/-}$ cells, and wt or 3xFlag-GS-TERT overexpressing TERT$^{-/-}$ cells using a protein titration (200, 40, and 8 ng).
(D) Telomere restriction fragment assay of telomere elongation in TERT$^{-/-}$ cells by overexpression of either wt or 3xFlag-GS-TERT at the AAVS1 locus. Days indicate time after genome-editing at the AAVS1 locus. An independent targeting experimental replicate for overexpression of wt and 3xFlag-GS-TERT gave the same relative TRAP activity (data not shown) and telomere length differences (one replicate shown at left, the other replicate at right). WT indicates the parental HCT116 cell line.

Figure 2.6

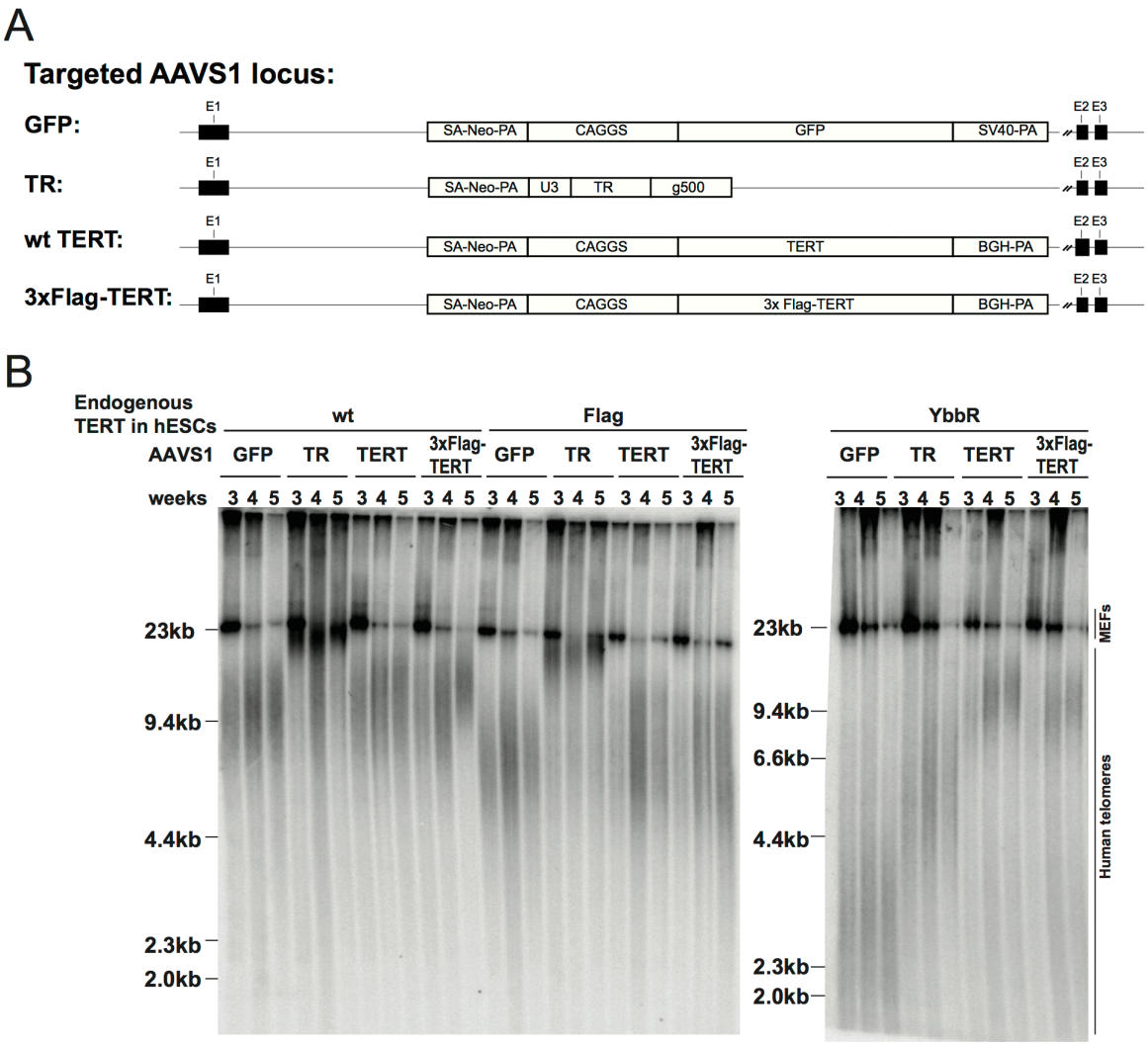


Figure 2.6
Overexpression of TERT and TR in tagged hESCs suggest potential post-assembly defects for N-terminally tagged telomerase
(A) Targeting schematic of GFP, TR, wt-TERT or Flag-TERT overexpression from the AAVS1 locus in endogenously targeted hESCs. g500 in the TR overexpression plasmid indicates 500bp downstream of genomic hTR locus.
(B) Telomere restriction fragment assay of targeted wt, Flag or YbbR hESCs overexpressing GFP, TR, wt-TERT or Flag-TERT at the AAVS1 locus. Samples were collected 3, 4 and 5 weeks (passages) after the targeting.

Figure 2.7

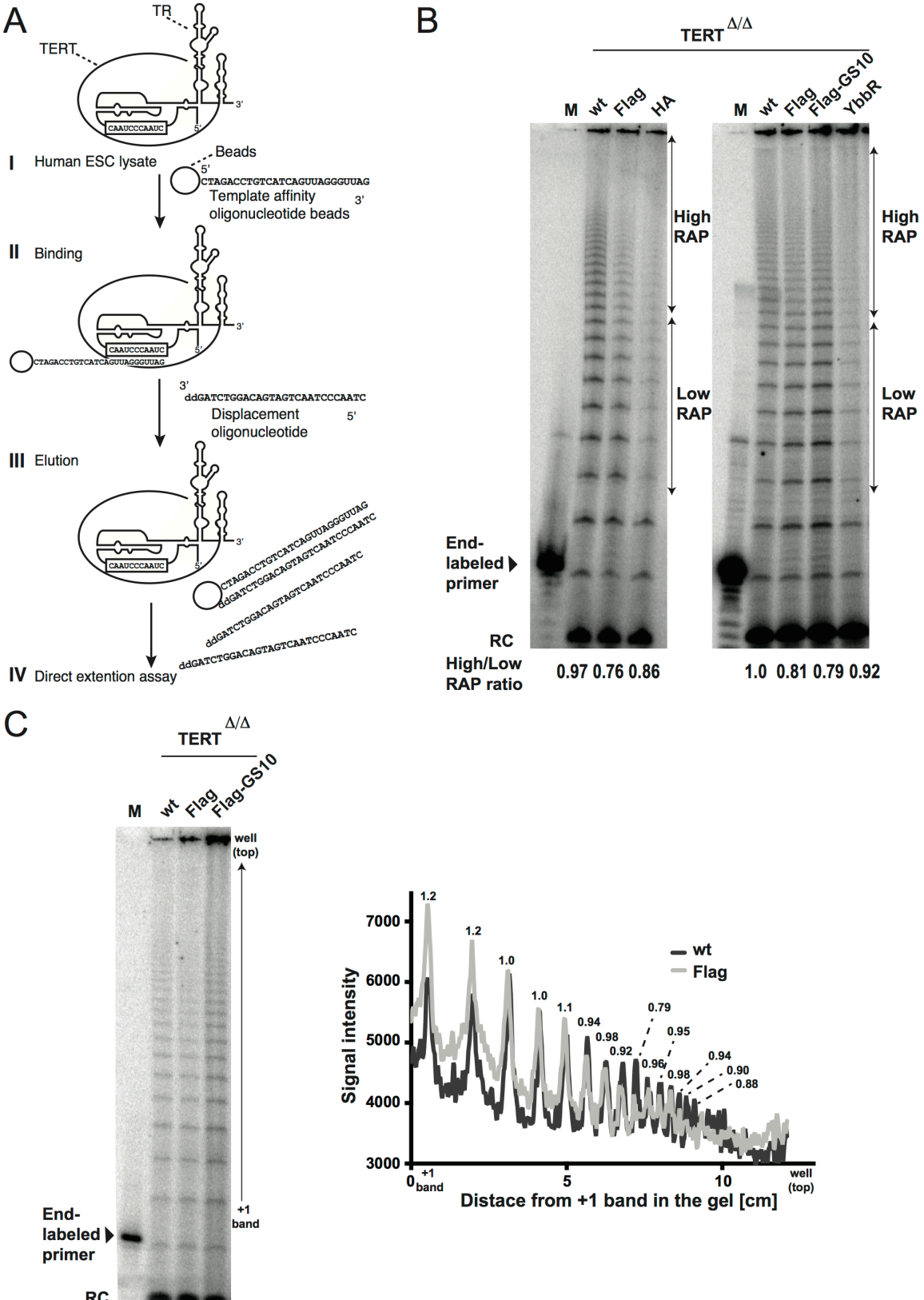

A
TR
TERT
CAAUCCCAAUC
I Human ESC lysate
Beads
CTAGACCTGTCATCAGUUAGGGUUAG
Template affinity oligonucleotide beads
II Binding
ddGATCTGGACAGTAGTCAATCCCAATC
Displacement oligonucleotide
III Elution
IV Direct extention assay
B
TERT Δ/Δ
M wt Flag HA
M wt Flag Flag-GS10 YbbR
High RAP
Low RAP
End-labeled primer
RC
High/Low RAP ratio
0.97 0.76 0.86
1.0 0.81 0.79 0.92
C
TERT Δ/Δ
M wt Flag Flag-GS10
well (top)
+1 band
End-labeled primer
RC
wt
Flag
Signal intensity
7000
6000
5000
4000
3000
0
+1 band
5
10
well (top)
Distace from +1 band in the gel [cm]
1.2
1.2
1.0
1.0
1.1
0.94
0.98
0.92
0.96
0.79
0.95
0.98
0.94
0.90
0.88

Figure 2.7
N-terminally tagged TERT is defective in repeat addition processivity
(A) Schematic overview of purification of endogenous telomerase by template affinity oligonucleotide purification as previously reported in (Wu et al., 2015). (I) Lysate form wt or tagged hESCs were prepared using hypotonic buffer. (II) The lysate was incubated with the telomerase template affinity oligonucleotide-conjugated beads. (III) After washing off unspecific binding on the beads, enriched telomerase was eluted using the displacement oligonuculeotide, which possesses complementary sequence to the template affinity oligonucleotide but cannot be elongated by telomerase due to its 3'-end 2'-3'-dideoxycytidine. (IV) The elution fraction was used for direct telomerase activity assay.
(B) Direct telomerase activity assay for wt or endogenously tagged hESCs. An 18 nt $(TTAGGG)_3$ primer was used as the substrate of the direct extension assay. This 18 nt substrate was used as an end-labeled marker. 12 nt $(TTAGGG)_2$ primer was used as the recovery control (RC) of ethanol precipitation. Telomerase activity represented in the region indicated by arrows was quantified and the ratio of high/low RAP is shown at the bottom of the gel.
(C) Direct telomerase activity assay as in (A) Independent lysates of wt and tagged TERT hESCs were prepared independently from (B) followed by telomerase template oligonucleotide purification and a direct extensions assay to measure telomerase activity. To visualize the RAP-defect the graph to the right shows the signal intensity of the gel to the left for wild-type cells superimposed with the signal intensities for Flag-TERT cells superimposed. Numbers above each peak indicate Flag/wt signal ratio.

Table 2.1
Summary of nomenclature, amino acid and DNA sequences used in this study

Nomenclature in the paper	Full name	Aminoacid sequence
Flag	3xFlag	MGSDYKDHDGDYKDHDIDYKDDDDKDI
Flag-GS10	3xFlag-GS10	MGSDYKDHDGDYKDHDIDYKDDDDKDIGSGSGSGSGSGSGSGSGSDI
Flag-GS20	3xFlag-GS20	MGSDYKDHDGDYKDHDIDYKDDDDKDIGSGSGSGSGSGSGSGSGSDIGSGSGSGSGSGSGSGSGSDI
HA	HA	MGSYPYDVPDYADI
HA-GS10	HA-GS10	MGSYPYDVPDYADIGSGSGSGSGSGSGSGSGSGSDI
YbbR	YbbR-TEV-3xFlag	MGSDSLEFIASKLAENLYFQSASMDYKDHDGDYKDHDIDYKDDDDKDI
Halo	Halo	MGSAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDD
		HVRFMDAFEALGLEEVVLVHDWGSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIE
		GTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLA
		KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGDI
Halo-GS10	Halo-GS10	MGSAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDD
		HVRFMDAFEALGLEEVVLVHDWGSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIE
		GTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLA
		KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGDIGSGSGSGSGSGSGSGSGSGSDI
3xFlag-GS (for AAVS1 over expression in HCT116)	3xFlag-GS	MGSDYKDHDGDYKDHDIDYKDDDDKDIGSGSGSGM
3xFlag (for AAVS1 over expression in hESCs)	3xFlag	MGSDYKDHDGDYKDHDIDYKDDDDKEFM

Table 2.2

Summary of the variety of epitope tags used in this study and the phenotypes of endogenous tagging

N-tag TERT	Size of insertion	Expression	Telomerase activity	Telomere length	Processivity	
Halo-GS10	966bp	Lower than wt*	Less active*	Shorter than wt*	-	* data not shown
Flag-GS20	210bp	Higher than wt	wt level	Shorter than wt	-	
YbbR	153bp	Lower than wt	Less active	Shorter than wt	-	
Flag-GS10	144bp	Higher than wt	~1.3 fold higher than wt	wt level	-	
Flag	78bp	wt	wt level	Shorter than wt	Low RAP	
HA	39bp	Higher than wt	Less active	Shorter than wt	Low RAP	

CHAPTER THREE

Cancer-associated TERT promoter mutations abrogate telomerase silencing

Based on Chiba et al., *eLife* 2015

ABSTRACT

Mutations in the human telomerase reverse transcriptase (TERT) promoter are the most frequent non-coding mutations in cancer, but their molecular mechanism in tumorigenesis has not been established. We used genome editing of human pluripotent stem cells with physiological telomerase expression to elucidate the mechanism by which these mutations contribute to human disease. Surprisingly, telomerase-expressing embryonic stem cells engineered to carry any of the three most frequent TERT promoter mutations showed only a modest increase in TERT transcription with no impact on telomerase activity. However, upon differentiation into somatic cells, which normally silence telomerase, cells with TERT promoter mutations failed to silence TERT expression, resulting in increased telomerase activity and aberrantly long telomeres. Thus, TERT promoter mutations are sufficient to overcome the proliferative barrier imposed by telomere shortening without additional tumor-selected mutations. These data establish that TERT promoter mutations can promote immortalization and tumorigenesis of incipient cancer cells.

INTRODUCTION

Activation of telomerase is the critical step for the immortalization of more than 90% of all human tumors(Counter et al., 1992; Greider and Blackburn, 1985; Kim et al., 1994). Non-coding mutations in the promoter of the catalytic subunit of telomerase (TERT) emerged recently as one of the most prevalent mutations in human cancer (Bojesen et al., 2013; Fredriksson et al., 2014; Horn et al., 2013; Huang et al., 2013; Killela et al., 2013; Weinhold et al., 2014). Interestingly, all TERT promoter mutations associated with cancer formation thus far generate novel binding sites for the ETS (E26 transformation-specific) family of transcription factors and are located close to the translational start site of TERT (e.g., -57A/C, -124C/T, and -146C/T) (Horn et al., 2013; Huang et al., 2013). Transient transfection experiments using ectopic TERT Luciferase-reporter constructs suggest that TERT promoter mutations can increase TERT transcription by 1.5 to 2 fold when assayed in tumor cells(Horn et al., 2013; Huang et al., 2013). To date, the physiological events that select for these specific mutations are still unclear, as they have been mostly investigated for their impact in tumor cell lines that are already immortal, maintain telomere length, and have aberrant karyotypes. These tumor cell lines have sufficient telomerase activity to maintain an immortal phenotype, but so do tumor cells without these TERT promoter mutations. Thus, changes in telomerase levels and telomere length provide incomplete information regarding the functional differences between cells that do or do not carry TERT promoter mutations.

In untransformed human tissues, telomerase activity is restricted to embryonic cells and some adult stem cell or progenitor compartments due to transcriptional silencing of TERT upon differentiation (Aubert, 2014; Gunes and Rudolph, 2013). As a consequence, differentiated somatic cells undergo progressive telomere shortening with cell division, which limits their proliferative capacity and has thus been proposed as a tumor suppressor mechanism (Wright et al., 1996). Critically short telomeres are detected as sites of DNA damage leading to cell death or replicative senescence (Palm and de Lange, 2008). Long-term inhibition of TERT (Herbert et al., 1999) or interference with telomerase recruitment to telomeres (Nakashima et al., 2013; Sexton et al., 2014) lead to cell death in telomerase-positive cancer and stem cells. Inversely, ectopic telomerase expression is sufficient to immortalize normal human fibroblasts by allowing them to bypass senescence (Bodnar et al., 1998; Morales et al., 1999). Since the discovery of telomerase reactivation in cancer, many *cis*-regulatory elements and corresponding transcription factors have been suggested to contribute to the regulation of TERT in healthy cells and its aberrant expression in tumor cells (Ducrest et al., 2002; Greenberg et al., 1999b; Kyo et al., 2008; Lin and Elledge, 2003). GWAS analysis identified a specific set of TERT promoter mutations in melanomas that all occur in a very small region close to the transcriptional start site and each results in novel putative TTCCGG- ETS binding sites (Horn et al., 2013; Huang et al., 2013). While ETS-factors are a large family of transcription factors that can recognize this binding site, recent data suggest that TERT promoter mutations are bound predominantly by GABP(Bell et al., 2015). This specificity does not appear restricted to melanomas as the same TERT promoter mutations have emerged as a major driver in a multitude of human solid tumors (Heidenreich et al., 2014), including glioblastomas, medulloblastomas, carcinomas of the bladder, urothelial cancer (Borah et al., 2015), thyroid and squamous cell carcinomas of

the tongue, as well as in liposarcomas and hepatocellular carcinomas(Heidenreich et al., 2014). Based on this tumor spectrum, TERT promoter mutations have been hypothesized to preferentially promote tumor progression in tissues with relatively low rates of self-renewal (Killela et al., 2013) Several studies have suggested that TERT promoter mutations can provide a biomarker to stratify human cancer subtypes(Borah et al., 2015; Heidenreich et al., 2014; Hinrichs et al., 2006). However, the mechanism by which these mutations promote tumor formation is unknown. The key outstanding questions are: 1) whether TERT promoter mutations are sufficient to immortalize cells and 2) why TERT promoter mutations occur in specific tumors subtypes.

Here we address these questions by genetically engineering human embryonic stem cells (hESCs) to carry the three most prevalent TERT promoter mutations in an isogenic background. The impact of these mutations was studied by measuring their effect on TERT expression, telomerase activity, and telomere length in stem cells as well as in differentiated cell types. We demonstrate that two out of three cancer-associated TERT mutations caused no effect and only the most prevalent promoter mutations mildly increased TERT levels in hESCs, which did not result in significantly increased telomerase activity. We find that increased TERT expression is not functionally linked to an increase in active telomerase, as TR, the telomerase RNA component, but not TERT is limiting in hESCs. However, the importance of these mutations in tumorigenesis becomes clear when hESCs are differentiated into normally telomerase-negative cells. Under these conditions all cancer-associated TERT mutations prevent repression of TERT, resulting in a retention of telomerase activity relative to wild-type differentiated cells. Ultimately, the resulting TERT expression led to aberrant telomerase enzymatic activity in terminally differentiated cells and abnormally long telomeres, thereby bypassing the telomere shortening tumor suppressor pathway.

RESULTS

Analyzing cancer-associated TERT promoter mutations in hESCs

We aimed to understand the molecular basis by which the cancer-associated TERT mutations impact telomerase biology. To address this question, we employed CAS9- (clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems 9) (Jinek et al., 2012) mediated genome editing to derive human pluripotent stem cells (WIBR#3) that carry TERT promoter mutations at the endogenous TERT locus. Initially we attempted conventional donor-based genome editing strategies with sgRNAs targeting sequences proximal to the targeting site. These attempts were however unsuccessful, likely due to the TERT promoter mutations being in a genomic region with ~80% GC content. This non-random base composition does not allow for the design of specific sgRNAs without a large number of potential off-targets. We tested several sgRNAs in proximity to TERT promoter mutations and found them to be toxic to cells shortly after transfection into cancer cells and human primary fibroblasts. We overcame this challenge by employing a two-step targeting approach (Figure 3.1A). In a first editing step we homozygously deleted a 1.5 kb region in the TERT gene using two sgRNAs that cut at positions -1462 and + 67 relative to the first ATG (Figure 3.1A). In a second editing step we reintroduced the deleted region either with or without the promoter mutations into the endogenous TERT locus (Figure 3.1A, B).

As the first deletion step removed the translational start site as well as some coding sequence of TERT, this targeting strategy resulted in telomerase-negative hESCs ($TERT^{\Delta/\Delta}$) (Figure 3.1C). Correct targeting was confirmed by Southern blot and PCR-sequencing of the genomic deletion (Figure 3.2A, B). As expected from our previous characterization of $TERT^{-/-}$ hESCs (Greider and Blackburn, 1985; Sexton et al., 2014), these cells proliferated normally for three to four months, followed by cell death due to progressive telomere shortening with no survivors after 140 days (Sexton et al., 2014). In a second targeting step we edited the newly formed genomic site with a specific sgRNA spanning the new junction (-1462 to +67) to reinsert and restore the deleted region with either the wild-type promoter or an altered region containing the most frequent TERT promoter mutations: -57A/C, -124C/T, or -146C/T (Figure 3.1B). This complementation approach restored the TERT gene and cellular viability of targeted cells. Accordingly, cells with a restored TERT gene gradually outcompeted none-rescued parental $TERT^{\Delta/\Delta}$ cells and lead to substantial telomere elongation by the time untargeted $TERT^{\Delta/\Delta}$ hESCs had died (Figure 3.1D). This complementation strategy therefore successfully generated hESCs that differed exclusively at the TERT locus by expressing TERT either from its wild-type promoter or from a promoter that contained one of the cancer-associated point mutations.

We first analyzed the impact of the TERT promoter mutations on TERT mRNA levels by qRT-PCR until cultures established stable TERT expression levels and all $TERT^{\Delta/\Delta}$ had died (Figure 3.1E). This analysis revealed that the most frequent TERT mutation (-124C/T) resulted in a 2-3-fold increase in TERT expression when compared to the isogenic wild-type control. This increase is in agreement with previous reports that evaluated these mutations using Luciferase reporter constructs (Horn et al., 2013; Huang et al., 2013). Noticeably, the two other mutations did not result in similarly increased TERT expression in hESCs. We confirmed this finding for an independent $TERT^{\Delta/\Delta}$ cell

line (Figure 3.3) and individual single cell-derived hemizygously targeted clones (Figure 3.4). Interestingly, the promoter mutation cell lines carrying the -124C/T mutation had elevated levels of TERT mRNA expression, without a equivalent increase in telomerase activity (Figure 3.1F).

TR, but not TERT is limiting for telomerase in hESCs

A lack of a significant change in telomerase activity despite increased TERT levels in hESCs that carry the -124C/T mutations suggested that TERT mRNA levels are not rate-limiting for telomerase activity in hESCs. Similar observations were made previously for some tumor cell lines in which telomerase activity is limited by levels of TR (Cristofari and Lingner, 2006; Xu and Blackburn, 2007), and might explain the tissue-specific impact of TERT and TR mutations in patients with dyskeratosis congenita(Armanios and Blackburn, 2012; Batista et al., 2011; Strong et al., 2011). Telomerase biogenesis is a complex biological process that has been shown in human pluripotent stem cells to depend on several activities that, when depleted, can become limiting(Batista and Artandi, 2013; Batista et al., 2011; Yang et al., 2008). To test the hypothesis that in wild type human pluripotent stem cells TR is the limiting factor for telomerase activity, we ectopically expressed TERT, TR, or both from the AAVS1 safe harbor locus (Hockemeyer et al., 2009; Hockemeyer et al., 2011) (Figure 3.5A). The introduction of such transgenes into this locus in isogenic settings overcomes concerns of random integration of the transgene. Overexpression levels were verified by western and northern blotting and qRT-PCR (Figure 3.5B,C; Figure 3.6A) and quantitative analysis showed that TERT mRNA was overexpressed >40 fold and TR levels by approximately 20 fold. TERT protein was detectable by immunoblotting when overexpressed, contrasted to the lack of detectable endogenous TERT protein. In addition, we determined telomerase activity levels (Figure 3.5D and Figure 3.6B,C) and telomere length changes in hESCs 36 days after targeting (Figure 3.5E). TR overexpression strongly increased telomerase activity and led to rapid telomere elongation in hESCs, while overexpression of TERT alone did neither. However, when differentiated into fibroblasts or neural precursor cells, we observed the inverse behavior. In this setting, telomerase activity levels were significantly increased when TERT was overexpressed while increased levels of TR did not affect telomerase activity (Figure 3.5D). This finding showed that in hESCs TERT levels were not limiting, and that increased TERT expression did not result in a significant increase of telomerase activity or telomere length. Hence, hESCs are unlikely to reveal the impact of the TERT promoter mutations. Therefore, observation of the effect of TERT promoter mutations requires the analysis of differentiated cells in which TERT down-regulation results in it becoming limiting for telomerase activity.

TERT promoter mutations abrogate TERT silencing and impair telomere shortening

We differentiated edited hESCs into embryonic bodies (EBs) and eventually into fibroblasts and determined TERT mRNA levels over a 15 day of differentiation period (Figure 3.7A). All cell lines differentiated with equal efficiencies, as evidenced by up-regulation of the differentiation marker COL1A1 and repression of OCT4 transcription (Figures 3.7B, C). Although TERT expression was successfully down-regulated in cells with wild-type TERT promoter, all three promoter mutation lines retained significant levels of TERT expression (Figure 3.7A). This failure of TERT transcriptional silencing

became apparent as early as 3 days after the induction of differentiation and accumulated into a four-fold increase in TERT expression in cells that carried the -57A/C or the -146C/T mutation and an 8-12-fold increase in cells in which transcription depended on the endogenous TERT promoter with the -124C/T mutation (Figures 3.7A, B). This failure to appropriately repress TERT transcription during EB differentiation became even more apparent when the cells were differentiated into fibroblast-like cells. As expected, TERT transcription was undetectable in differentiated wild-type fibroblasts. In contrast, fibroblasts with the cancer-associated promoter mutations showed high levels of TERT expression (Figure 3.7D). This difference was not due to impaired differentiation of cells with the TERT promoter mutations, as these cells had silenced OCT4 and appropriately induced COL1A1 expression (Figure 3.7D). Importantly, while telomerase activity is not detectable in wild-type fibroblasts, the aberrant TERT expression resulted in robust telomerase activity in fibroblasts that contained the promoter mutations (Figure 3.7E). As before, we confirmed these findings in an independent $TERT^{\Delta/\Delta}$ cell line (Figures 3.8A-D) and in individual single cell-derived targeted clones (Figures 3.8E, F). Furthermore we confirmed that this failure to repress TERT expression persists in fibroblasts as late as 45 days after differentiation (Figures 3.9).

The failure to silence telomerase could be specific to the fibroblast differentiation paradigm or a more general defect during differentiation. To address this issue, we first generated neural precursor cells (NPCs) using the highly robust dual SMAD inhibition protocol(Chambers et al., 2009), establishing NPCs that can be maintained in culture for extended periods of time with low levels of telomerase expression. These NPCs can be further differentiated towards terminally differentiated non-proliferating post mitotic neurons that are characterized by the expression of the pan-neural marker proteins TUJ1 and NEUN. Independent of their genotype, all cells were able to differentiate into NPCs and neurons showing equal down-regulation of OCT4 expression and induction of neuronal marker genes (Figures 3.10A-C). However, a striking difference became apparent in TERT levels as both NPCs and neurons that carried the TERT promoter mutations failed to repress TERT transcription (Figures 3.10A, C and Figures 3.11) and showed robust telomerase activity (Figure 3.10D). Even when neurons were maintained in the presence of a mitotic inhibitor, the promoter mutations led to elevated TERT mRNA and telomerase activity levels, suggesting that telomerase activity can accumulate in slowly and non-dividing cells as late as 1 month after induction of terminal differentiation (Figures 3.10C, D).

Cancer-associated TERT promoter mutations result in telomerase levels equal to those found in immortal tumor cell lines

Next, we assessed the impact of the aberrant telomerase activity of TERT promoter-mutation-containing cells by directly comparing the telomerase levels in fibroblasts and NPCs that carried the promoter mutations to the telomerase activity found in three established tumor cell lines (Figures 3.12A, B). Remarkably, telomerase activity levels in -124C/T NPCs were equivalent to the activity found in immortal Hela S3 cells and about 50% of the activity found in hESCs, HCT116, and 293T cells. Importantly, telomerase activity in these cells was greatly increased relative to wild type cells. Moreover, -124C/T fibroblasts had about 50% of the telomerase activity measured in HeLa S3 cells, 30% of that found in 293T cells, and 25% of that of HCT116 colon

carcinoma cells. These findings suggested that TERT promoter mutations induce telomerase levels that are sufficient to enable immortalization or at least significantly delay telomere length-induced senescence. Finally, we analyzed the functional consequences of increased TERT expression by evaluating telomere length changes in hESCs, NPCs, and fibroblasts derived from the isogenic set of TERT promoter-edited cell lines (Figures 3.12C, D, and Figure 3.13A). All differentiated cell lines with cancer-associated promoter mutations showed an increase in telomere length compared to the wild-type controls.

TERT promoter mutations suppress telomere shortening in in vivo tumors

To further explore the *in vivo* relevance of these findings in the context of long-term differentiation as well as tumor progression, cells with TERT promoter mutations were assayed for teratoma tumor formation in immune-compromised mice (Figure 3.12E and Figure 3.13B). For this assay all cell lines were injected subcutaneously into NOD/SCID mice, allowing pluripotent cells to differentiate and form a teratoma comprised of cells derived from all three germ layers. All cell lines injected formed teratomas of approximately equal size, were explanted simultaneously (75 days after injection), and analyzed for their telomere length. Using this unbiased approach, we again found that cells with TERT promoter mutations carried aberrantly long telomeres, with the -124C/T mutation having the strongest defect in silencing telomerase activity and retaining almost identical telomere length as undifferentiated hESCs (Figure 3.12C, E and Figure 3.13B). These data demonstrated the causal relationship between the TERT promoter mutations and telomere maintenance and showed that the TERT promoter mutations can up-regulate TERT levels sufficiently to suppress telomere erosion without additional tumor-selected changes.

DISCUSSION

A key challenge in cancer research is to understand how mutations that sequentially occur in normal cells eventually produce a tumor. For noncoding mutations identified by GWAS, this is a particular challenge. Here we were able to dissect how the most frequent noncoding mutations in human cancer exert their tumorigenic effect. We are able to do so because cancer genomics have identified candidate mutations, genome editing is facile and robust, and we tested the effects in an otherwise wild-type background, thus being able to attribute a phenotypic effect specifically to a single genetic change.

Using genome editing of the endogenous TERT locus we generated a panel of three hESC lines that differed exclusively at a single position in the TERT promoter associated with cancer. Analyzing the impact of these mutations in hESCs, we showed that the most frequent mutation, -124C/T, increased TERT mRNA levels in hESCs by about 2-3 fold. However, neither the -57A/C nor the -146C/T mutation led to an increase in TERT transcription, despite the fact that these mutations generate the same putative ETS-binding motif of TTCCGG. This suggests a strong positional effect between the location of the ETS mutation and the core transcription initiation machinery. The possibility of such context-dependent positional constraints between the TERT promoter mutations and the core transcriptional machinery is further supported by the fact that a single point mutation at position -89 (C/G, -31 bp upstream of the TSS) would result in the same TTCCGG sequence. The fact that this mutation has as of now not been reported to be associated with cancer despite it being between the -57 and -124 sites is likely due to the need of the core transcription factors to bind to this site. It is intriguing to speculate why all promoter mutations are located in close proximity to the TSS; it seems possible that TERT transcriptional regulation is closely linked with the core transcriptional machinery rather than regulated through the canonical positioning of transcription factors along an extended promoter. The experimental approach established in this study of genome editing the TERT promoter provides an experimental system to uncover *cis*-regulatory elements that are necessary for telomerase expression in stem cells and its transcriptional regulation upon differentiation.

Increased expression of TERT mRNA in -124C/T containing hESCs did not lead to a significant increase in telomerase activity or pronounced telomere lengthening, establishing that in hESCs TR levels, but not TERT levels, are limiting for telomerase assembly and telomere lengthening. Therefore, immortal hESCs are as uninformative with regard to cancer-associated TERT mutations as immortal tumor tissue or cell lines. However, whereas upon differentiation wild-type hESCs efficiently silence TERT transcription, resulting in loss of telomerase activity and telomere shortening, the cancer-associated TERT promoter mutations were sufficient to maintain expression of TERT and resulted in telomerase activity levels comparable to immortal cancer cell lines. These experiments uncover that the underlying cancer-causing mechanism is likely a failure to repress telomerase upon differentiation into somatic cells. It is remarkable that TERT promoter mutations are sufficient to up-regulate TERT expression without additional cancer-selected changes in the genome such as increased levels of ETS factors.

TERT promoter mutations are not frequently found in leukemias and colorectal cancers (Heidenreich et al., 2014). Direct evidence (Chiu et al.; Schepers et al., 2011) as

well as the pathology of the telomerase-related disease dyskeratosis congenita in which patient with mutations in the telomere maintenance pathway present with bone marrow failure as well as lung, intestinal, and skin pathologies show that TERT is expressed in these highly proliferative tissues and is required for their long-term self-renewal capacity and ability to maintain tissue homeostasis (Armanios and Price, 2012; Aubert et al., 2012).

Tumor-initiating events in these cancers predominantly drive proliferation pathways that spur formation of hyperplasia and niche-independent proliferation that allow incipient cancer cells to outcompete their neighbors (Barker et al.; Magee et al.; Merlos-Suarez et al.; Zhou et al.). In this setting, mutations in the TERT promoter or alterations in the telomerase biogenesis pathway might be at first neutral, not providing a direct proliferative advantage as telomeres are still long or telomerase is active (Figure 3.14A). During the genesis of these tumors, telomere shortening might present a challenge at a later stage when cells have already outcompeted their neighbors.

In contrast, tumor-initiating cells that are thought to not directly arise from a canonical telomerase-positive stem cell compartment (e.g. liposarcomas), that undergo high numbers of divisions after differentiating (e.g. neural-crest derived melanocytes), or that have to reenter a proliferation cycle in response to chronic injury (e.g. urothelial cells and hepatocytes) could be challenged by the telomere-dependent proliferative barrier comparatively early in their progression. In these cell types TERT promoter mutations will provide an immediate and strong proliferative advantage over neighboring cells. In this case telomerase activation occurs in cells with in which telomerase is absent or low, an otherwise mostly intact genome, and therefore will be present in most tumor cells and detected as a frequent and thus early event (Figure 3.14B).

It is important to note that this model depicts the very extreme cases of a TERT-positive adult stem cell with long telomeres contrasted to the expected outcome of a TERT promoter mutation in a telomerase-negative cell with short telomeres. Likely this sharp distinction between canonical telomerase-positive stem cell compartments and telomerase-negative compartments is rather continuous *in vivo.* Telomerase expression, telomere length, and the number of cell divisions will differ between tissues and with age and therefore the benefit of the TERT promoter mutation will be complexly graded. Given this, it will be critical to determine exactly which cells of the human body are telomerase-positive, when and how telomerase is silenced upon differentiation, and how many divisions cells undergo in human tissue after becoming telomerase-negative.

Telomerase inhibition has been proposed as a target for cancer therapies. We demonstrate that TERT promoter mutations are sufficient to de-repress TERT, providing a potential target to inhibit TERT expression and telomerase activity.

In order to identify therapeutic approaches specific to these promoter mutations, a model system in which TERT is dysregulated solely by these mutations is necessary. Our model system fulfills this requirement and allows for a direct assessment of any potential inhibition by measuring TERT expression following differentiation. In contrast, this approach will be challenging in cancer cells, as TERT mRNA levels, telomerase levels, and telomere length vary dramatically regardless of whether they carry any of the TERT promoter mutations. Further mechanistic studies in such tumor cells are also challenged by the high frequency of concurrent TERT copy number variations, promoter polymorphisms, and cancer-associated dysregulation of factors implicated in TERT

regulation such as MYC. As such, it will be challenging to evaluate the effectiveness of such an inhibitor due to these potentially compensatory effects arising from these misregulations. As such, it is imperative to test any potential therapeutic approach directed at these promoter mutations in a model system that only carries these mutations in an otherwise wild-type background, such as the model system described here. Specifically targeting the TERT promoter mutations is an attractive approach, as TERT promoter mutations are exclusive to the tumor cells and are not present in surrounding normal tissue. Therefore, any intervention that is targeted specifically against their mode of operation is expected to affect tumor cell survival, but not the telomerase-positive adult stem cells of the patient.

Figure 3.1

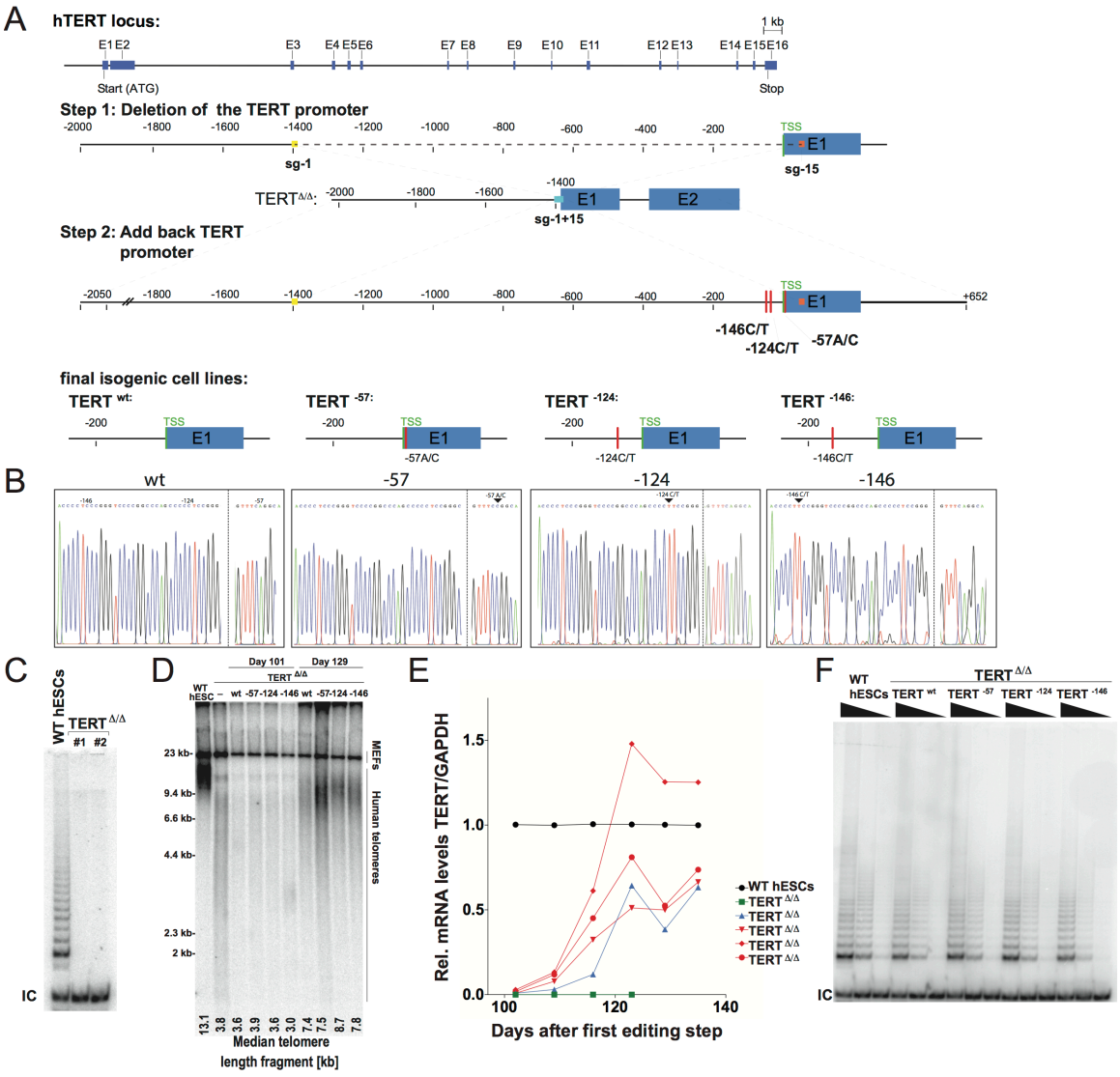

A
hTERT locus:
1 kb
E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12 E13 E14 E15 E16
Start (ATG)
Stop
Step 1: Deletion of the TERT promoter
-2000 -1800 -1600 -1400 -1200 -1000 -800 -600 -400 -200
TSS
E1
sg-1
sg-15
TERT Δ/Δ:
-1400
E1
E2
sg-1+15
Step 2: Add back TERT promoter
-2050 -1800 -1600 -1400 -1200 -1000 -800 -600 -400 -200 +652
TSS
E1
-146C/T
-124C/T
-57A/C
final isogenic cell lines:
TERT wt:
TERT -57:
TERT -124:
TERT -146:
-200
TSS
E1
-57A/C
-124C/T
-146C/T
B
wt
-57
-124
-146
C
WT hESCs
TERT Δ/Δ
#1 #2
IC
D
Day 101
Day 129
TERT Δ/Δ
WT hESC
wt -57 -124 -146 wt -57 -124 -146
23 kb-
9.4 kb-
6.6 kb-
4.4 kb-
2.3 kb-
2 kb-
MEFs
Human telomeres
13.1 3.8 3.6 3.9 3.6 3.0 7.4 7.5 8.7 7.8
Median telomere length fragment [kb]
E
Rel. mRNA levels TERT/GAPDH
1.5
1.0
0.5
0.0
100
120
140
Days after first editing step
WT hESCs
TERT Δ/Δ
TERT Δ/Δ
TERT Δ/Δ
TERT Δ/Δ
TERT Δ/Δ
F
WT hESCs
TERT Δ/Δ
TERT wt
TERT -57
TERT -124
TERT -146
IC

Figure 3.1
Generation of isogenic TERT promoter mutation-containing hESCs reveals a modest increase of TERT expression only for the -124C/T mutation
(A) Schematic overview of the two-step approach used to genome-edit TERT promoter mutations in hESCs. First, TERT knock-out cell line ($TERT^{\Delta/\Delta}$) that lacks 1.5 kb upstream and 66 bp downstream of the 1st ATG was established using two CAS9/sgRNAs (sg-1 and sg-15). Second, an sgRNA against the newly synthesized NHEJ-derived junction (-1462 and +67: sg1+15; see Figure 1- figure supplement 1B) were co-electroporated with donor plasmids containing the deleted regions with or without the cancer-associated TERT promoter mutations.
(B) Sequence analysis of targeted cells confirmed successful restoration and introduction of the TERT promoter mutations.
(C) Telomeric repeat amplification protocol (TRAP) assay of whole cell extracts from $TERT^{\Delta/\Delta}$ hESC lines (n=2) using 200 ng protein. $TERT^{\Delta/\Delta}$ #1 and #2 cells were collected at day 89 and day 146 after the first editing respectively. IC: internal control.
(D) Telomere restriction fragment assay of wild-type (WT), $TERT^{\Delta/\Delta}$, and the targeted hESCs over a time course after targeting (day 0: first editing step, day 73: second editing). $TERT^{\Delta/\Delta}$ #1 cells are telomerase-deficient, undergo telomere shortening and die around day 120 unless they regain telomerase activity through the second targeting step. At the first time point (day 101), the majority of the cells are untargeted $TERT^{\Delta/\Delta}$ cells, therefore telomere length is heterogeneous and short. This short telomere length results in reduced hybridization intensity with the TTAGGG radioactive probe. In contrast at the second time point (day 129), uncomplemented $TERT^{\Delta/\Delta}$ #1 died due to progressive telomere shortening and the targeted populations are enriched. In this targeted population the restoration of telomerase resulted in substantial telomere elongation and an overall increase in telomere signal intensity. 2 μg of genomic DNA after digestion with MboI and AluI were loaded in each lane. Quantification of the average telomere length signal is indicated at the bottom of the gel. Throughout all figures we refer to non-targeted wild-type WIBR#3 hESCs as WT. We refer to wild-type cells generated by reintroducing the wild-type promoter into $TERT^{\Delta/\Delta}$ as wt.
(E) Relative expression levels of TERT mRNA by mutant and wt promoter-containing hESCs over a time course after targeting measured by quantitative RT-PCR. Expression is relative to WT hESCs (black line). Expression of TERT was normalized to GAPDH. Also shown is $TERT^{\Delta/\Delta}$ cells (green line) until day 123. This is the last time point in which RNA could be isolated before $TERT^{\Delta/\Delta}$ cultures died.
(F) TRAP assay of whole cell extracts from WT and promoter mutation-containing hESCs (day 147) using decreasing amount of protein (200, 40, 8 ng).

Figure 3.2

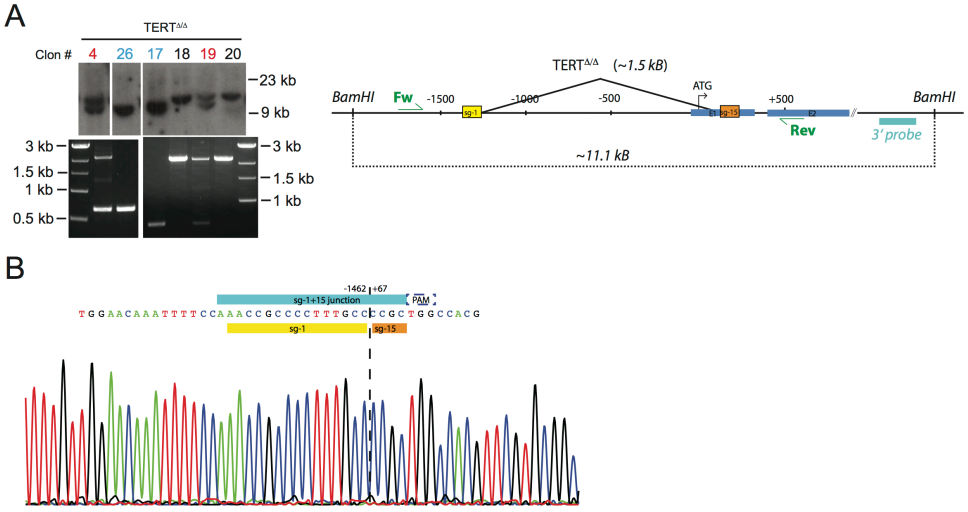


Figure 3.2
Genotyping of TERT$^{\Delta/\Delta}$ hESCs prior to the second targeting to insert mutated promoter sequences and independent confirmation of promoter mutation experiments
(A) Southern blot analysis for TERT$^{\Delta/\Delta}$ hESCs. Genomic DNA isolated form individual clones (n=120) was digested with BamHI and hybridized with the 3' probe (the top panel). The correctly targeted allele appears as 9.5 kb and untargeted wild type allele is 11 kb. Homozygous targeted hESC clones are shown in blue, heterozygous targeted clones are in red, and untargeted clones are in black. The parental cell lines used for the second targeting, TERT$^{\Delta/\Delta}$ and TERT$^{\Delta/\Delta\#2}$**,** are clone #26 and #17 respectively. The correct deletion events were also confirmed by PCR using external primers (bottom panel).
(B) NHEJ-derived junction of the deleted region of the homozygous targeted lines TERT$^{\Delta/\Delta}$ and TERT$^{\Delta/\Delta\ \#2}$ was determined by sequencing. sgRNA for the second targeting was designed across the junction (sg-1+15).

Figure 3.3

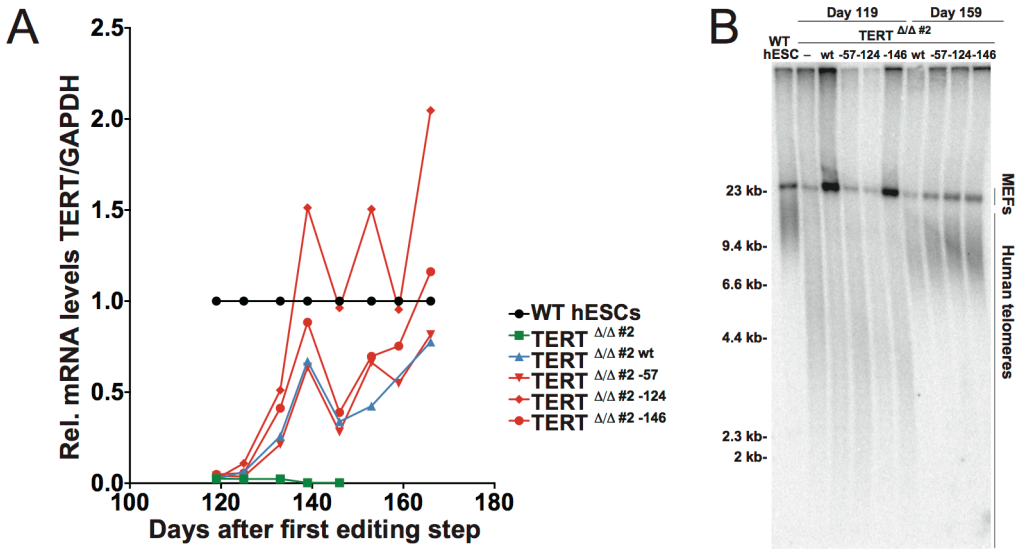


Figure 3.3
Independent confirmation of promoter mutation experiments (shown in Figure 3.1C,D) using an independent TERT$^{\Delta/\Delta\#2}$ cell line
(A) Relative expression levels of TERT mRNA by mutant and wt promoter-containing hESCs (TERT$^{\Delta/\Delta\ \#2}$) over a time course after targeting measured by quantitative RT-PCR. Expression of TERT was normalized to GAPDH.
(B) Telomere restriction fragment assay of WT, TERT$^{\Delta/\Delta\ \#2}$ and the targeted hESCs over a post-targeting time course (day 0: first editing; day 86: second editing).

Figure 3.4

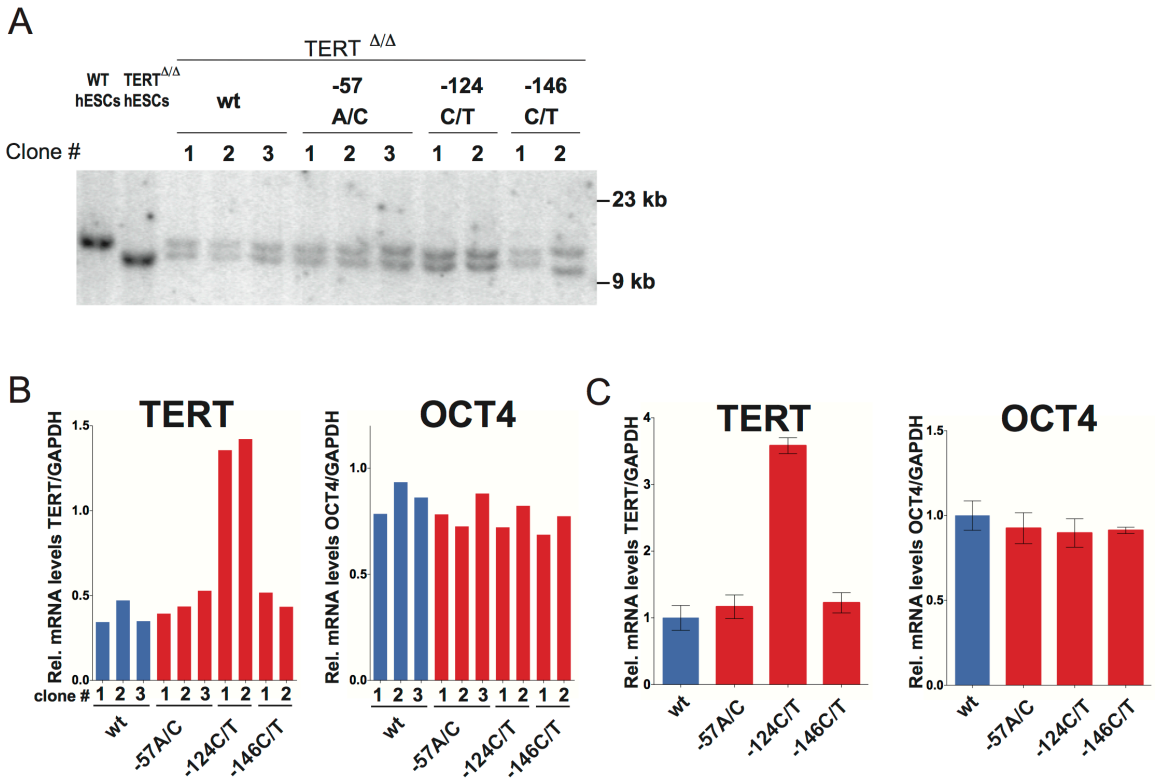


Figure 3.4
Clonal analysis of TERT promoter mutation hESCs confirmed results from bulk analysis
(A) Southern blot analysis for TERT$^{\Delta/\Delta}$ hESCs clones. Correct targeting appeared as 11 kb band at the size of WT hESCs and untargeted allele as 9.5 kb at the size of parental TERT$^{\Delta/\Delta}$ hESCs.
(B) Quantitative RT-PCR of TERT and OCT4 in individual clones of the targeted hESCs. Expression levels are shown relative to WT hESCs and normalized to GAPDH.
(C) Average expression of data shown in (B). Expression levels are compared to TERT$^{\Delta/\Delta}$ wt hESC clones. Error bars represent the SEM.

Figure 3.5

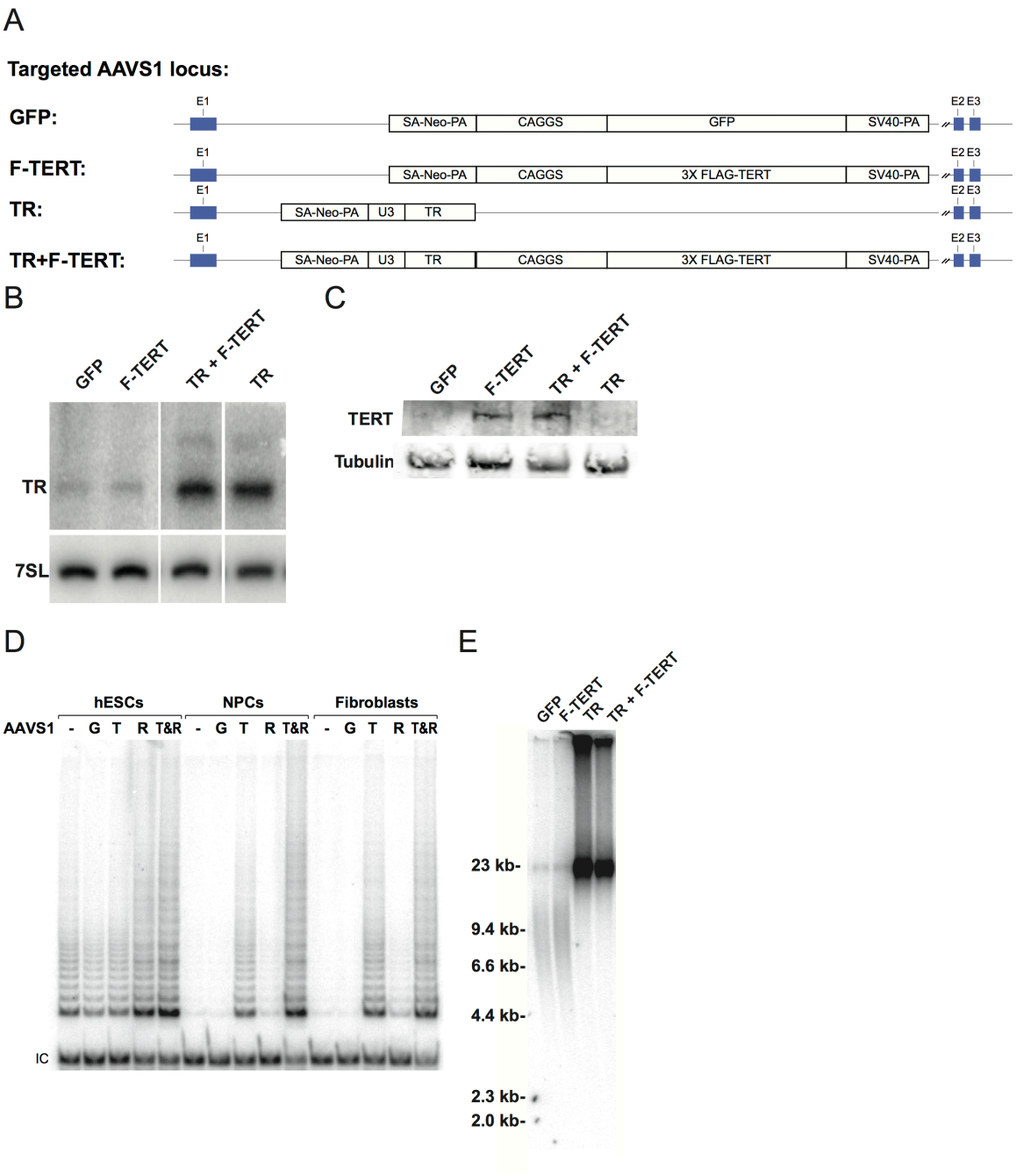

A
Targeted AAVS1 locus:
GFP:
F-TERT:
TR:
TR+F-TERT:
E1
E2 E3
SA-Neo-PA
CAGGS
GFP
3X FLAG-TERT
SV40-PA
U3
TR
B
GFP
F-TERT
TR + F-TERT
TR
7SL
C
TERT
Tubulin
D
hESCs
NPCs
Fibroblasts
AAVS1
- G T R T&R
IC
E
23 kb-
9.4 kb-
6.6 kb-
4.4 kb-
2.3 kb-
2.0 kb-

Figure 3.5
Telomerase activity is restricted by levels of TERT in differentiated cells while TR is limiting in wild-type hESCs
(A) Targeting schematic of GFP, 3XFLAG-TERT (F-TERT), TR, and F-TERT+TR overexpression from the AAVS1 locus in wild-type hESCs.
(B) Northern blot detection of total TR and 7SL in targeted hESC lines. TR runs as a doublet in UREA PAGE.
(C) SDS-PAGE immunoblot of total TERT and tubulin proteins in editing hESC lines from whole cell extract.
(D) TRAP assay of whole cell extracts from NPCs and fibroblast-like cells differentiated from GFP (G), F-TERT (T), TR (R), or F-TERT+TR (T&R) overexpressing hESCs using 200 ng protein.
(E) Telomere restriction fragment assay of GFP, F-TERT, TR, and F-TERT+TR overexpressing hESCs.

Figure 3.6

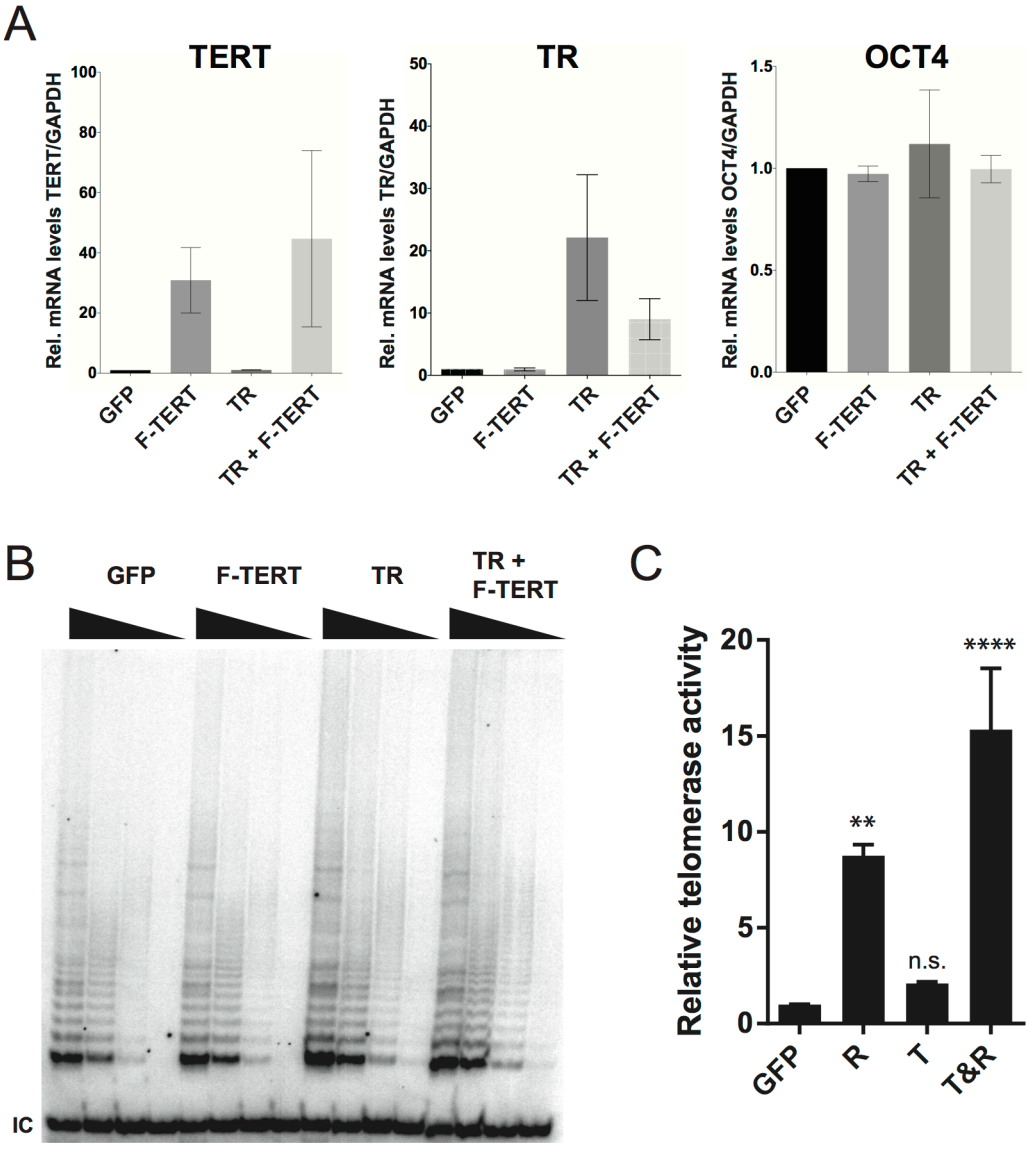


Figure 3.6
Quantification of TERT and TR expression levels and telomerase activity in the overexpression hESCs
(A) Quantitative RT-PCR of TERT, TR, and OCT4 in GFP, F-TERT, TR, or F-TERT+TR overexpressing hESCs. Expression level is relative to GFP hESCs and normalized to GAPDH. Error bars represent the SEM of three biological replicates taken one week apart.
(B) TRAP assay of whole cell extracts from GFP, F-TERT, TR, or F-TERT+TR overexpressing hESCs using decreasing amount of protein (250, 50, 10, 2 ng).
(C) Relative telomerase activity of the hESC lines was assayed by QTRAP. Values were set as fold activity relative to the GFP control line (n=3, ANOVA with Tukey's test, SEM bars). R: TR, T: F-TERT and T&R: F-TERT+TR

Figure 3.7

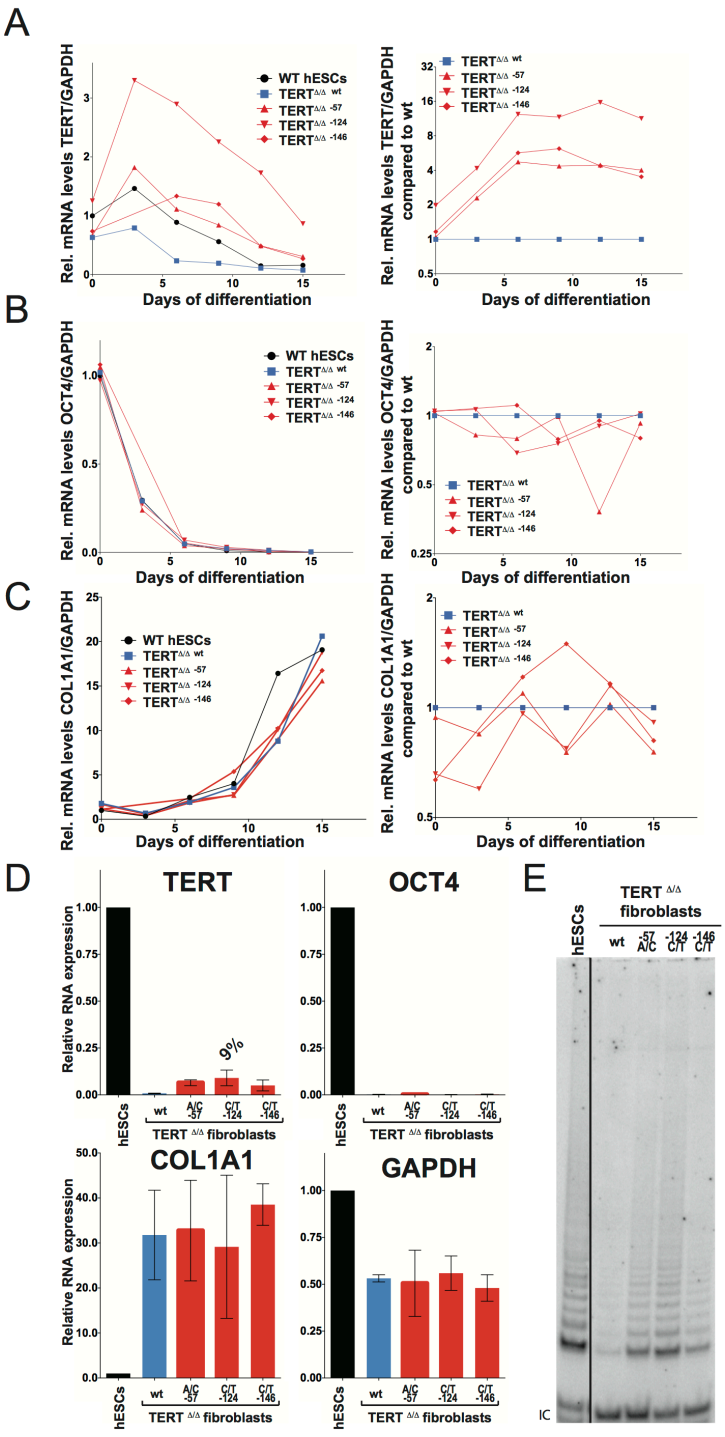

A
B
C
D
E
Rel. mRNA levels TERT/GAPDH
Rel. mRNA levels TERT/GAPDH compared to wt
Rel. mRNA levels OCT4/GAPDH
Rel. mRNA levels OCT4/GAPDH compared to wt
Rel. mRNA levels COL1A1/GAPDH
Rel. mRNA levels COL1A1/GAPDH compared to wt
Days of differentiation
WT hESCs
TERT Δ/Δ wt
TERT Δ/Δ -57
TERT Δ/Δ -124
TERT Δ/Δ -146
TERT
OCT4
COL1A1
GAPDH
Relative RNA expression
9%
hESCs
wt
A/C -57
C/T -124
C/T -146
TERT Δ/Δ fibroblasts
TERT Δ/Δ fibroblasts
IC

Figure 3. 7
Fibroblasts carrying cancer-associated TERT promoter point mutations failed to silence TERT expression upon differentiation and have telomerase activity
(A), (B) and (C) Relative expression level of TERT, OCT4 or COL1A1 in the promoter-mutated hESC-derived fibroblasts compared to WT hESCs over a time course of differentiation (left panel). Relative expression level of TERT, OCT4 or COL1A1 compared to $TERT^{\Delta/\Delta}$ and WT fibroblasts over a time course of differentiation. The right panel shows the same data as in the left panels, normalized to $TERT^{\Delta/\Delta\ wt}$ fibroblasts. Expression of TERT, OCT4, or COL1A1 was normalized to GAPDH.
(D) Quantitative RT-PCR of TERT, OCT4, COL1A1, and GAPDH in the fibroblasts carrying the promoter mutations 24 days after differentiation. Expression level is relative to WT hESCs.
(E) TRAP assay of whole-cell extracts from WT hESCs, and the fibroblasts carrying the TERT promoter mutations (24 days after differentiation) using 2 μg of protein.

Figure 3.8

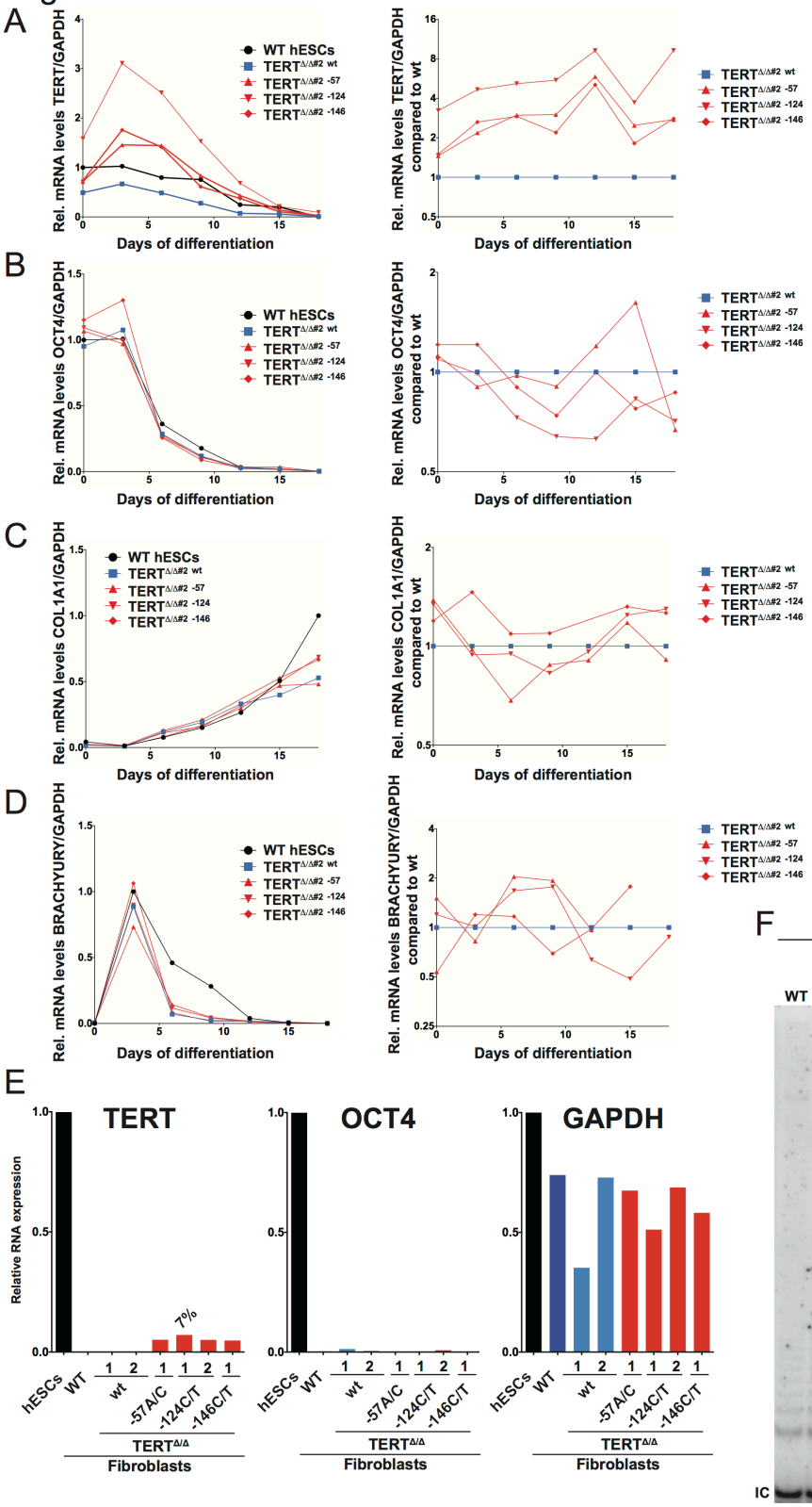

A
B
C
D
E
F
Rel. mRNA levels TERT/GAPDH
Rel. mRNA levels TERT/GAPDH compared to wt
Rel. mRNA levels OCT4/GAPDH
Rel. mRNA levels OCT4/GAPDH compared to wt
Rel. mRNA levels COL1A1/GAPDH
Rel. mRNA levels COL1A1/GAPDH compared to wt
Rel. mRNA levels BRACHYURY/GAPDH
Rel. mRNA levels BRACHYURY/GAPDH compared to wt
Days of differentiation
WT hESCs
TERT Δ/Δ #2 wt
TERT Δ/Δ #2 -57
TERT Δ/Δ #2 -124
TERT Δ/Δ #2 -146
Relative RNA expression
TERT
OCT4
GAPDH
7%
hESCs
WT
wt
-57A/C
-124C/T
-146C/T
TERT Δ/Δ
Fibroblasts
Fibroblasts
TERT Δ/Δ
wt
-57
-124
-146
IC

Figure 3.8
Independent confirmation of the failure of TERT repression and telomerase activity upon fibroblast differentiation shown in Figure 3 using an independent TERT$^{\Delta/\Delta\ \#2}$ cell line. The results obtained by the bulk analysis were also confirmed by clonal analysis of fibroblasts carrying the TERT promoter mutations
(A) and (B) Relative expression levels of TERT and OCT4 in the promoter-mutated hESC-derived fibroblasts compared to WT hESCs over a time course of differentiation (left panels). The right panels show the same data as in A, normalized to TERT$^{\Delta/\Delta\ \#2\ wt}$ fibroblasts.
(C) Relative expression level of COL1A1 in the promoter-mutated hESC-derived fibroblasts compared to WT fibroblasts (day 15) over a time course of differentiation (left panels). The right panels show the same data as in A, normalized to TERT$^{\Delta/\Delta\ \#2\ wt}$ fibroblasts.
(D) Relative expression level of BRACHYURY (T) in the promoter-mutated hESC-derived fibroblasts compared to WT embryonic bodies (day 3) over a time course of differentiation (left panels). The right panels show the same data as in A, normalized to TERT$^{\Delta/\Delta\ \#2\ wt}$ fibroblasts.
(E) Quantitative RT-PCR of TERT, OCT4, and GAPDH in individual clones of fibroblasts carrying the mutations. Expression levels are shown relative to WT hESCs.
(F) TRAP assay of whole cell extracts from fibroblasts differentiated from clonal hESCs carrying the TERT promoter mutations using 2 μg of protein.

Figure 3.9

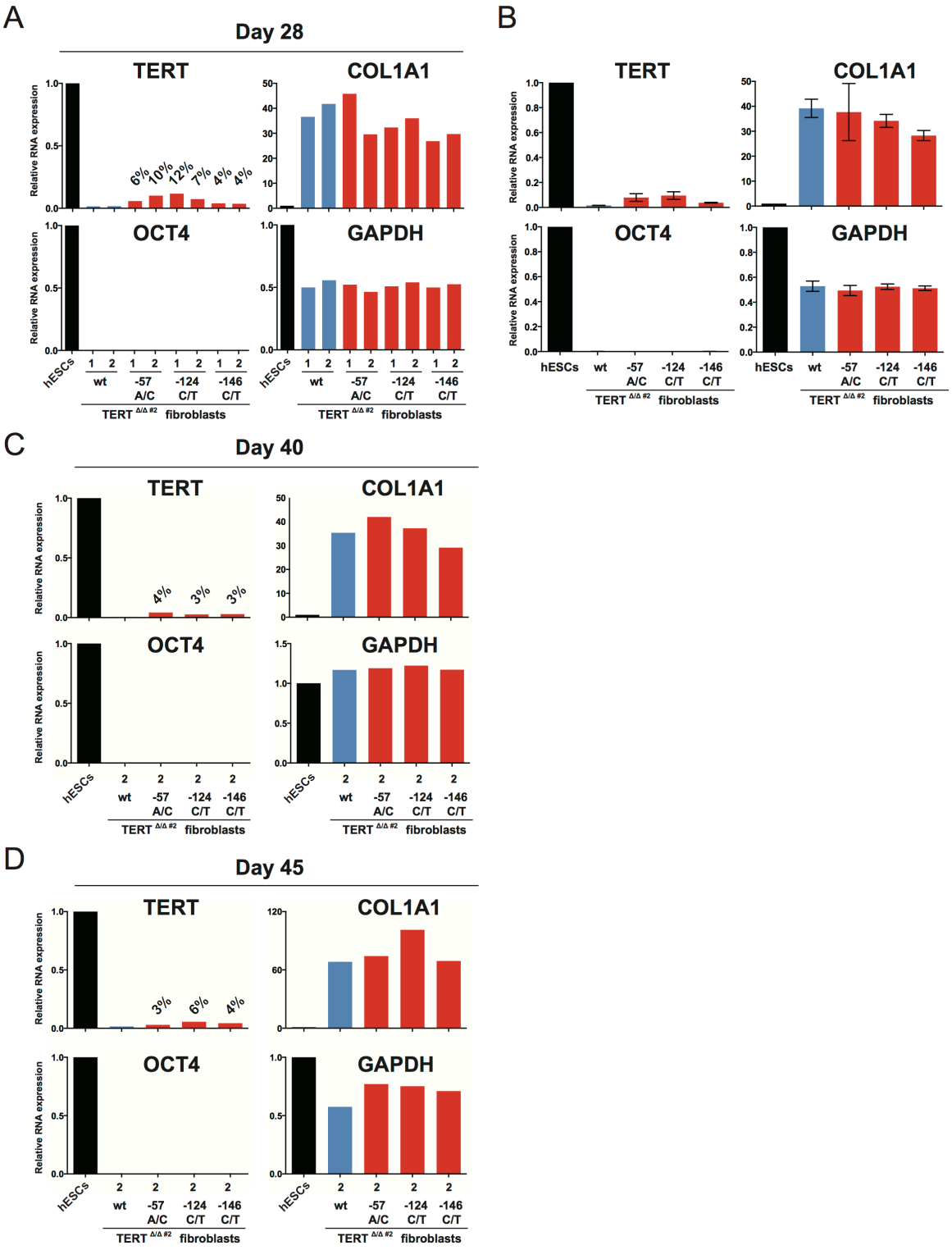

A
Day 28
TERT
COL1A1
OCT4
GAPDH
Relative RNA expression
6%
10%
12%
7%
4%
4%
hESCs
wt
-57 A/C
-124 C/T
-146 C/T
TERT Δ/Δ #2 fibroblasts
B
C
Day 40
4%
3%
3%
D
Day 45
3%
6%
4%

Figure 3.9
The failure of TERT repression in fibroblasts was retained throughout long-term culture
(A), (C) and (D) Relative expression level of TERT, OCT4, COL1A1, and GAPDH in individual clones of fibroblasts carrying the mutations at days 28, 40, and 45 after differentiation. Expression levels are shown relative to WT hESCs.
(B) Average expression data shown in (A). Expression levels are compared to WT hESC clones. Error bars represent the SEM.

Figure 3.10

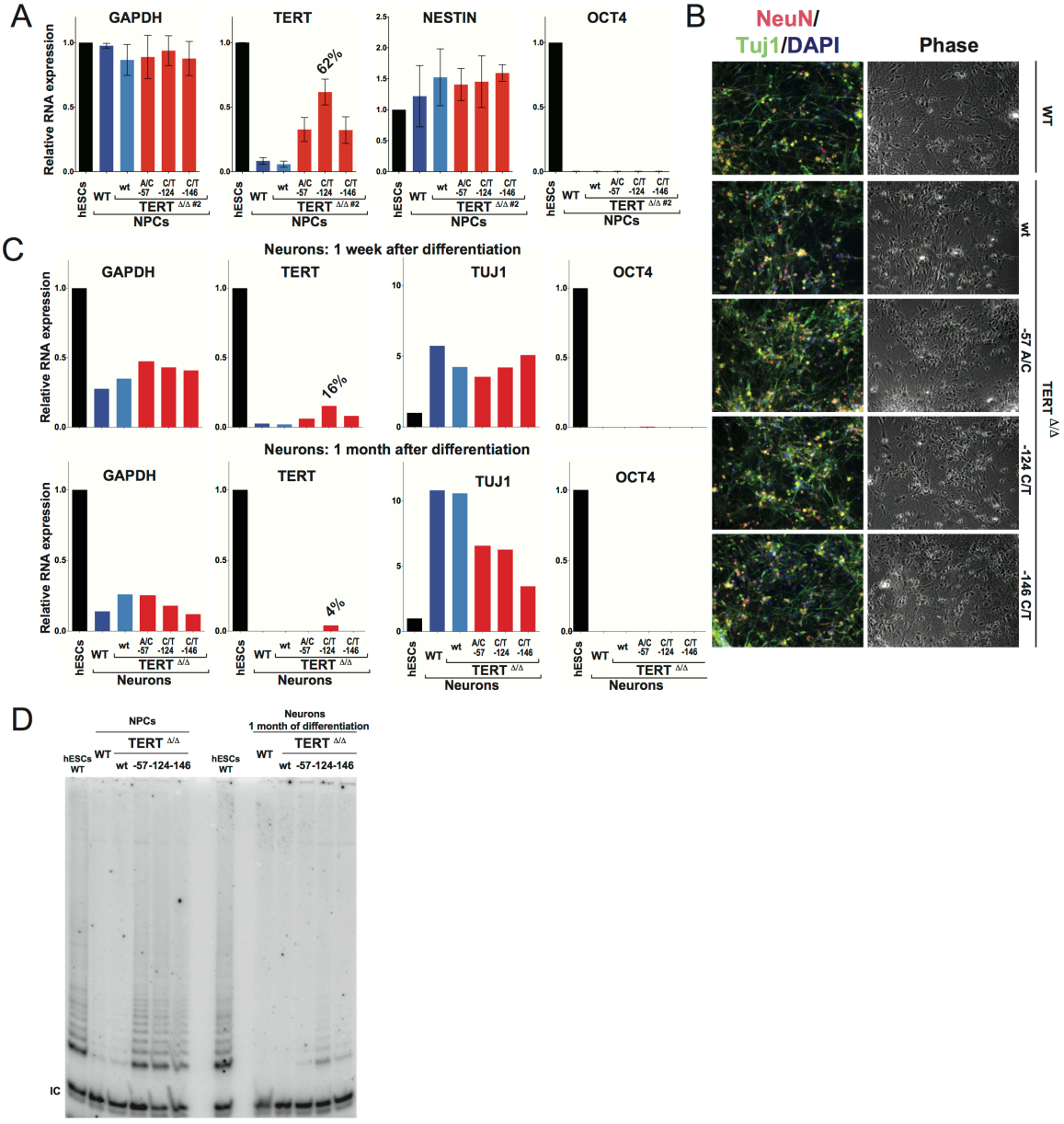

A
GAPDH
TERT
NESTIN
OCT4
Relative RNA expression
62%
hESCs
WT
wt
A/C -57
C/T -124
C/T -146
TERT Δ/Δ #2
NPCs
B
NeuN/
Tuj1/DAPI
Phase
WT
wt
-57 A/C
-124 C/T
-146 C/T
TERT Δ/Δ
C
Neurons: 1 week after differentiation
TUJ1
16%
Neurons: 1 month after differentiation
4%
TERT Δ/Δ
Neurons
D
NPCs
Neurons
1 month of differentiation
-57-124-146
IC

Figure 3.10
Neural precursors and neurons differentiated from the promoter mutation hESCs failed to repress TERT and telomerase activity
(A) Quantitative RT-PCR of GAPDH, TERT, NESTIN, and OCT4 in the neural precursors carrying the promoter mutations 20-25 days after differentiation from hESCs. Expression levels are relative to the WT hESCs.
(B) Phase-contrast and immunofluorescence images of neurons differentiated from wild-type hESCs or the TERT promoter mutation-containing hESCs. Shown are cells 28 days after neural induction from NPCs and treated with mitotic inhibitor for 16 days. The left panel shows IF staining against NeuN (red), Tuj1 (green), and DAPI staining (blue).
(C) Quantitative RT-PCR of GAPDH, TERT, TUJ1, and OCT4 in the neurons carrying the promoter mutations. The top panel shows expression levels of neurons 7 days after neuronal differentiation from NPCs. The bottom panel shows expression levels of neurons 28 days after induction of neuronal differentiation from NPCs and treated with mitotic inhibitor for 16 days. Expression level is relative to the WT hESCs.
(D) TRAP assay of whole cell extracts from NPCs (35 days after differentiation from hESCs) and neurons (28 days after neuronal differentiation from NPCs and treated with mitotic inhibitor for 16 days) using 1 μg protein.

Figure 3.11

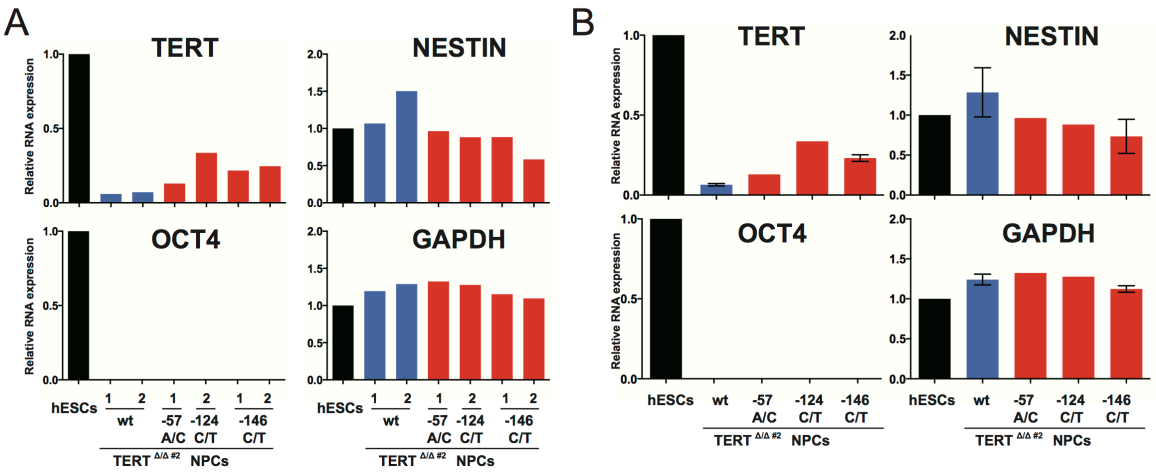


Figure 3.11
Clonal analysis of TERT promoter mutation NPCs confirmed results from bulk analysis
(A) Quantitative RT-PCR of TERT, OCT4, NESTIN, and GAPDH in NPCs differentiated from individual clones of the targeted hESCs (28 days after differentiation from hESCs). Expression levels are shown relative to WT hESCs.
(B) Average expression of data shown in (A). Expression levels are compared to WT hESCs. Error bars represent the SEM.

Figure 3.12

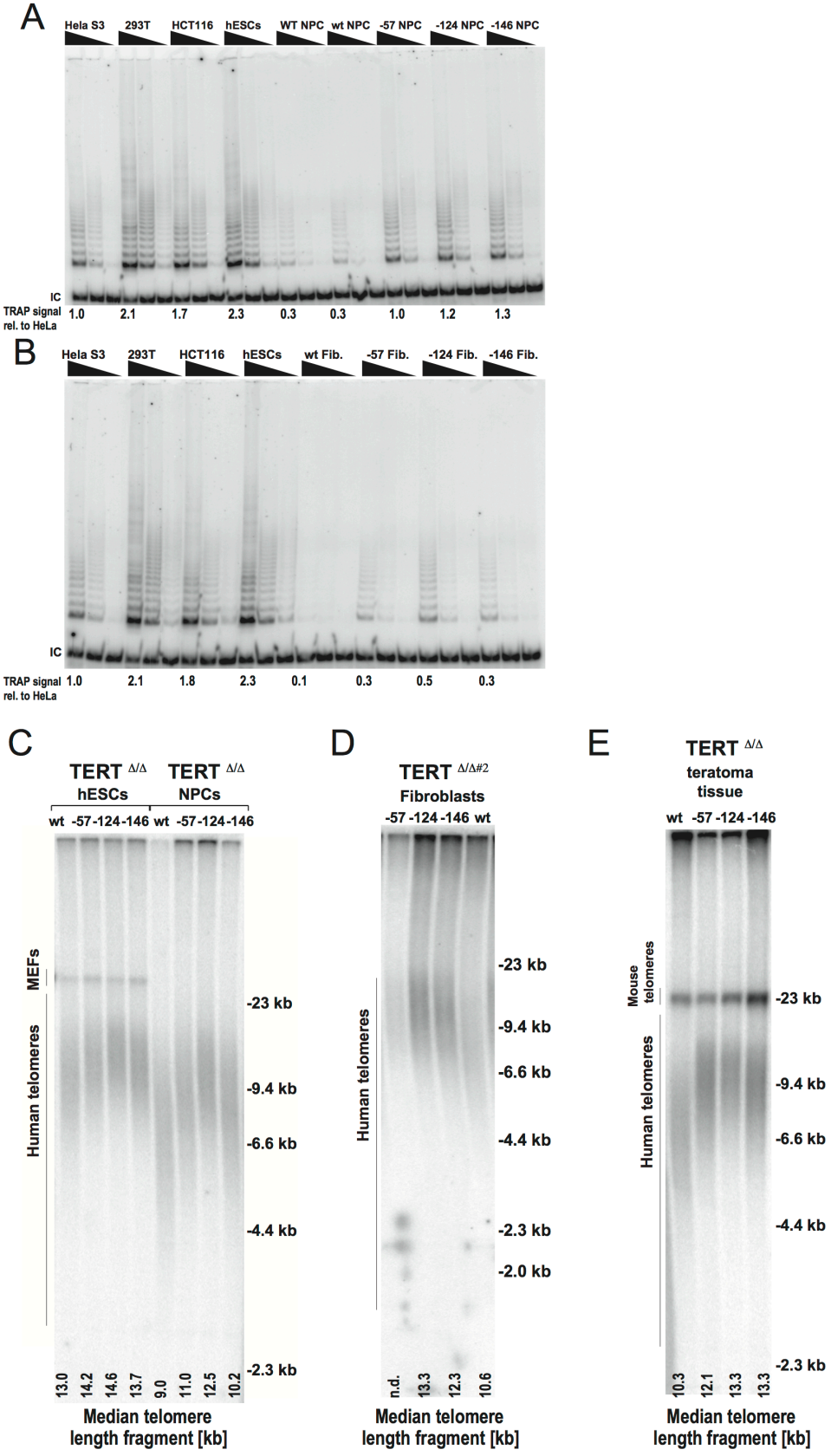

A
Hela S3
293T
HCT116
hESCs
WT NPC
wt NPC
-57 NPC
-124 NPC
-146 NPC
IC
TRAP signal rel. to HeLa
1.0 2.1 1.7 2.3 0.3 0.3 1.0 1.2 1.3
B
Hela S3
293T
HCT116
hESCs
wt Fib.
-57 Fib.
-124 Fib.
-146 Fib.
IC
TRAP signal rel. to HeLa
1.0 2.1 1.8 2.3 0.1 0.3 0.5 0.3
C
TERT Δ/Δ hESCs
TERT Δ/Δ NPCs
wt -57 -124 -146 wt -57 -124 -146
MEFs
Human telomeres
-23 kb
-9.4 kb
-6.6 kb
-4.4 kb
-2.3 kb
13.0 14.2 14.6 13.7 9.0 11.0 12.5 10.2
Median telomere length fragment [kb]
D
TERT Δ/Δ#2 Fibroblasts
-57 -124 -146 wt
Human telomeres
-23 kb
-9.4 kb
-6.6 kb
-4.4 kb
-2.3 kb
-2.0 kb
n.d. 13.3 12.3 10.6
Median telomere length fragment [kb]
E
TERT Δ/Δ teratoma tissue
wt -57 -124 -146
Mouse telomeres
Human telomeres
-23 kb
-9.4 kb
-6.6 kb
-4.4 kb
-2.3 kb
10.3 12.1 13.3 13.3
Median telomere length fragment [kb]

Figure 3.12

Fibroblasts and neural precursors carrying cancer-associated TERT promoter point mutations showed comparable telomerase activity to cancer cell lines, and telomere length was maintained over long-term culture and tumor development

(A) and (B) TRAP assay of whole-cell extracts from cancer cell lines (HeLaS3, 293T, and HCT116), WT hESCs, and the NPCs or fibroblasts carrying the TERT promoter mutations (24 days after differentiation for fibroblasts and 20 days for NPCs) using decreasing amount of protein (200, 40, 8 ng). For comparison, TRAP samples in (A) and (B) were prepared simultaneously and samples from cancer cell lines are identical in (A) and (B). TRAP signals relative to HeLa S3 were quantified and are shown at the bottom of the gels.

(C) Telomere restriction fragment assay of the hESCs and NPCs (65 days after differentiation from hESCs). Median telomere length signals were quantified and shown at the bottom. It is important to note the telomere shortening in NPCs and fibroblasts in wild type cells exceeds the initial telomere length difference found in the hESCs.

(D) Telomere restriction fragment assay of the fibroblasts (30 days after differentiation from hESCs). Median telomere length signals were quantified and shown at the bottom.

(E) Telomere restriction fragment assay of teratoma tumor tissue generated from wt and promoter-mutation containing hESCs (75 days after injection). Median telomere length signals were quantified and shown at the bottom.

Figure 3.13

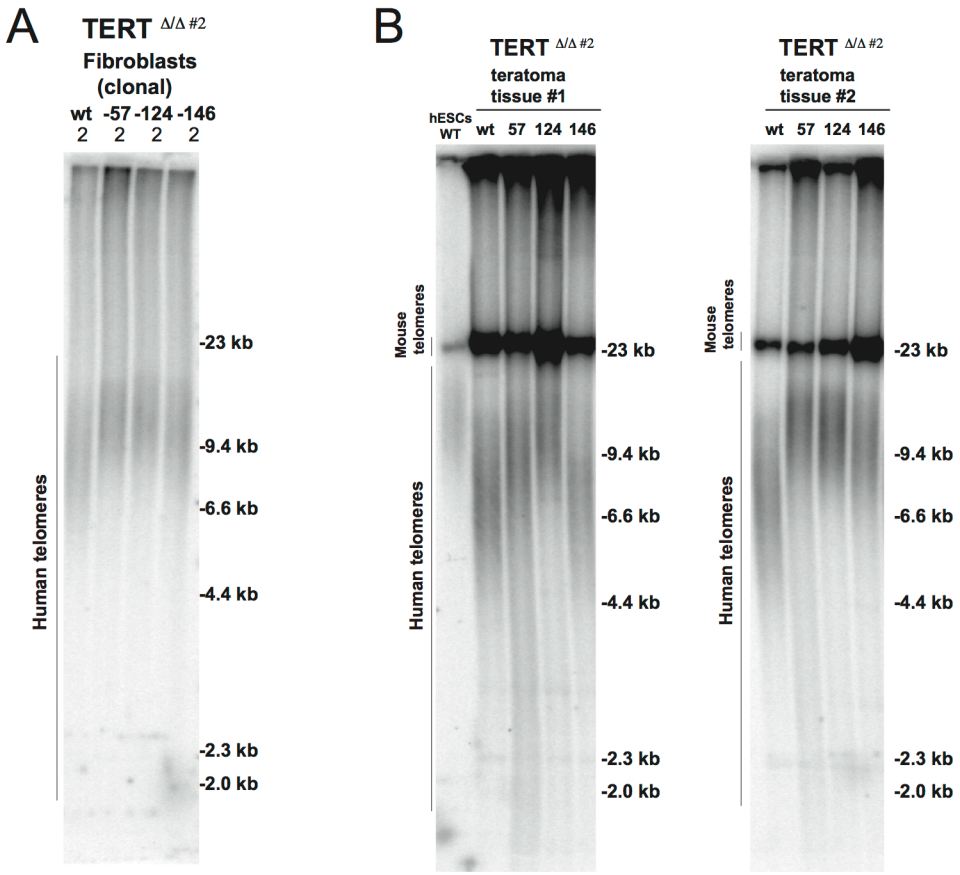
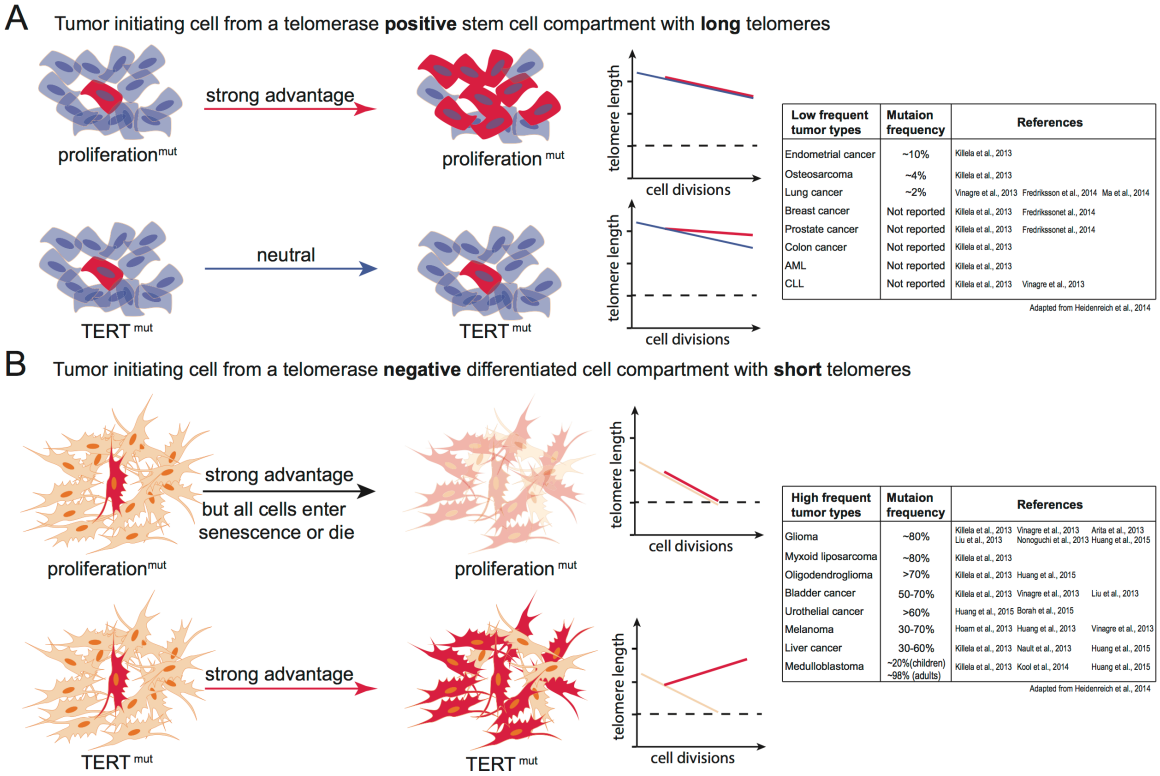


Figure 3.13
Fibroblasts and teratoma tissue carrying cancer-associated TERT promoter point mutations maintained telomere length over long-term culture and tumor development
(A) Telomere restriction fragment assay of the fibroblasts (36 days after differentiation from hESCs).
(B) Telomere restriction fragment assay of teratoma tumor tissue generated from wt and promoter-mutation containing hESCs. Teratoma tissues in the group #1 were explanted 84 days after injection except -57 mutant samples (64 days after injection). Teratoma tissues in the group #2 were explanted 56 days after injection.

Figure 3.14



Low frequent tumor types	Mutaion frequency	References
Endometrial cancer	~10%	Killela et al., 2013
Osteosarcoma	~4%	Killela et al., 2013
Lung cancer	~2%	Vinagre et al., 2013 Fredriksson et al., 2014 Ma et al., 2014
Breast cancer	Not reported	Killela et al., 2013 Fredrikssonet al., 2014
Prostate cancer	Not reported	Killela et al., 2013 Fredrikssonet al., 2014
Colon cancer	Not reported	Killela et al., 2013
AML	Not reported	Killela et al., 2013
CLL	Not reported	Killela et al., 2013 Vinagre et al., 2013

Adapted from Heidenreich et al., 2014

High frequent tumor types	Mutaion frequency	References
Glioma	~80%	Killela et al., 2013 Vinagre et al., 2013 Arita et al., 2013 Liu et al., 2013 Nonoguchi et al., 2013 Huang et al., 2015
Myxoid liposarcoma	~80%	Killela et al., 2013
Oligodendroglioma	>70%	Killela et al., 2013 Huang et al., 2015
Bladder cancer	50-70%	Killela et al., 2013 Vinagre et al., 2013 Liu et al., 2013
Urothelial cancer	>60%	Huang et al., 2015 Borah et al., 2015
Melanoma	30-70%	Hoarn et al., 2013 Huang et al., 2013 Vinagre et al., 2013
Liver cancer	30-60%	Killela et al., 2013 Nault et al., 2013 Huang et al., 2015
Medulloblastoma	~20%(children) ~98% (adults)	Killela et al., 2013 Kool et al., 2014 Huang et al., 2015

Adapted from Heidenreich et al., 2014

Figure 3.14
Model explaining the tumor spectrum associated with TERT promoter mutations
Shown are the differential outcomes of a cell acquiring a cancer-associated TERT promoter mutation or a proliferation-inducing mutation dependent on telomere length of the cell.
(A) In a cell with long telomeres and telomerase activity, a proliferation-promoting mutation will result in a strong proliferative advantage and can act as the tumor-initiating event. Cells with long telomeres arise from tissues that have a telomerase positive stem cell compartment such as the hematopoietic or intestinal system. In contrast, mutations in the TERT promoter do not provide a proliferative advantage, they are neutral and do not promote tumor formation. Cell states are depicted on the left; cells that acquire mutations are shown in red. A schematic depicting telomere length changes as a function of the number of cell divisions is shown on the right. The dashed line indicates the critical telomere length at which cells are subjected to the Hayflick limit and stop proliferating or die. The red line indicates the telomere length changes predicted for cells that acquire either a proliferation-promoting mutation (top) or a TERT promoter mutation (bottom). The blue line indicates the telomere length changes predicted for wild-type cells. Indicated is a case where telomere shortening is suppressed by the TERT promoter mutations. However, since these cell already have long telomeres and/or naturally express telomerase, telomeres in neither wild-type cells or cells acquiring a proliferation inducing mutation will shorten to the point that the cells are subjected to the Hayflick limit.
(B) Schematic as shown in (A) but for a telomerase negative cells with short telomeres. A proliferation-promoting mutation will also provide a growth advantage in telomerase-negative differentiated cells with short telomeres, however, these cells will enter replicative senescence or die. In contrast, a cell with short telomeres acquiring a TERT promoter mutation can bypass the Hayflick limit (dashed lines), immortalize, and outcompete its neighboring cells. Cell states are depicted on the left; cells that acquire mutations are shown in red. Schematic depicting telomere length changes as a function of the number of cell divisions is shown on the right. The orange line indicates the telomere length changes predicted for wild-type cells. The table to the right shows the frequency of TERT promoter mutations found in different types of tumors (adapted form (Heidenreich et al., 2014)). Table includes references that report the specific tumor subtypes and frequencies used to generate this table.

CHAPTER FOUR

Mutations in the promoter of the telomerase gene *TERT* contribute to tumorigenesis by a two-step mechanism

Based on Chiba et al., *Science* 2017

ABSTRACT

TERT promoter mutations (TPMs) are the most common non-coding mutations in cancer. The timing and consequences of TPMs have not been fully established. Here we show that TPMs do not prevent telomere attrition, resulting in cells with critically short and unprotected telomeres. Immortalization by TPMs requires a gradual upregulation of telomerase, coinciding with telomere fusions. These data suggest that TPMs contribute to tumorigenesis by promoting immortalization and genomic instability in two phases. In an initial phase, TPMs do not prevent bulk telomere shortening but extend cellular life-span by healing the shortest telomeres. In the second phase, the critically short telomeres lead to genome instability and telomerase is further upregulated to sustain cell proliferation.

INTRODUCTION

Genomic instability is a hallmark of cancer (Hanahan and Weinberg, 2011) as it accelerates the genetic evolution of neoplastic cells. Genomic instability can be a continuous feature of cancer cells such as in mismatch repair-deficient cells, or it can be a transient episode such as during telomere crisis. Telomere shortening restricts the replicative lifespan of most somatic cells (Harley et al., 1990). Once telomeres have been exhausted, cells permanently arrest or die, due to the triggering of DNA damage checkpoints. Cells with inactivated checkpoints can avert this fate(Shay, 2016; Smogorzewska and de Lange, 2004) and continue to proliferate until the point when telomeres become critically short, resulting in end-to-end fusions of chromosomes, which result in chromosomal rearrangements and DNA copy number changes(Feldser et al., 2003). During this period of telomere crisis, massive chromosomal instability leads to the death of most cells, due to catastrophic DNA damage or genotypes incompatible with survival. Signatures of telomere-driven genomic instability and crisis, such as telomere fusion events are detected in a variety of cancer samples and cell lines, giving testimony to a past period of telomere crisis(Capper et al., 2007; Gisselsson et al., 2001; Letsolo et al., 2010; Lin et al., 2014; Lo et al., 2002; Nones et al., 2014; Plentz et al., 2004; Roger et al., 2013; Simpson et al., 2015) and reviewed in (Maciejowski and de Lange, 2017)). The massive genomic instability during telomere crisis is not conducive to tumor formation, but can trigger the selection of cells that have acquired a mechanism to re-establish telomere maintenance. One common mechanism involves activation of telomerase(Kim et al., 1994), the telomere-elongating enzyme (Greider and Blackburn, 1985; Greider and Blackburn, 1987), which is repressed in most differentiated cells (Cong et al., 2002). Re-activation of telomerase expression in neoplastic cells commonly occurs through point mutations or structural rearrangements in the *TERT* promoter, leading to re-activation of telomerase expression. The three most prevalent *TERT* promoter mutations (TPMs) are at nucleotide positions -57 A>C (-57), -124 C>T (-124) and -146 C>T (-146) relative to the translation start site (Horn et al., 2013; Huang et al., 2013; Killela et al., 2013). All cancer-associated TPMs generate *de novo* E26 transformation-specific (ETS) transcription factor binding sites and have been associated with increased TERT expression (Fredriksson et al., 2014). Several ETS transcription factors, including GABPα, have been demonstrated to mediate the increased TERT expression in cells with TPMs(Bell et al., 2015; Li et al., 2015; Min and Shay, 2016; Stern et al., 2015).

It is poorly understood at what time during cancer progression TPMs occur - before or after telomere crisis - and how the resulting activation of telomerase affects telomere length and the timing of telomere crisis. Recent studies in melanoma and glioblastoma found that tumors with TPMs are associated with decreased telomere length(Ceccarelli et al., 2016; Hayward et al., 2017). Found in established cancers, TPMs can be a negative prognostic indicator, suggesting that they arise late in progression(Borah et al., 2015; Nagore et al., 2016). However, a recent study in melanomas has found TPMs in the majority of pre-neoplastic lesions that did not yet fulfill the histopathological criteria for melanomas (Shain et al., 2015). These pre-neoplastic lesions did not show marked chromosomal aberrations, whereas the melanomas that arose from these lesions tended to show multiple copy number changes

that could have been attributed to telomere crisis. This observation raises the possibility that at least in some settings TPMs are present before telomere crisis.

Further complexity in the link between telomeres and cancer comes from seemingly contradictory observations regarding the relationship between telomere length and cancer risk. Experiments in mice show that telomere shortening in the presence of defective DNA damage checkpoints robustly increases cancer formation(Artandi et al., 2000; Artandi and DePinho, 2000). In humans, mutations in the telomerase pathway can lead to premature telomere shortening in highly proliferative tissues, eventually resulting in telomere-driven genomic instability, bone marrow failure, and lymphoma (Armanios and Price, 2012; Nelson and Bertuch, 2012). These findings suggest that constitutionally short telomeres may truncate the time leading to genomic instability and thereby increase cancer risk. Contradicting such a model is the finding of multiple population studies that short telomere length is correlated with a decreased risk for cancer (Bojesen et al., 2013; Han et al., 2009; Iles et al., 2014; Nan et al., 2011; Rode et al., 2016). These latter findings suggest that short telomeres exert a tumor suppressive effect, possibly because they constrain the number of population doublings renegade cells can undergo before they die or stop dividing, due to replicative senescence (Aubert, 2014; Cesare and Karlseder, 2012).

Here we genetically engineer TPMs into human embryonic stem cells, differentiate them into fibroblasts and show that TPMs are required but are not sufficient for their immortalization. Surprisingly, we find that telomeres in cells with TPMs continue to shorten and ultimately promote telomere-driven genomic instability as evidenced by telomere-fusions. Based on these observations we propose a refined model for the role of telomere length and TPMs in cancer formation and genomic instability.

RESULTS

TPMs immortalize cell populations in a checkpoint independent manner

TERT overexpression immortalizes normal somatic cells (Bodnar et al., 1998; Morales et al., 1999). In cancer, TPMs result in moderately increased TERT expression (Fredriksson et al., 2014), but it is not clear whether the mutations are sufficient to counteract telomere shortening and sustain unlimited cell proliferation. To explore the long-term effects of TPMs on telomere length and genomic stability in a genetically defined setting, we engineered the three most frequent TPMs *de novo* into human embryonic stem cells (hESCs) using CAS9 mediated genome editing (Chiba et al., 2015) (Fig. 4.1A and Fig. 4.2A-D). We started the "experimental clock" by differentiating the parental cell lines into fibroblasts, which are normally telomerase-negative. We assayed the proliferative capacity of hESC-derived fibroblasts with and without a single TPM using three distinct conditions: (i) fibroblasts transduced with Simian virus 40 large T antigen (SV40 TAg, inactivating pRb and p53 signaling) (Fig. 4.1B, 4.2B), (ii) fibroblasts with intact cell cycle and DNA damage checkpoints (Fig. 4.1C), and (iii) fibroblasts with inactivated cell cycle and DNA damage checkpoints, achieved by deletion of p14/p16 function ($CDKN2A^{\Delta/\Delta}$) (Fig. 4.1D, Fig. 4.2C). In all cell lines the TPMs extended the proliferative capacity significantly past the proliferative barrier of wild-type cells. Cells with TPMs proliferated without signs of crisis or a strong decrease in doubling rate at the time when wild-type cells arrested in a telomere length dependent manner (Bodnar et al., 1998) (Fig. 4.1B-D, Fig. 4.2E and 4.2F). Overall, these data demonstrate that TPMs promote the immortalization of bulk cell populations.

Telomeres shorten despite the presence of TPMs but eventually stabilize

The finding that TPMs can immortalize human fibroblasts raised the question of whether they are sufficient to counteract telomere shortening. We measured telomere length over time using Southern blotting of telomere restriction fragments. In wild-type cells, telomeres shortened progressively with a rate of approximately 40-60 base pairs/PD, which is in agreement with previous reports for human telomerase-negative fibroblasts (Figures 4.1E, F, G and 4.3A, B) (Harley et al., 1990; Karlseder et al., 2002). Surprisingly, in cells with TPMs telomeres initially also shortened with rates similar to wild-type cells. However, the rate of telomere shortening decreased after about 70 PDs when telomeres stabilized at a length of 2.5-4.5 kb for all TPM cell lines (Figures 4.1E, F, G and 4.3A, B). Remarkably, the stabilized mean telomere length in the TPM cells was shorter than the telomeres in wild-type control cells at the time of their arrest (Figures 4.1E, F, G and 4.3A, B). The analysis of the telomere length distribution of cells with TPMs revealed that the mean length shifted from long to short telomeres and then stabilized (Fig. 4.1H and Fig. 4.3C), suggesting that short telomeres were preferentially maintained, while long telomeres continued to shorten.

TPMs cannot fully suppress telomere-driven genomic instability

Unprotected short telomeres can lead to genomic instability through telomere fusion events, which have been detected in a variety of tumor samples and cell lines (Artandi et al., 2000; Capper et al., 2007; Feldser et al., 2003; Lin et al., 2014; Maciejowski and de Lange, 2017; Plentz et al., 2004). To assess whether the

chromosome ends are protected in cells with TPMs, we used a PCR-based method to quantify telomere-telomere fusion events over the course of a year after cell differentiation(Capper et al., 2007). We detected fusions in SV40 TAg cells with the -124 and -146 TPMs when telomere length stabilized (Fig. 4.4A and 4.4B and Fig. 4.5A and 4.5B). These fusion events were suppressed by overexpression of TERT (Fig. 4.5C), showing that they were driven by telomere attrition. Cells with the -57 TPM showed fewer fusions, presumably due to their slightly longer telomeres (Fig. 4.1E). Thus, TPMs not only promote cancer cell immortalization but might also drive cancer cell evolution via dysregulation of telomere protection.

Increase of TERT expression associates with telomere shortening

To determine the effect of TPMs on telomerase levels, we assayed enzymatic activity and TERT mRNA expression over time (Fig. 4.4C, Fig. 4.5D and 4.5E). Shortly after differentiation, telomerase activity and TERT expression were higher in cells with TPMs than in wild-type cells, consistent with prior reports (Chiba et al., 2015). 50 days after differentiation, telomerase levels fell below the assay's limit of detection in both cell types (Fig. 4.4C and Fig. 4.5D). Nevertheless, cells with the TPM proliferated at a similar rate as cells overexpressing TERT (Fig. 4.6A-C), demonstrating that TERT expression levels were not limiting for proliferation when telomeres were long.

We investigated whether the continued telomere shortening during the early phase of the experiment was due to complete absence of TERT expression, or whether TERT was expressed at low levels that was insufficient to maintain telomere length. To distinguish between these possibilities, we hypothesized that low levels of TERT expression could be functionally revealed by making the scarce telomerase more efficient at elongating telomeres. Telomerase positive cells are highly sensitive to the expression of POT1ΔOB (Loayza and De Lange, 2003), an allele of the shelterin protein POT1 (Baumann et al., 2002), that leads to telomere elongation in telomerase-positive cancer and stem cells, but not in telomerase-negative fibroblasts. Exploiting this feature, we expressed Myc-POT1ΔOB in wild-type cells and -124 TPM cells, prior to the upregulation of telomerase to detectable levels (Fig. 4.6D-G). POT1ΔOB expression did not alter proliferation rates (Figure S2C) or induce telomerase activity (Figure S2E) in either wild-type or -124 cells. Yet, expression of POT1 Δ OB induced significant telomere elongation in cells with TPMs (Figure 4.6D). These results demonstrate that TPMs result in telomerase activity that is below the detection limit of our method before telomeres get short. Once telomere length in TPM cells had stabilized, telomerase expression and activity rose to 5-10% of that found in hESCs (Fig. 4.4C and Fig. 4.5D). These observations were consistent across all three TPM cell lines, independent of checkpoint status.

TPMs are not a binary switch towards cellular immortality but increase the fraction of cells that gain immortality

The finding that progressive telomere shortening in cells with TPMs results in telomere fusion events led us to hypothesize that a fraction of cells fails to upregulate telomerase sufficiently and will fail to become immortal. To assess the potential of individual cells within a population to stabilize their telomeres and immortalize, we

isolated subclones of -124 TPM ($CDKN2A^{\Delta/\Delta}$) and wild-type ($CDKN2A^{\Delta/\Delta}$) cells at day 104 after differentiation (Figure 4.7A). This time point is prior to the time at which cells with TPMs upregulate TERT expression and wild-type cells enter replicative senescence. We isolated 11 clones from wild-type and 27 clones from cells carrying the -124 TPM (Figure 4.7B). Both sets of clones grew in parallel for 45 days before all wild-type clones ceased to proliferate. All 27 clones with TPMs grew into cell lines that could be analyzed for telomere length (Figure 4.7D). Six cell lines were picked for long-term analysis (Figure 4.7C). Five out of six clones proliferated more than 70 PDs beyond the proliferative capacity of wild-type cells (Figure 4.7C). However, one of the six clones, stopped proliferating with morphology and culture characteristics of cells in crisis. The clones that continued dividing had comparable proliferation rates and showed progressive telomere shortening with ultimate upregulation of telomerase similar to the corresponding bulk population (Figures 4.7E, 4.7F and 4.7G). The increased telomerase activity observed in the bulk population therefore was not the result of selection of pre-existing cells with higher expression levels in the starting population, but rather was the consequence of an additional event associated with the time when telomeres became critically short. We conclude that while a substantial fraction of cells with TPMs stabilize their telomeres and become immortal, TPMs are not deterministic for cellular immortalization.

Next, we compared the levels of telomerase in immortalized TPM cells to the levels in established cancer cells. We noted that the levels found in hESCs or in HEPG2 cells, a well-established cancer cell line carrying the -124 TPM 5-10 fold higher than the minimal telomerase levels required for immortality in our long-term cultured TPM fibroblasts (Figure 4.8A). Moreover, we noticed that despite the high telomerase levels in cancer cell lines telomeres are short (Figure 4.8B) suggesting that cancer cell lines have telomerase levels in excess of the minimally required activity for telomere homeostasis.

Short telomeres and low telomerase levels are protective against immortalization by TPMs

At the time point of the acquisition of a TPM telomeres did not immediately stop shortening. The time lag until telomerase activity increased sufficiently to maintain all telomeres could provide a rationale for the cancer-protective function of constitutionally short telomeres, and explain how the cellular context influences the immortalization process. We hypothesize that: (i) telomere length at the time when a cell acquires a TPM determines the number of cell divisions a cell can undergo before telomeres become critically short and (ii) once telomeres have become critically short the ability to upregulate telomerase determines whether a cell with a TPM becomes immortalized or ceases to proliferate.

To test these hypotheses, we attenuated the effects of TPMs on TERT transcription by disrupting the cooperation between GABPα/β and TPMs(Akıncılar et al., 2016; Bell et al., 2015; Stern et al., 2015). Initial attempts to directly knockout GABPα failed, likely due to an essential telomere-independent function in hESCs (Figures 4.10A and B). Using an alternative approach we mutated the three endogenous ETS-factor binding sites in the *TERT* promoter (E3), which have been proposed as binding sites of the GABPα/β heterodimer (Figure 4.9A). Analysis of TERT expression and telomere length in these hESCs revealed that the endogenous ETS binding sites contribute to

TERT transcription (Figures 4.9B, C and 4.10C, E and F). However, the endogenous ETS sites did not act cooperatively but rather additively with the TPMs, as cells with TPM-E3 could still increase TERT transcription (Figure 4.9C and 4.10D).

Nevertheless, hESCs with a TPM and the E3 mutation (E3-124 and E3-146) had lower TERT expression and stably maintained shorter telomeres in comparison to hESCs with only a TPM (-124 and -146) (Fig. 4.9B, C and 4.10C-F). From this allelic series we generated SV40 Tag-transduced fibroblasts and monitored proliferation, telomere length, and frequency of telomere fusions (Fig. 4.9B, and 4.9D-E). Fibroblasts without the TPM, but with the three ETS mutations (wt-E3), had the shortest telomeres and died shortly after differentiation. In accordance with our first hypothesis, -124-E3 and -146-E3 cells with constitutionally shorter telomeres showed a high frequency of fusion events, much earlier than -124/-146 cells (Fig. 4.9D and 4.10G). Both -124-E3 and -146-E3 cells initially proliferated despite their short telomeres (Fig. 4.9E), arguing that they retained low levels of telomerase activity. Although they harbored TPMs, both -124-E3 and -146-E3 cells upregulated telomerase to only low levels; these cells entered crisis and did not become immortalized, confirming our second hypothesis.

Therefore the cellular context – starting telomere length and the ability of a cell to upregulate telomerase – determine if a cell becomes immortal as well as the amount of telomere driven genomic instability that it will encounter prior to immortalization.

DISCUSSION

In the classic model of cellular immortalization, telomerase is reactivated during telomere crisis in cells with inactivated DNA damage checkpoints required to withstand pro-apoptotic and cell-cycle arresting signals from uncapped telomeres (Figure 4.11 left). Marked genomic instability during this period provides a strong selective pressure for cells with reactivated telomerase. This model is difficult to reconcile with the observation that constitutively short telomeres are protective against cancer (Bataille et al., 2007; Bojesen et al., 2013; Han et al., 2009; Iles et al., 2014; Nan et al., 2011; Rode et al., 2016), as it would predict that cells with short telomeres would reach telomere exhaustion and crisis at an earlier time point, thereby accelerating the selection of immortalized and genomically altered cells. A further inconsistency is a recent finding in melanoma that TPMs undergo positive selection already at a pre-neoplastic stage, arising before genetic alterations that disrupt cell cycle- and DNA damage- checkpoints (Shain et al., 2015). This latter observation indicates that replicative senescence is a tumor-suppressive mechanism that acts relatively early during the evolution of some cancers such as melanoma. Telomere shortening, as a proliferative barrier to transformation from nevi to melanoma, has been questioned because the telomeres in melanoma are short, whereas nevi have long telomeres (Michaloglou et al., 2005). Alternative mechanisms implicated in the growth arrest associated with nevi include oncogene-induced senescence, a phenomenon observed in vitro where exogenous expression of activated oncogenes such as BRAF V600E results in hyperactivation of the MAP-kinase pathway and subsequent cell cycle arrest (Michaloglou et al., 2005; Serrano et al., 1997). Additional implicated mechanisms are based on the observation that proliferation-inducing mutations also induce replication stress resulting in a DNA damage response that can restrict the proliferative potential of incipient cancer cells including those of melanocytic nevi (Bartkova et al., 2006; Di Micco et al., 2006; Halazonetis et al., 2008; Serrano et al., 1997).

Our findings provide both insights into the relationship between the timing of TPM acquisition and telomere exhaustion as well as indicate that replicative senescence presents a barrier early during the evolution of some cancers. We show that cells with TPMs continue to undergo progressive bulk telomere shortening until telomeres become critically short. At this point some cells with TPMs upregulate telomerase expression, which allows them to evade replicative senescence and to continue to proliferate. We conclude that TPMs are necessary but not sufficient to maintain telomere length and convey cellular immortality. Additionally our finding, of increased telomere fusions in cells that became immortalized in this way, indicates that TPM cells proliferate for an extended period with partially deprotected telomeres.

Based on these observations we propose the following revised model that reconciles the seemingly contradictory roles of telomere length in cancer (Figure 4.11 right): TPMs arise randomly during early phases of tumorigenesis where they initially provide no selective advantage. The low expression levels of telomerase resulting from TPMs is insufficient to prevent telomere shortening. Once telomeres become critically short, cells with TPMs have an advantage and expand within the neoplasm, as their siblings without TPMs enter replicative senescence or die of apoptosis. The low levels of telomerase expression allow stabilization of only these telomeres that become critically short, whereas other telomeres continue to shorten (Britt-Compton et al., 2009; Goldman

et al., 2005; Hemann et al., 2001; Teixeira et al., 2004). As the number of chromosomes with critically short telomeres increases, telomerase activity becomes rate-limiting, resulting in selective pressure for telomerase upregulation. During this phase, telomerase expression levels and total enzymatic activity are marginal, leaving some telomeres uncapped and promoting telomere fusions. This genomic instability is predicted to be sustained, but at a low and stochastic level, compared to the acute and pronounced genomic instability during telomere crisis. Cells with increased telomerase activation are selected, but have critically short telomeres and have acquired DNA copy number changes or structural rearrangements when they emerge from this phase. According to our model, the acquisition of a TPM would not act as a binary switch that provides instant immortality with stabilized telomeres, but would allow for a phase during which the telomerase activity of cells within a population can gradually ramp up. Until telomerase levels are sufficiently high to protect all telomeres, the population goes through a period of sustained, low-level genomic instability that facilitates the selection of cells with additional, growth- and survival-promoting genetic alterations. This model can provide an explanation for the genome-wide copy number changes found in *TERT* promoter mutant melanomas that arise after the emergence of TPMs (Shain et al., 2015). It would also account for why patients with constitutively short telomeres have smaller nevi (Bataille et al., 2007) and a decreased melanoma risk (Bojesen et al., 2013; Han et al., 2009; Iles et al., 2014; Nan et al., 2011; Rode et al., 2016), whereas patients with germline shelterin complex mutations such as a POT1(Robles-Espinoza et al., 2014; Shi et al., 2014) or TPP1 (Aoude et al., 2015), which result in longer telomeres, have an increased melanoma risk. This is because pre-neoplastic lesions in patients with constitutively long telomeres would reach replicative senescence later, thereby increasing the number of partially transformed cells that are at risk of acquiring additional pathogenic mutations.

In summary, telomere exhaustion acts as a critical barrier early during the neoplastic transformation of some cancers, because it limits the numeric expansion of early neoplastic cells at a precancerous stage. Constitutive telomere length likely shifts this barrier, thereby increasing cancer risks in individuals with longer telomere length. Gradually increasing telomerase levels in cells with a TPM are influenced by additional and yet to be characterized factors. They become selected for during a sustained growth phase in which telomerase activation is marginal and results in low-level genomic instability. This phase may provide a therapeutic opportunity in which cancer precursor cells are particularly sensitive to telomerase inhibition (Gellert et al., 2005).

Figure 4.1

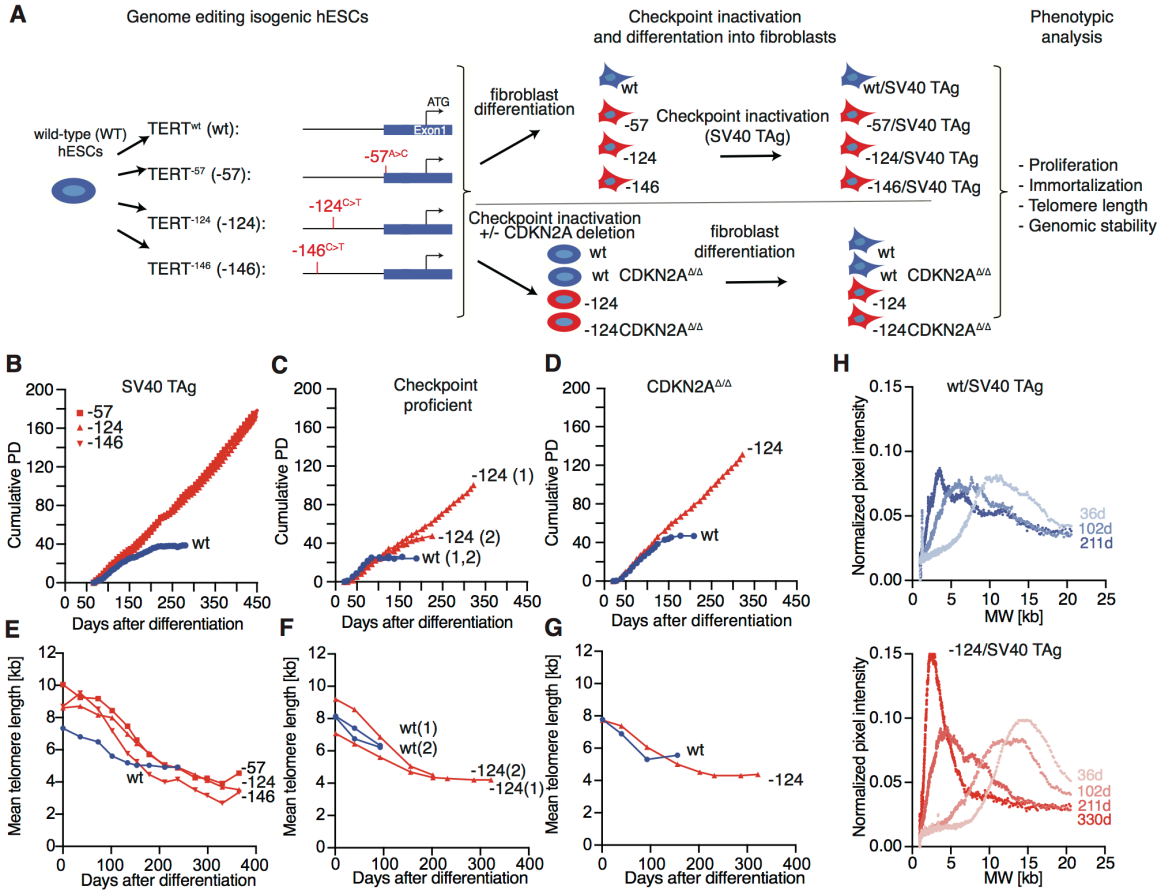


Figure 4.1
TPMs support cellular immortalization *in vitro* but do not prevent telomere shortening
(A) Experimental overview: Isogenic hESCs with the TPMs were differentiated into fibroblasts. To inactivate cell cycle and DNA damage checkpoints, either CDKN2A function was deleted in hESCs prior differentiation or fibroblasts were infected with SV40 TAg.
(B-D) Growth curves of cumulative PDs over days after differentiation. (B) SV40 TAg fibroblasts with (red, -57, -124, -146) or without (blue, wt) TPMs (C) DNA damage checkpoint proficient cells with (red, -124) or without (blue, wt) TPM. (1), (2) indicate two independent experiments. (D) CDKN2A$^{\Delta/\Delta}$ cells with (red, -124) or without (blue, wt) TPM
(E-G) Quantification of mean telomere length over time after differentiation.
(H) Accumulation of shorter telomeres over time shown by visualization of telomere length distribution of images shown in Figure 4.3.A. Quantification of the normalized pixel intensity over molecular weight per lane for the indicated time points after differentiation.

Figure 4.2

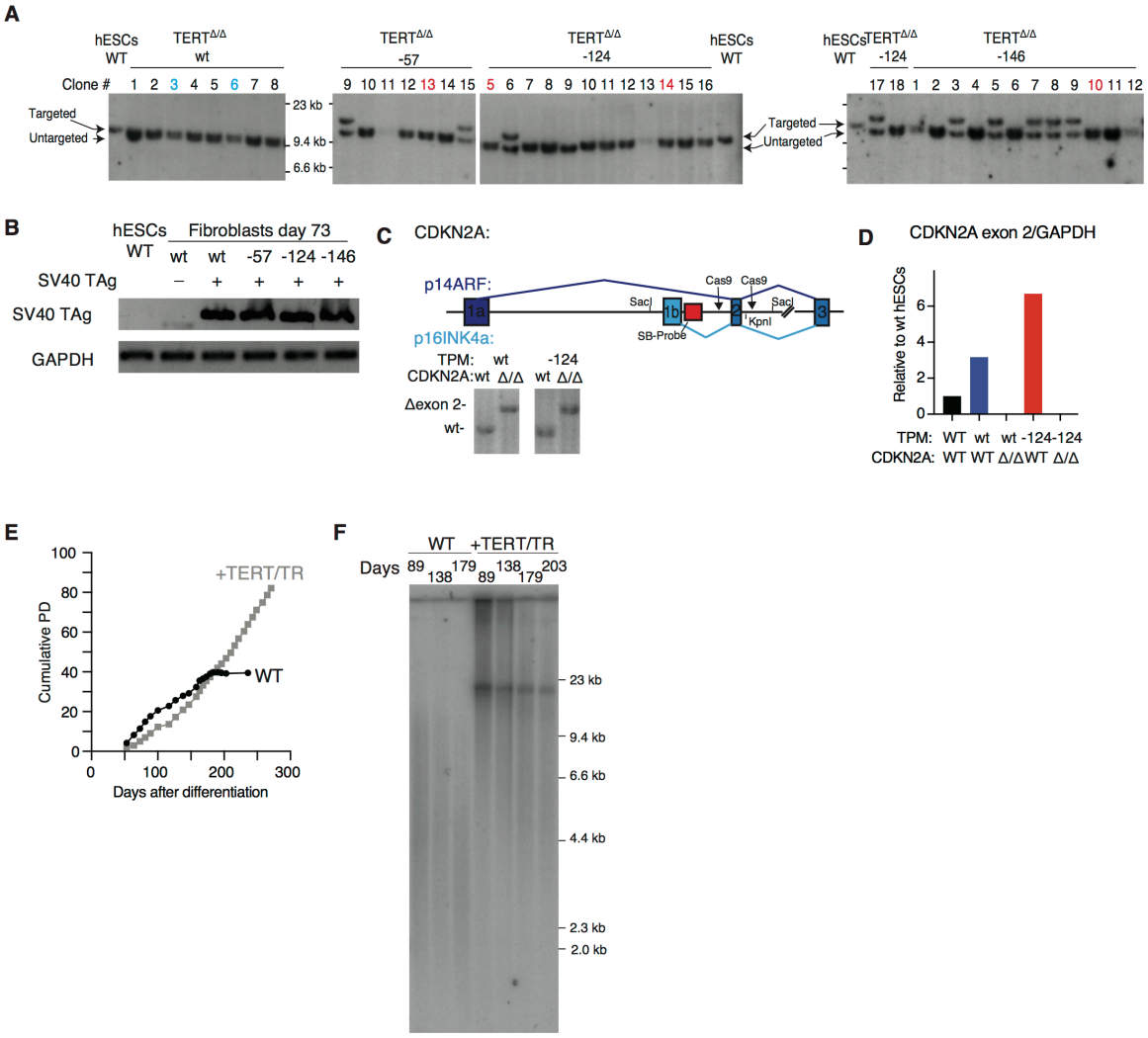

A
hESCs WT
TERTΔ/Δ wt
TERTΔ/Δ -57
TERTΔ/Δ -124
hESCs WT
hESCs WT
TERTΔ/Δ -124
TERTΔ/Δ -146
Clone # 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 5 6 7 8 9 10 11 12 13 14 15 16 17 18 1 2 3 4 5 6 7 8 9 10 11 12
Targeted
Untargeted
23 kb
9.4 kb
6.6 kb
Targeted
Untargeted
B
hESCs WT
Fibroblasts day 73
wt wt -57 -124 -146
SV40 TAg – + + + +
SV40 TAg
GAPDH
C
CDKN2A:
p14ARF:
p16INK4a:
Cas9 Cas9
SacI
SacI
KpnI
SB-Probe
TPM: wt -124
CDKN2A:wt Δ/Δ wt Δ/Δ
Δexon 2-
wt-
D
CDKN2A exon 2/GAPDH
Relative to wt hESCs
TPM: WT wt wt -124-124
CDKN2A: WT WT Δ/ΔWT Δ/Δ
E
Cumulative PD
+TERT/TR
WT
Days after differentiation
F
WT +TERT/TR
Days 89 138 179 89 138 179 203
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

Figure 4.2
Genotyping and characterization of cells used in this study
(A) Genotyping of single cell derived isogenic hESC clones with and without TPMs by Southern blotting as described in Figure 2.3B. The correctly targeted allele is 11 kb and untargeted is 9.6 kb. The highlighted correctly targeted clones were used for further analysis (wt-6 and -124-14 for checkpoint proficient cells and for the deletion of $CDKN2A^{\Delta/\Delta}$; wt-3, -57-13, -124-5 and -146-10 for the SV40 TAg experiments)
(B) Expression of SV40 TAg and GAPDH after transduction.
(C) Targeting schematic for the generation of CDKN2AΔ/Δ hESCs. Deletion of exon2 of p16 and p14 in the CDKN2A locus was obtained by the simultaneous expression of two Cas9/sgRNAs. For Southern blot analysis, genomic DNA was digested with SacI and KpnI.
(D) Confirmation of CDKN2A exon 2 deletion by measuring relative mRNA expression of exon 2.
(E) To confirm that arrest of the wild-type cells was due to progressive telomere shortening, TERT and TR, the RNA component of telomerase, were constitutively overexpressed from the AAVS1 locus in hESCs. In fibroblasts, overexpression of telomerase was sufficient to rescue the arrest phenotype and support indefinite proliferation. Growth curves show cumulative PDs over days after differentiation (Wild-type: grey, Overexpression of TERT/TR: black).
(F) Telomere length analysis of WT cells and TERT/TR overexpressing cells.

Figure 4.3

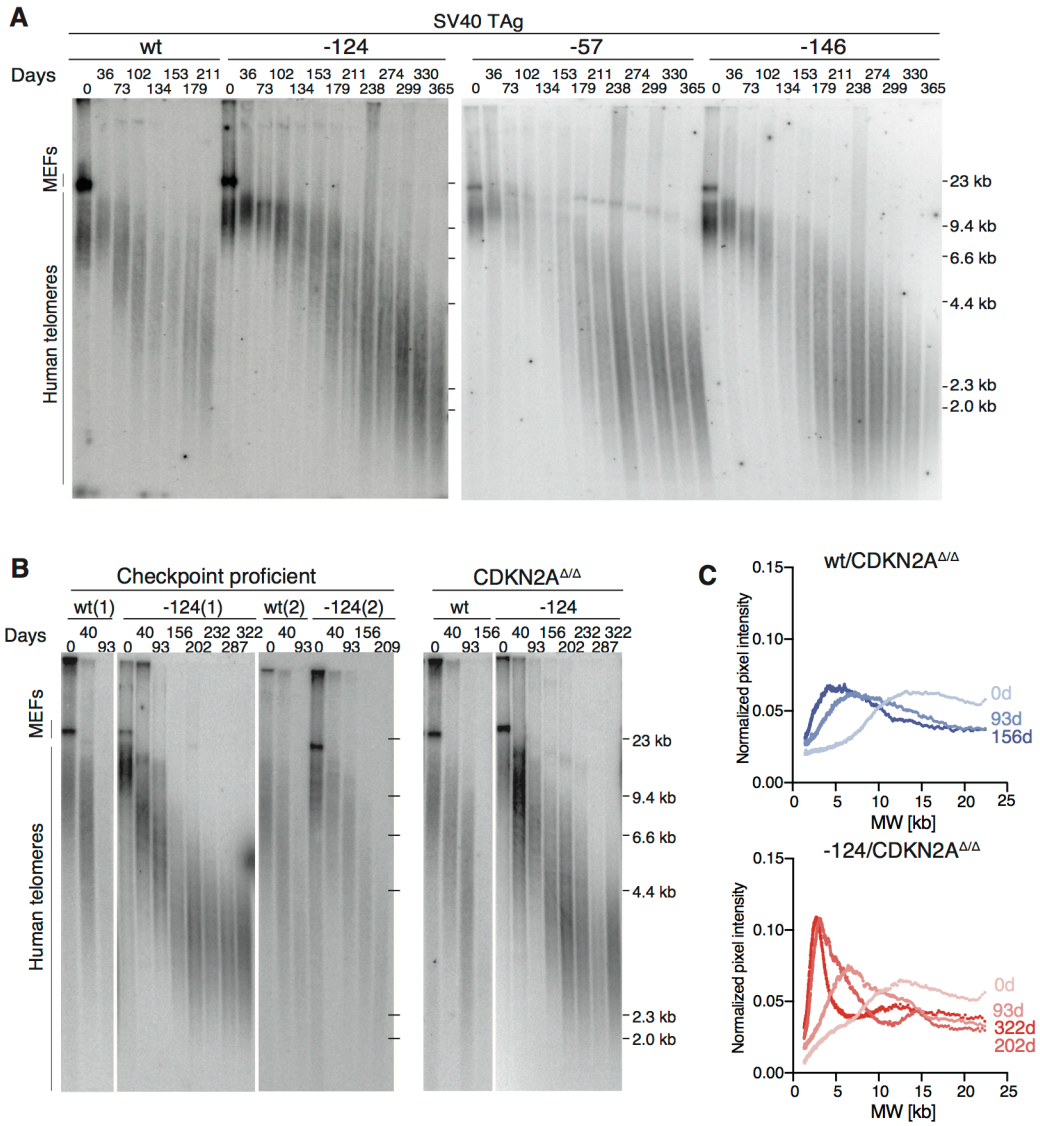


Figure 4.3
Telomeres shorten in the presence of TPMs in both checkpoint proficient and deficient cells

(A) Analysis of telomere length in SV40Tag fibroblasts. Day 0 refers to hESCs at the day of differentiation initiation. hESCs are cultured on mouse embryonic fibroblasts (MEFs), the telomeric signal of these MEFs is indicated. Quantification of mean telomere length is shown in Figure 4.1E.

(B) Analysis of telomere length over time of the cells and experiment shown in Figure 4.1C and 4.1D. From the left: Checkpoint proficient cells with or without -124 TPM (two independent -124 TPM clones are shown), CDKN2A$^{\Delta/\Delta}$ cells with or without -124 TPM. The days post-differentiation are shown above each lane.

(C) Accumulation of shorter telomeres over time shown by visualization of telomere length distribution of images shown in panel (B). Quantification of the normalized pixel intensity over molecular weight per lane for the indicated time points after differentiation.

Figure 4.4

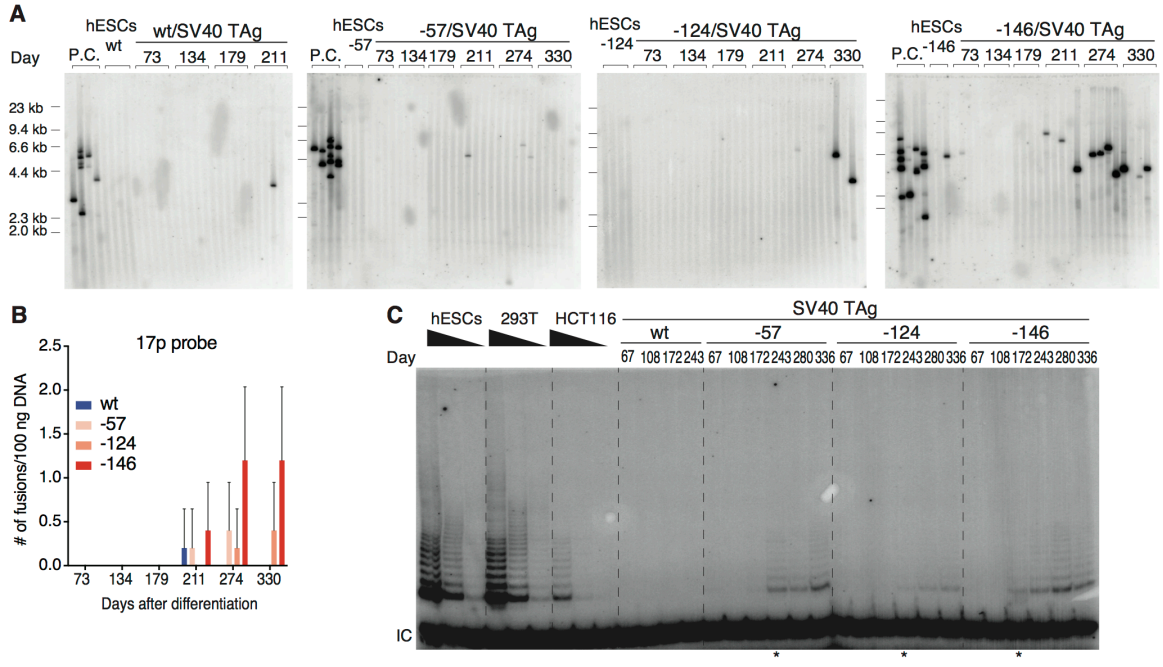


Fig. 4.4

TPMs do not fully protect against genomic instability and mutant cells gradually increase telomerase expression

(A) Detection of interchromosomal telomere fusions over time in SV40 TAg fibroblasts by PCR of specific subtelomeric regions. Fusions were detected by probes against the 17p subtelomeric regions(Capper et al., 2007). DNA from cells in crisis served as a positive control (P.C.: TERT, p53 and p16 triple knockout fibroblasts cultured into crisis).

(B) Quantification of fusion events shown in (A).

(C) Telomerase activity assay of the indicated cell lines over time. IC: internal control. * indicates time points when telomerase activity became detectable.

Figure 4.5

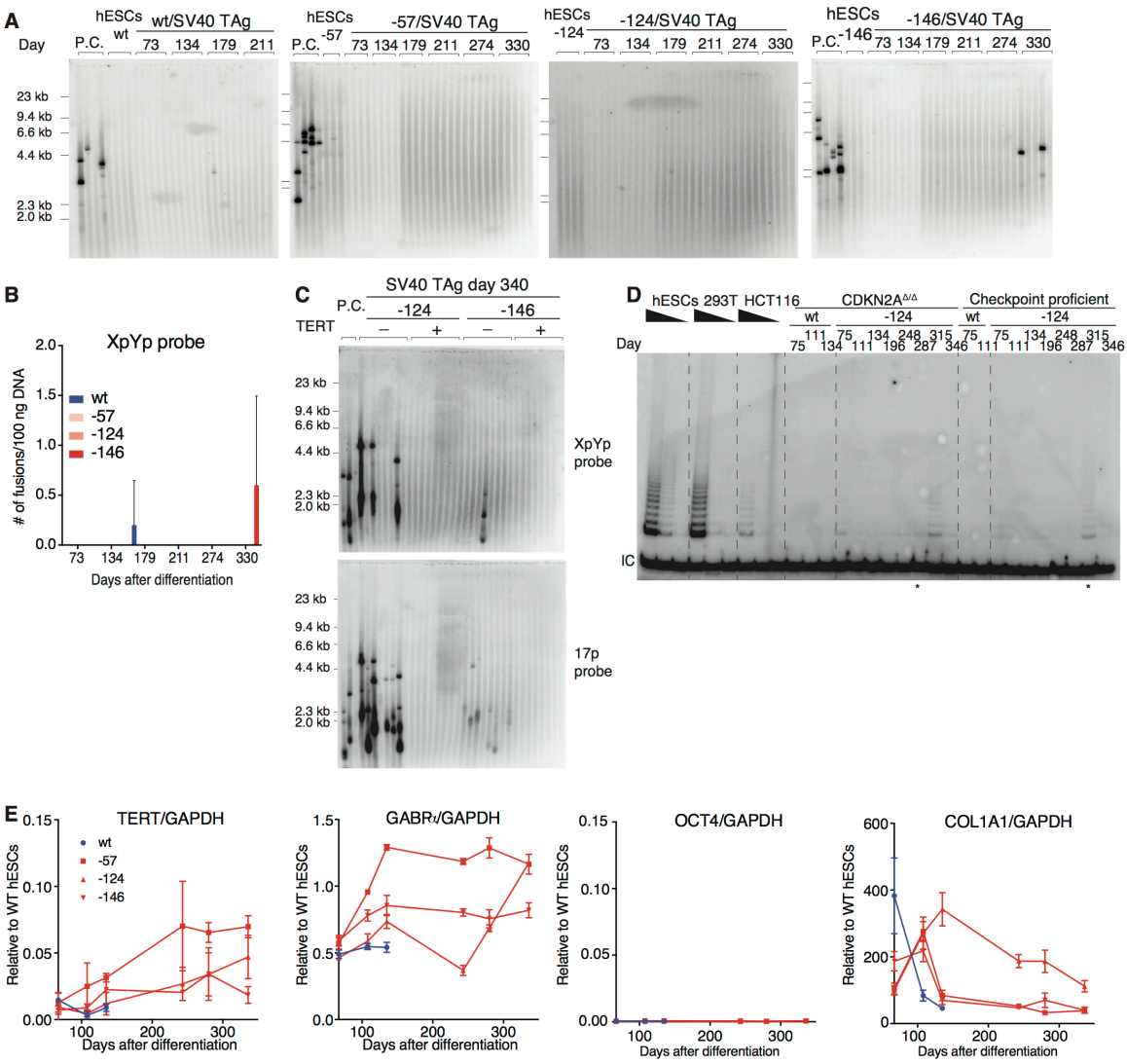

A
hESCs wt/SV40 TAg
hESCs -57/SV40 TAg
hESCs -124/SV40 TAg
hESCs -146/SV40 TAg
Day
P.C.
wt
-57
-124
-146
73 134 179 211 274 330
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
B
XpYp probe
# of fusions/100 ng DNA
wt
-57
-124
-146
Days after differentiation
C
SV40 TAg day 340
TERT
XpYp probe
17p probe
D
hESCs 293T HCT116
CDKN2A Δ/Δ
Checkpoint proficient
IC
E
TERT/GAPDH
GABRA1/GAPDH
OCT4/GAPDH
COL1A1/GAPDH
Relative to WT hESCs
Days after differentiation

Figure 4.5
Gradual telomerase increase is checkpoint independent and via upregulation of TERT mRNA expression
(A) Detection of interchromosomal telomere fusions over time in SV40 TAg fibroblasts by PCR of specific subtelomeric regions. Fusions were detected by probes against the XpYp subtelomeric regions(Capper et al., 2007). DNA from cells in crisis served as a positive control (P.C.: TERT, p53 and p16 triple knockout fibroblasts cultured into crisis).
(B) Quantification of fusion events shown.
(C) TERT overexpression suppressed telomeric fusions in TPM cells, demonstrating that fusions are driven by telomere attrition. Telomere fusion PCR was performed in TPM cells with or without overexpression of TERT at day 340. -124 and -146 TPM cells were retrovirally transduced at day 289 with TERT.
(D) Telomerase activity assay of the cells shown in Figure 4.1C-D over time. IC: internal control for the assay, * indicates time points when telomerase activity becomes detectable.
(E) Relative expression levels of TERT, GABPα, OCT4 and COL1A1 mRNA in SV40 Tag cells over time. Expression is relative to WT hESCs and normalized to GAPDH. Error bars show the standard error of the mean (SEM), n = 3.

Figure 4.6

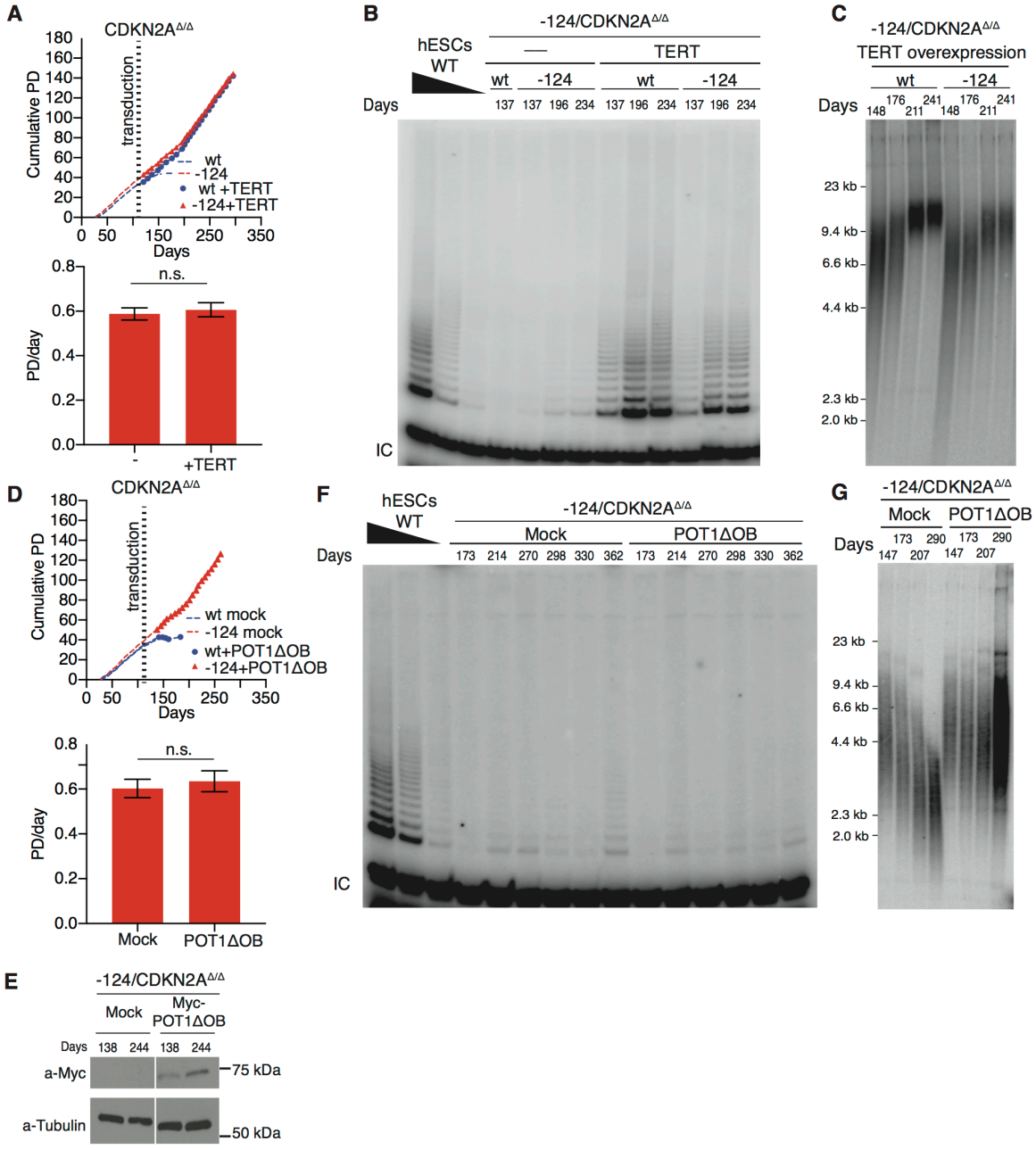
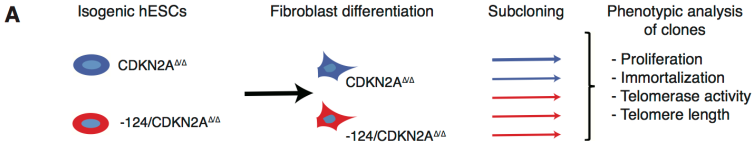

A
CDKN2A$^{\Delta/\Delta}$
Cumulative PD
transduction
wt
-124
wt +TERT
-124+TERT
Days
PD/day
n.s.
-
+TERT
B
-124/CDKN2A$^{\Delta/\Delta}$
hESCs WT
—
TERT
wt
-124
wt
-124
Days
137 137 196 234 137 196 234 137 196 234
IC
C
-124/CDKN2A$^{\Delta/\Delta}$
TERT overexpression
wt
-124
Days
148 176 211 241 148 176 211 241
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
D
CDKN2A$^{\Delta/\Delta}$
Cumulative PD
transduction
wt mock
-124 mock
wt+POT1ΔOB
-124+POT1ΔOB
Days
PD/day
n.s.
Mock
POT1ΔOB
E
-124/CDKN2A$^{\Delta/\Delta}$
Mock
Myc-POT1ΔOB
Days
138 244 138 244
a-Myc
75 kDa
a-Tubulin
50 kDa
F
hESCs WT
-124/CDKN2A$^{\Delta/\Delta}$
Mock
POT1ΔOB
Days
173 214 270 298 330 362 173 214 270 298 330 362
IC
G
-124/CDKN2A$^{\Delta/\Delta}$
Mock
POT1ΔOB
Days
173 290 173 290
147 207 147 207
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

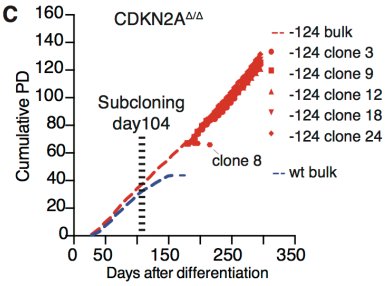
Figure 4.6
TERT expression is not limiting for proliferation of TPM cells with sub-threshold telomerase activity
(A) Growth curve and proliferation rate of uninfected and TERT overexpressing cells shown as cumulative PDs over days after differentiation. Cells were infected on day 108 after differentiation (dashed line). PD/day were calculated after each passage between day 129 and 296. Error bars show SEM two-sided student t-test, $p = 0.66$, $n = 24$. No significant difference in growth rate was observed indicating that telomerase levels were not limiting for proliferation in TPM cells while telomeres were long.
(B) Telomerase activity of TERT overexpressing cells. Uninfected cells were compared with TERT overexpressing cells at 137, 196, and 234 days after differentiation.
(C) Telomere length analysis showed telomere elongation by TERT overexpression in wt and -124 TPM cells.
(D-G) Telomerase positive cells are highly sensitive to the expression of POT1ΔOB (Loayza et al., 2003), an allele of the shelterin protein POT1 (Baumann and Cech, 2001), that leads to telomere elongation, but not in telomerase-negative cells. Exploiting this feature, we investigated whether telomere shortening in early phase was due to complete absence of TERT expression or TERT was expressed at low levels that were insufficient to maintain telomere length.
(D) Cumulative PDs of cells retrovirally infected with POT1ΔOB or mock infected cells. No difference in proliferation rate of PD/day was observed after each passage between day 145 and 262. Error bars: SEM, two-sided student t-test, $p = 0.61$, $n = 19$. Cells were infected on day 103 after differentiation.
(E) Western blot analysis of retrovirally transduced cells with Myc-POT1ΔOB.
(F) Telomerase activity of POT1ΔOB and mock-infected cells at indicated time points after differentiation.
(G) Telomere length analysis of POT1ΔOB and mock-infected cells showed expression of POT1ΔOB induced significant telomere elongation in cells with TPMs, indicating telomerase activity that was below the detection limit of our method.

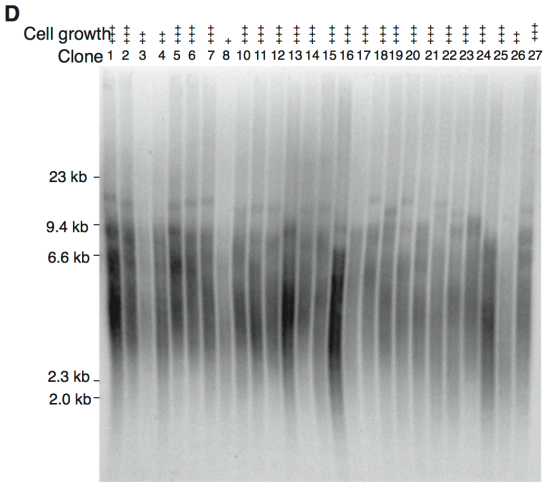
Figure 4.7

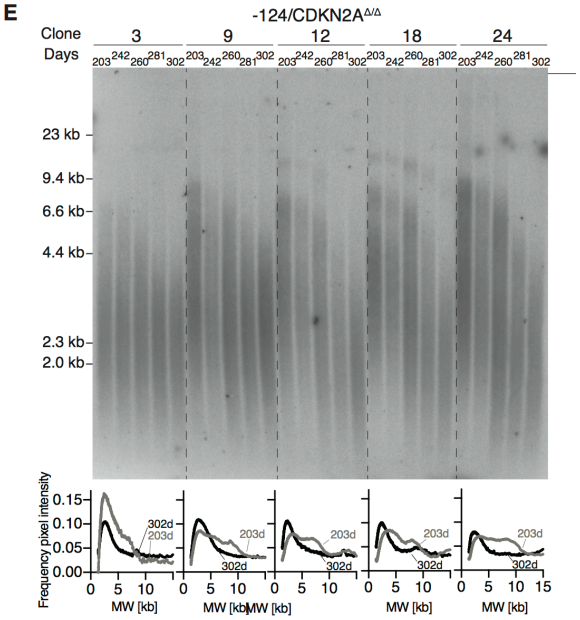


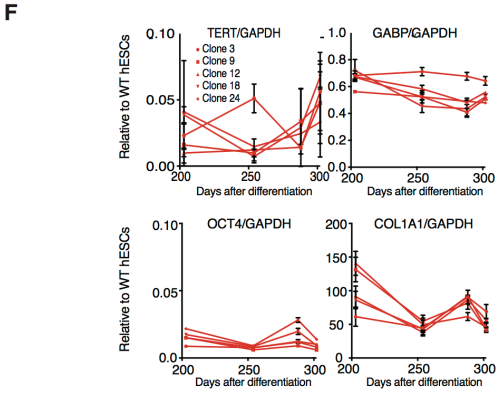
B

Density	Visible outgrowth wt/CDKN2A$^{\Delta/\Delta}$	Visible outgrowth -124/CDKN2A$^{\Delta/\Delta}$
0.5 cells per well	11 of 96	27 of 96
1 cell per well	10 of 96	38 of 96
2 cell per well	17 of 96	62 of 96
4 cell per well	47 of 96	90 of 96









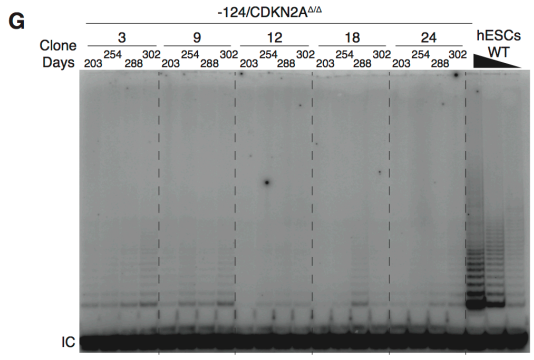


Figure 4.7
A large fraction of cells with a TPM are poised to immortalize and exhibit the same telomere length dynamics as the bulk population, yet a small fraction of individual cell clones failed
(A) Experimental overview: To assess the potential of individual cells within a population to stabilize their telomeres and immortalize, we isolated subclones of -124 TPM ($CDKN2A^{\Delta/\Delta}$) and wild-type ($CDKN2A^{\Delta/\Delta}$) cells at day 104 after differentiation, before the upregulation of telomerase.
(B) Table of frequency of successful subcloning by limiting dilution. We isolated 11 clones from wild-type and 27 clones from cells carrying the -124 TPM. Both sets of clones grew in parallel for 45 days before all wild-type clones ceased to proliferate.
(C) Telomere length analysis of all TPM clones at day 149. Evaluation of cell growth is indicated above each clone ranging from poor growth (+) to excellent growth (+++). Six cell lines were chosen for long-term analysis.
(D) Growth curve showing cumulative PDs over time of the six of the subcloned lines. Five out of six clones proliferated more than 70 PDs beyond the proliferative capacity of wild-type cells. However, one of the six clones (#8), stopped proliferating with morphology and culture characteristics of cells in crisis.
(E) Telomere length analysis of the clones analyzed for proliferation. Plotted below the autoradiogram are the telomere length distributions (frequency of pixel intensity over molecular weight) of the earliest and latest time point (Day 203, grey line and 302, black line).
(F) Relative expression levels of TERT, GABPα, OCT4, COL1A1 mRNA in each clone between days 203 and 302. Expression was normalized by GAPDH. Error bars show SEM, n = 3.
(G) Telomerase activity assay of the TPM -124 clones at indicated time points after differentiation.

Figure 4.8

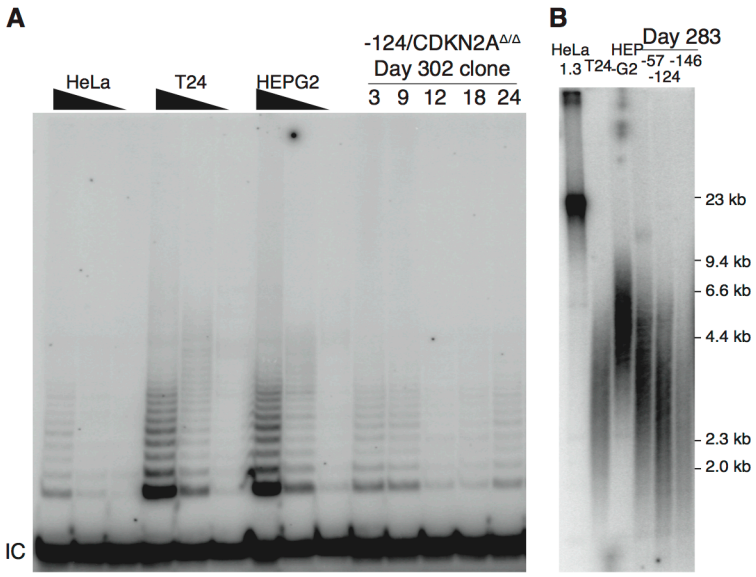


Figure 4.8
Telomerase activity and telomere length are not correlated in established cancer cell lines
(A) Comparison of telomerase activity between cancer cell lines and clones of -124 CDKN2A$^{\Delta/\Delta}$ at day 302. For cancer cell lines, 200, 40, 8 ng of lysate were used for the assay. For -124 CDKN2A$^{\Delta/\Delta}$, 200 ng of lysate was used.
(B) Comparison of telomere length between the indicated cancer cell lines and SV40 Tag transduced fibroblasts with a TPM at day 283.

Figure 4.9

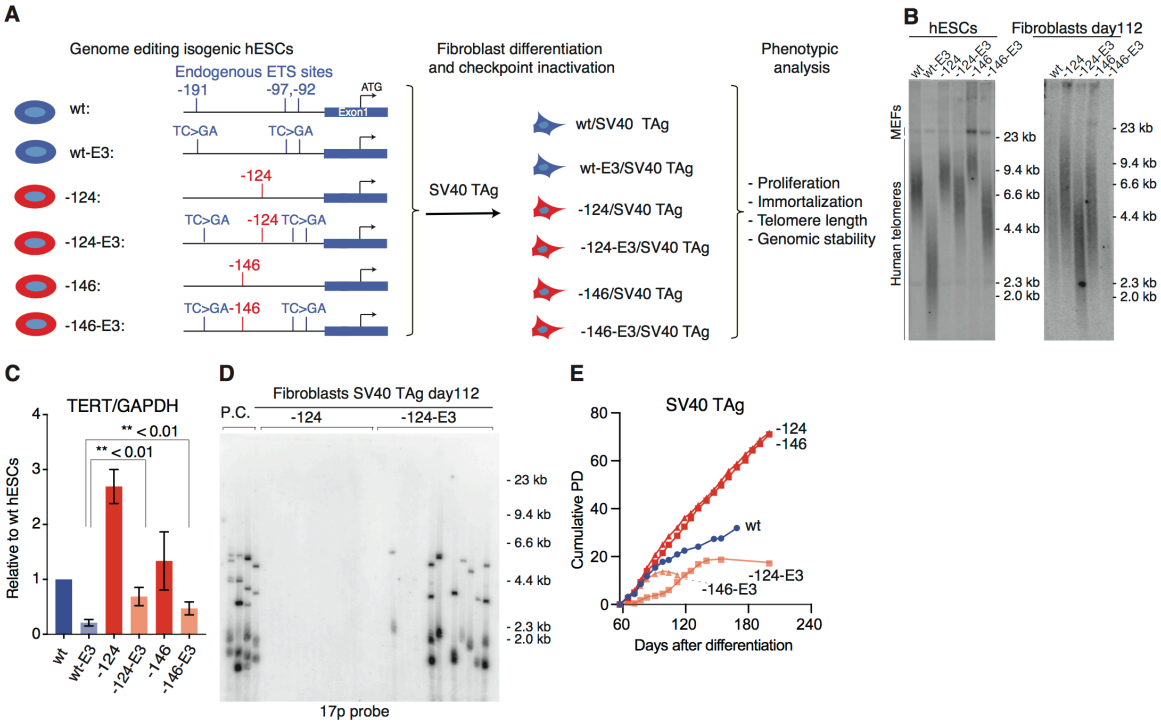


Figure 4.9

Short telomeres and low telomerase levels protect cells from immortalization by TPMs

(A) Schematic overview of allelic series of cells carrying TPMs (red) and/or endogenous ETS site mutations (E3: mutated TC>GA, blue) in the TERT promoter. E3 refers to the simultaneous mutations of all three endogenous ETS sites (-191, -97 and -92 from the translational start site: ATG).

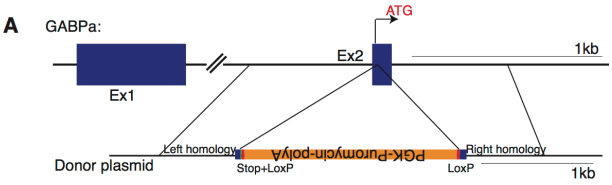
(B) Telomere length analysis for hESCs and fibroblasts with TPMs and ETS mutation combinations.

(C) TERT expression normalized to GAPDH relative to wt hESC. Error bars: SEM, two-sided student t-test, n = 3.

(D) Telomere fusions detected in -124 and -124 E3 fibroblasts with 17p.

(E) Growth curve of fibroblasts with or without TPMs and/or ETS mutations.

Figure 4.10



hESCs	# of clones genotyped	# of clones heterozygous targeted	# of clones homozygous targeted
WT	23	12 (52%)	0
TERT$^{\Delta/\Delta}$ wt	18	6 (33%)	0
TERT$^{\Delta/\Delta}$ -57	14	4 (29%)	0
TERT$^{\Delta/\Delta}$ -124	15	5 (33%)	0
TERT$^{\Delta/\Delta}$ -146	14	5 (36%)	0

B

GABPa$^{+/-}$ hESCs clone	Sequence of non-integrated alleles	Status
Reference seq.	cATGACTAAAAGAGAAGCAGAGGAGCTGATA (sgRNA / PAM)	—
WT-9	ATGACTAAAAGAGAAGCAGAGGAGCTGATA	wild type
wt-6	ATGACTAAANGAGAAGCAGAGGAGCTGATA	wild type
-57-4	ATGACTAAANGAGA------GGAGCTGATA	6 bp deletion
-57-18	ATGACTAAAAGAGA---------GCTGATA	9 bp deletion
-57-25	ATGACTAAAAGAGA---------GCTGATA	9 bp deletion
-124-2	ATGACTAAAAGAGAAGCAGAGGAGCTGATA	wild type
-146-15	ATGACTAAANGAGAAGCAGAGGAGCTGATA	wild type
-146-28	ATGACTAAAAGAGA------GGAGCTGATA	6 bp deletion

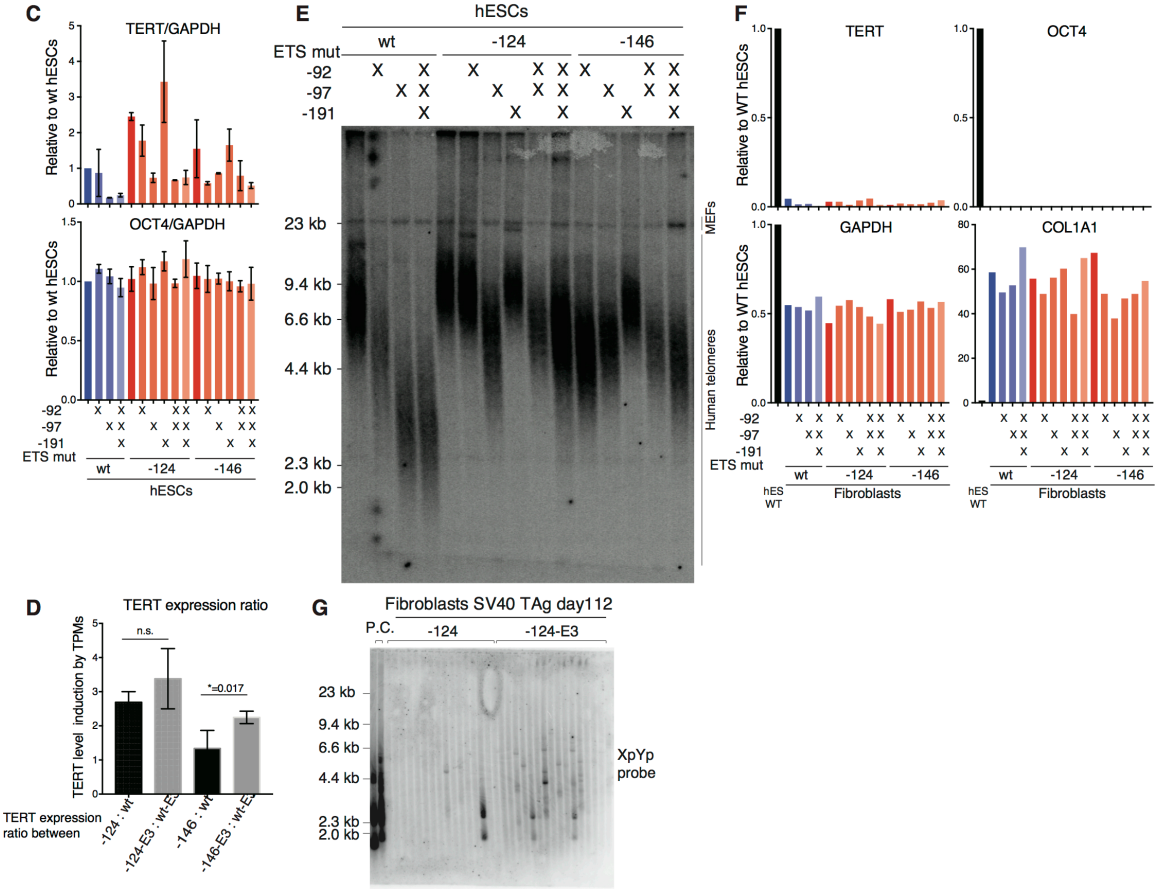


Figure 4.10
Modulation of TERT expression by attempted GABPα knockout and mutation of endogenous ETS sites in the TERT promoter
(A) Schematic overview of GABPα knockout strategy and frequency of successful targeting determined by Southern blot genotyping.
(B) PCR genotyping of non-integrated alleles of GABPα+/- clones. Note: All alterations of the non-targeted allele are in-frame deletions. These in-frame deletions are expected to retain GABPα function, suggesting an essential role for GABPα in hESCs. Using an alternative approach, we mutated the three endogenous ETS-factor binding sites in the TERT promoter (E3), which have been proposed as binding sites of the GABPα/β heterodimer.
(C) Relative expression levels of TERT and OCT4 for the indicated combinations for mutations in the endogenous ETS sites with or without TPMs. Expression levels are relative to hESCs and normalized to GAPDH. Mutations in the ETS sites decrease TERT expression in wt hESCs, indicting a contribution of these ETS sites to TERT transcription even in the absence of the TPMs. Moreover, the endogenous ETS sites did not act cooperatively but rather additively with the TPMs, as TPMs were able to increase TERT transcription in the presence of the triple ETS mutation.
(D) Fold change of TERT expression for the indicated comparisons (Error bars: SEM, two-sided student t-test, n = 3). TPMs can equally upregulate TERT expression in the absence of the endogenous ETS sites.
(E) Telomere length analysis of hESCs carrying individual and combinatorial mutations in the endogenous ETS sites with or without TPMs.
(F) Relative expression levels of TERT, OCT4, GAPDH and COL1A1 in fibroblasts differentiated from hESCs. Samples were collected at day 21 after differentiation.
(G) Telomere fusions detected in -124 and -124 E3 fibroblasts with probe XpYp in subtelomeric regions at day 112.

Figure 4.11

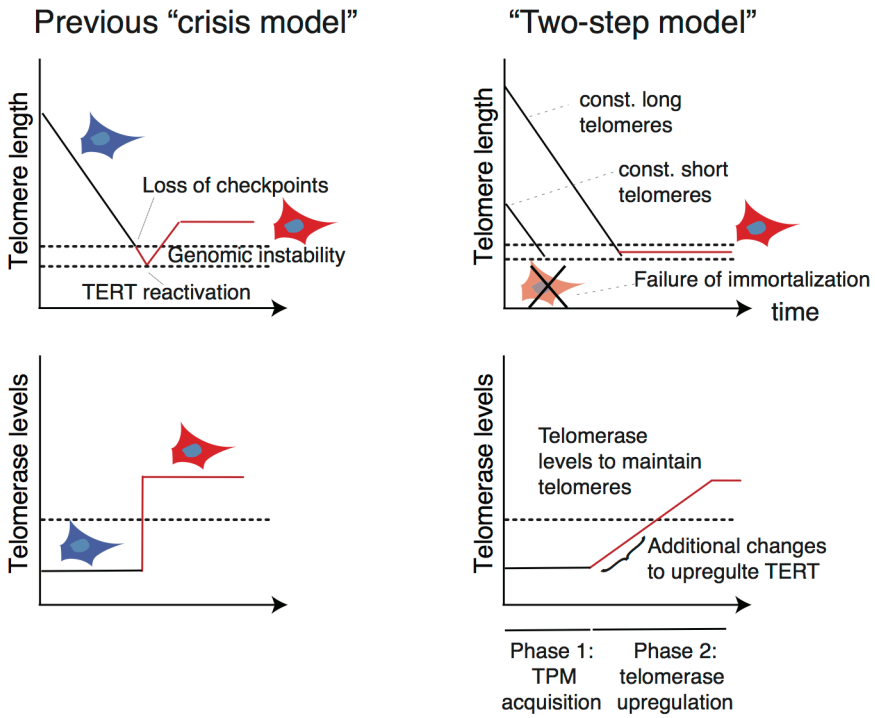


Figure 4.11
A biphasic model of cellular immortalization by cancer associated TERT promoter mutations
A revised model for cell immortalization by TPMs. In the classic crisis model, TERT reactivation occurs with genomic instability, elongates and stabilizes telomeres from critically short length (left panels). This model falls short to explain the protective role of short telomeres: Telomerase reactivation is a single event. In this model the frequency of this step is expected to increase when telomeres shorten – with a high frequency of short telomeres the reactivation of telomerase should become more frequent.
In the revised biphasic model (right panels) TPMs can be acquired at any time before crisis (phase 1), but this initially does not prevent bulk telomere shortening and telomere driven genomic instability (phase 2). In phase 2 a fraction of cells will fail to immortalize, however the cells that continue to proliferate cycle with short telomeres and until additional events trigger the upregulation of telomerase levels. During phase 1 constitutionally short telomeres are protective because they limit how many times a tumor initiating cell can divide before it has to upregulate telomerase expression. In phase 2 cells can acquire telomere driven genomic instability.

CHAPTER FIVE

Conclusions

Telomere maintenance by telomerase is a key requirement for the long-term genomic integrity of stem cells and germ cells. In somatic cells, TERT silencing leads to telomere shortening, which functions as a tumor suppression mechanism by restricting the replicative capacity of a cell. In contrast, in cancer cells, telomerase activity enables maintenance of telomeres over long-term proliferation. In general, short telomeres associate with lack of telomere protection and genomic instability leading to tumorigenesis. However, population genetic studies showed individuals with longer telomeres tend to have higher cancer risk. These seemingly opposite observations highlight the complexity of telomere length and timing of telomerase activity acquisition and tumorigenic outcomes. Thus, it is essential to understand how telomerase is regulated in normal cells and how changes of telomerase regulation contribute to tumorigenesis. In this work, I have first aimed to understand behaviors of the endogenous telomerase in human embryonic stem cells. Second, we characterized the most frequent non-coding cancer mutations discovered at the TERT promoter and revealed its function in long-term telomere maintenance.

Understanding the regulation of the endogenous telomerase has been challenging due to its low cellular abundance. Ectopic overexpression of TERT results in dramatic elongation of telomeres making it difficult to study physiological telomere length regulation. To tackle this challenge, we epitope-tagged endogenous telomerase for biochemical and imaging analysis. Tagging telomerase with Halo-tag was of particular interest because Halo-TERT could allow us to study in vivo actions of telomerase with single molecule resolution. Unfortunately, all attempts to insert the Halo tag into TERT locus resulted in dramatic reduction of the TERT expression (~5% of wild type TERT expression). Halo-TERT hESCs have very short telomeres and show characteristics of telomere dysfunction including morphological changes and cell death. A smaller tag such as YbbR showed milder defect in TERT expression (~50% of wild type) and Flag tag showed almost no transcriptional defects. These results suggest intrinsic topology for efficient transcription and/or mRNA stability is altered by insertion of artificial sequence in length dependent manner. However, the molecular mechanism that leads to the reduction of TERT expression is not clear. To perform in vivo telomerase imaging under physiological telomerase level and telomere length, it is important to determine the regulatory elements of TERT and then modulate impairment of TERT expression caused by insertion of an epitope tag. Other defects associated with the N-terminal tagging of telomerase are impaired processivity and shorter telomeres compared to the isogenic wild type cells. The defect in processivity was subsequently confirmed by another group(Schmidt et al., 2018). As described in chapter 2, the N-terminal tagging could impact the function of the TEN domain effecting processivity as well as recruitment of telomerase to telomeres. This study unmasked defects associated with the epitope-tagged telomerase, which have been overlooked in previous ectopic overexpression systems. Genome editing allows the study endogenous regulations, yet careful characterization of established cells and genetic controls are essential. In addition, further characterization of

telomerase by biochemical and structural approaches (Nguyen et al., 2018) will facilitate proper labeling of telomerase for in vivo live imaging of telomerase.

Another important aspect of telomerase regulation is to elucidate its role during tumorigenesis. Telomere maintenance in cancer is classified two ways: telomerase-dependent (~90% of tumor) or telomerase-independent recombination based alternative lengthening of telomeres (ALT ~10%)(Shay and Bacchetti, 1997). Previous studies have suggested there are contributions of proto-oncogenic transcription factors such as c-Myc in TERT expression in cancer cells. However, genetic characterizations causing the upregulation of TERT by those factors are cell type and context specific. hESCs are genetically intact and regulation of TERT upon differentiation into tissue stem cells and somatic cells recapitulates the physiological different levels of TERT expression among various cell types. Thus, hESCs can be a suitable system to study TERT expression in both cancer and normal cell context in genetically defined manner. Recent genome wide sequencing studies identified TPMs as the most frequent cancer-associated non-coding mutation(Heidenreich et al., 2014). Interestingly, TPMs are more commonly found in tumors derived from low self-renewing cells(Killela et al., 2013). By introducing TPMs in hESCs using genome editing, we revealed that cell-acquired TPMs aberrantly express TERT in somatic differentiation. This phenotype can be explained in two ways: TPMs can counteract TERT silencing mechanism in somatic cells, or alternatively that TPMs can act as activator binding site in the TERT promoter. These possibilities can be tested by introducing TPMs in somatic cells using genome editing. Several studies reported GABPa/b is recruited to TPMs as an activator using the endogenous ETS sites in the TERT promoter as dimerization scaffold(Bell et al., 2015; Stern et al., 2015). However, our results show that disruption of dimerization by endogenously mutating the endogenous ETS sites in the TERT promoter in the presence of a TPM can still increase TERT expression. We also revealed that those endogenous ETS sites are important TERT expression in the wild type hESCs. This suggests that either GABPa/b can use upstream or downstream endogenous ETS binding sites or other ETS transcription factors can compensate loss of activation by GABPa. The majority of ETS transcription factors act as activators but at least three factors in the TEL- and ERF- subfamily are reported as repressors (Sharrocks, 2001). It is meaningful to investigate which ETS factor is involved in TERT gene regulation in physiological wild type promoters and how TPMs expropriate TERT expression during tumor formation. In addition, differential ETS expression profiles in different tissue/cell types might be able to explain the difference of TPM frequency in tumor types. -124 and -146 are sporadic mutations and -57 is found in familial form of melanoma. Interestingly, all of them create the same ETS binding site yet -57 has not discovered as a sporadic mutation. -57 locates one nucleotide downstream of the transcription start site in the 5'-UTR. This positional difference raises a possibility that -124, -146 and -57 could fulfill TERT upregulation by distinct mechanisms. Recently, mutations in the 5'-UTR were identified in clear cell renal cell cancer(Mitchell et al., 2018). Fully understanding the mechanism of TERT expression by TPMs requires further investigation. If we can elucidate molecular mechanisms governing TERT expression by TPMs, it may lead to therapeutic approaches to target disruption of TERT expression associated with TPMs.

Besides different cancer types of TPMs, previous studies were only able to take snapshot of TPM's phenotype once tumor formation is completed and have never

addressed if TPMs are sufficient to maintain telomeres to make cancer cells immortal. We addressed this question using our genetically defined system. TPMs can immortalize a bulk population of cells but surprisingly TPMs do not prevent telomere shortening and genomic instability in long-term tissue culture. A type of melanoma derived from adjacent nevus acquires TPMs during tumor progression. To confirm our in vitro findings in vivo, telomere length in TPM melanoma and precursor nevus was compared within the same section of a patient sample. Analysis of patient derived melanoma samples confirmed that TPMs do not support telomere maintenance in vivo(Chiba et al., 2017). We found that gradual increase of TERT expression is required for immortalization of TPM cells. These results suggest that TPMs alone are not sufficient to immortalize cells and additional changes to upregulate TERT expression are required for immortalization by TPMs. These results led us to propose a new two-step model by TPM in tumorigenesis that could not be explained by the crisis model (discussed in Chapter 4). However, there are still questions to be answered. First, what are the changes that increase TPM-associated TERT expression over time? The timing of gradual increase of TERT expression coincides with detection of telomere fusions. This observation raises a possibility that DNA damage responses at stabilized short telomeres might contribute to the gradual increase of telomerase activity. Previous studies suggested that ATM and ATR localizing at telomeres recruit telomerase, resulting in telomere elongation (Lee et al., 2015; Tong et al., 2015). However, our data indicates that the gradual increase of telomerase activity in TPMs is caused by an increase in TERT transcripts. So far, no clear connection between DNA damage signal at telomeres and TERT expression has been reported. Another potential mechanism is telomere position effect-over long distance cooperating with TPMs (Akıncılar et al., 2016; Kim et al., 2016). Overexpression of POT1ΔOB elongates telomeres in a telomerase dependent manner. If TPMs rely on telomere position effects to increase TERT expression, the timing of gradual telomerase activity increase should be delayed by elongated telomeres by POT1ΔOB overexpression. Modulation of telomere length did not significantly change the timing of upregulated telomerase activity suggesting that the contribution of telomere position effect is not definitive. To genetically test the contribution of telomere position effect on TERT expression via TPMs during telomere shortening, deletion of the region coordinating the looping of the end of chromosome 5 to the TERT locus may provide the conclusive answer(Akıncılar et al., 2016). The most important experiment to understand changes of TERT expression over time is to determine what changes trigger gradual increase at the TERT locus. One possibility for regulation is epigenetic change at the TERT locus. Studies have showed the association of gene activating epigenetic marks such as H3K4me2/3 and TERT expression via TPMs(Stern et al., 2015). Reduction of CpG island methylation of the TERT promoter in TPM cancer cell lines compared to cancer cell lines with the wild type promoter has also been detected(Stern et al., 2017). It is not clear if these epigenetic changes cause gradual increase of TERT expression or they are results of increase of transcription level by a transcription factors associating with TPMs. Time course analysis of changes in chromatin at the TERT locus might provide new insights of how TPMs increase TERT expression level during tumorigenesis and how it connects to signaling associated with telomere shortening over the time course.

Another important point to fully understand the role of TPMs in cancer, is the relationship with other cancer mutations. In melanoma, BRAF, NRAS, CDKN2A and

TP53 are highly frequently mutated along with TPMs. In this work, we recapitulated this order of events. In the traditional crisis model, acquisition of TERT expression is selected after loss of checkpoints. In the crisis model model, loss of checkpoints is a critical step to overcome telomere induced senescence. Previous studies showed TPMs can occur before the loss of check points(Shain et al., 2015) and in this work, our results lead to our proposition of a new two-step immortalization process by TPMs (Figure 4.11, right panel). Interestingly, we found that even without loss of checkpoints, cells become immortal by TPMs. However, one of the checkpoint-proficient TPM cells failed to be immortalized. The cells that failed to immortalize showed overall longer telomeres compared to immortalized cells and telomerase activity in checkpoint proficient TPM cells is lower than checkpoint deficient TPM cells. Comparisons between the immortal and mortal checkpoint-proficient TPM cells might provide a new insight of an unexpected connection between DNA checkpoints, telomere maintenance and TERT expression by TPMs.

BRAF V600E mutation is also very frequently found in melanoma. The timing of BRAF V600E mutation is at the early stage of tumorigenesis, before or coincident with TPM acquisition. It is important to test the contribution of the BRAF mutation in TPM cell's proliferation, telomere maintenance and TERT expression over the course of tumor formation. Our preliminary data suggests introduction of BRAF V600E mutation into TPM cells increase TERT expression and proliferation rate of the cell yet do not prevent telomere shortening (data not shown). A study using melanoma cancer cell lines carrying TPMs and BRAF V600E showed that inhibition of the RAS-ERK pathway reduced recruitment of Pol II to the TERT promoter and subsequently TERT expression level (Li et al., 2016). Conversion of a TPM promoter to the wild type promoter in a cell line using genome editing resulted in tolerance for inhibition of the RAS-ERK pathway. However, the level of Pol II occupancy does not change between the wild type promoter and TPM promoter. One of the substrates of ERK2 kinase is SP1, which modulates its DNA binding specificity (Merchant et al., 1999). SP1 is suggested to be involved in TERT expression in cancer cells with the wild type promoter as well as embryonic stem cells (Cheng et al., 2015; Kyo et al., 2000). Thus, it is still not clear if BRAF V600E specifically acts on TPMs., especially because some of the ETS factors are direct targets of the MAPK signaling cascade(Sharrocks, 2001; Wasylyk et al., 1998). Moreover, timing and consequence of BRAF V600E and TPM acquisition in telomere maintenance, telomerase activity and tumor forming capacity cannot be investigated in established cancer cell lines. Our genetically defined in vitro modeling system enables researchers to test these important questions.

Previously, to immortalize primary cells overexpression of TERT has been commonly used. In this work, we showed that one nucleotide change at the TERT promoter can immortalize a fibroblast. Immortalization by TERT overexpression results in a robust increase in telomerase activity. Surprisingly, the level of telomerase activity required for immortalization of TPM cells is much lower than expected (5~20% of the activity observed in human embryonic stem cells or most cancer cell lines tested). This observation highlights importance of tight silencing of TERT in somatic cells because small residual expression of TERT could result in immortalization of a cell. It also raises the question why cancer cells express telomerase levels that are beyond what is required for cellular immortality.

A complication of understanding TERT regulation is different tissue and cell types shows variable TERT expression. For example, pluripotency genes such as OCT4 or SOX2 are regulated as binary switch (on/off) between pluripotent stem cells and somatic cells. However, in case of TERT, between pluripotent stem cells and somatic cells, TERT regulation is the binary mode, yet different adult stem cells and progenitor cells possess moderate TERT expression level. Currently, molecular mechanisms and genetic elements governing cell type specific TERT regulation have not been identified. Understanding the physiological TERT regulation can also provide insights how TPMs overwrite the TERT silencing of the wild type TERT promoter. Using human pluripotent stem cells and genome editing, this challenging task might be able to be accomplished.

In this work, it was surprising that one nucleotide substitution of a non-coding region can immortalize cells in unexpected telomere length maintenance manner. In the early days of modern biology, non-coding regions were considered “junk” and studies have been focused on coding regions of tumor suppressors and oncogenes. TPMs are the good example of how important it is to take regulatory elements of genes for account to understand the fundamental biology of cancer. Genome wide studies have revealed many new genetic interactions in cancer, however it is very important to elucidate how those genes contribute to tumorgenesis at the molecular level to discover unexpected roles in cancer biology.

Materials and methods

hESC culture

Genome-editing experiments were performed in WIBR#3 hESCs (Lengner et al., 2010), NIH stem cell registry # 0079. Cell culture was carried out as described previously (Soldner et al., 2009). Briefly, all hESC lines were maintained on a layer of inactivated mouse embryonic fibroblasts (MEFs) in hESC medium (DMEM/F12 [Lifetech]) supplemented with 15% fetal bovine serum [Lifetech], 5% KnockOutTM Serum Replacement [Lifetech], 1 mM glutamine [Lifetech], 1% non-essential amino acids [Lifetech], 0.1 mM β-mercaptoethanol [Sigma], 1000 U/ml penicillin/streptomycin [Lifetech], and 4 ng/ml FGF2 [Lifetech]). Cultures were passaged every 5-7 days either manually or enzymatically with collagenase type IV [Lifetech] (1.5 mg/ml) and gravitational sedimentation by washing 3 times in wash media (DMEM/F12 [Lifetech] supplemented with 5% fetal bovine serum [Lifetech], and 1000 U/ml penicillin/streptomycin [Lifetech]).

Gene targeting in hESCs

All targeting experiments were preformed as previously described (Hockemeyer et al., 2009; Hockemeyer et al., 2011). CAS9 and all sgRNAs were expressed using the px330 plasmid (Cong et al., 2013). Endogenously N-terminal tagged TERT hESC lines and cancer associated TERT promoter mutation containing cell lines were generated by two targeting steps. First, 1-2×10^7 hESCs were co-electroporated with 15µg of two CAS9 plasmids targeting -1418 to -1399 bp (aaccgccccttgccctag) and +110 to+129 bp (taccgcgaggtgctgccgc) from the TSS and 7.5 µg of a GFP-expression plasmid was electroporated along with px330. Cells were sorted for GFP fluorescence 72 hours after electroporation. Single-cell derived hES colonies were isolated and their targeting was confirmed by Southern blotting and PCR followed by sequencing. 120 clones were analyzed and three homozygous targeted hESC lines ($TERT^{\Delta/\Delta}$) were obtained. For the second targeting, px330 plasmids were designed with sgRNAs against the newly formed NHEJ-derived junction site in $TERT^{\Delta/\Delta}$ cells and electroporated with 35 µg of a repair plasmid. For endogenously N-terminal tagging, the deleted wild type TERT promoter and coding element (wt) or an insertion of an epitope tag after the first ATG of TERT (3xFlag (Flag), 3x Flag-GS10 (Flag-GS10), 3x Flag-GS20 (Flag-GS20), HA or YbbR-TEV-3xFlag (YbbR)) were used as a donor. For TERT promoter mutation, the wild type TERT promoter element (wt) or the respective TERT promoter mutations (57A/C, 124 C/T, 146C/T) were used. After the second targeting, cells were continuously passaged. Over a period of 120 days all $TERT^{\Delta/\Delta}$ lines that did not undergo the second targeting step died due to critically short telomeres. However, cells that were correctly targeted in the second targeting step regained TERT expression and outgrew untargeted cells. These cells were analyzed in bulk or as single cell derived clones, after the parental $TERT^{\Delta/\Delta}$ control culture, that did not undergo the second targeting step, had completely died. Targeting of individual clones was confirmed by Southern blot analysis.

For *CDKN2A* $^{\Delta/\Delta}$ hESC lines, Exon2 of *CDKN2A* was deleted using accattctgttctctctggc and cgcggaaggtccctcaggtg as sgRNA target sites.

Gene targeting in HCT116

TERT gene disruption was performed as published previously (18). Cells were transfected with plasmids expressing zinc finger nucleases (ZFNs) targeting exon 1 of TERT, along with a donor plasmid expressing a PGK-promoter-driven hygromycin resistance cassette. Transfection was performed using lipofectamine 3000 [Lifetech] per manufacture instructions. Targeted cells were selected using 300 µg/ml hygromycin [Thermo]. $TERT^{-/-}$ clonal cells were selected and assayed for the complete loss of telomerase activity using TRAP (protocol below). AAVS1 targeting was performed as previously described (18). Cells were transfected with a plasmid expressing ZFNs to target the AAVS1 locus, along with a donor plasmid containing TERT cDNA and a splice-acceptor NEO resistance cassette. Targeted cells were selected using 250 µg/ml G418 [Thermo].

Southern blotting and PCR genotyping

Southern blot analysis was performed as previously described (Hockemeyer et al., 2011; Soldner et al., 2009) using an external 3'- probe for TERT (6280 bp - 6846 bp downstream of the TERT 1st ATG), an external 5'- probe (5481 bp - 5006 bp upstream of the TERT 1st ATG) and an internal 5'-probe (1813 bp - 1206 bp upstream of the TERT 1st ATG). Primers used for PCR genotyping are located in an internal region of the donor plasmid due to inefficient PCR amplification of GC-rich sequences (Fw: ggccgattcgacctctct , Re: ctccttcaggcaggacacct). CDKN2A deletion was confirmed by Southern blotting with using probe amplified from genomic DNA with primers (Fw: ggggaaatgatgttggcttagaatcct, Re:caatgaagtccttcgtcttggtca).

Differentiation to fibroblast-like cells

For the formation of embryonic bodies (EBs) hESC colonies were grown on petri dishes in fibroblast medium (DMEM/F12 [Lifetech]) supplemented with 15% fetal bovine serum [Lifetech], 1 mM glutamine [Lifetech], 1% non-essential amino acids [Lifetech], and penicillin/streptomycin [Lifetech]). After 9 days EBs were transferred to tissue culture dishes to attach. Fibroblast-like cells were passaged with Trypsin EDTA ([Lifetech], 0.25%), triturated into a single-cell suspension and plated on tissue culture dishes. Cultures were maintained in fibroblast media and passed every 6 days.

Differentiation to neural precursor cells and neurons

Before differentiation to NPCs, hESCs were cultured under feeder-free conditions on matrigel [Corning]-coated plates in E8 media (DMEM/F12 [Lifetech]) supplemented with 64 µg/ml L-ascorbic acid [Sigma], 19.4 µg/ml insulin [Sigma], 14 µg/l sodium selenite [Sigma], 543 ng/l sodium bicarbonate [Sigma], 1000 U/ml penicillin/streptomycin [Lifetech], 100 ng/ml FGF2 [Lifetech], and 10.7 µg/ml Transferrin [Sigma]). hESCs were passaged with accutase [Invitrogen] and triturated to a single-cell solution and plated on matrigel-coated plates at 50,000 cell/cm^2. The dual SMAD inhibition protocol for the differentiation of hESCs to NPCs was adapted from a previous study(Chambers et al., 2009). Differentiation was induced when cells reached 90-100% confluency.

NPCs were maintained in N2 media (50% DMEM/F12 [Lifetech], 50% Neurobasal Media [Lifetech] supplemented with 0.75% BSA (w/v) [Sigma], N2 Supplement [Lifetech], 20 ng/ml insulin [Sigma], 1 mM glutamine [Lifetech], 1000 U/ml

penicillin/streptomycin [Lifetech], 25 ng/ml FGF2 [Lifetech] and 40 ng/ml EGF [R&D systems]) and passaged every 5 days. For the terminal differentiation to neurons NPCs were plated at 50,000 cells/cm^2 on matrigel-coated plates in N2B27 media (50% DMEM/F12 [Lifetech], 50% Neurobasal Media [Lifetech] supplemented with 0.75% BSA (w/v) [Sigma], N2 Supplement [Lifetech], B27 Supplement [Lifetech], 1 mM glutamine [Lifetech], 1000 U/ml penicillin/streptomycin [Lifetech]). Neurons were treated with 250 nM mitotic inhibitor (Cytosine-β-D-arabinofuranoside [Sigma]).

qRT-PCR

RNA was extracted with TRIzol [Lifetech] and treated with DNaseI [NEB]. 600 ng RNA were converted to cDNA with the iScript Reverse Transcriptase [BioRad] and random and poly A priming. TR cDNA was prepared by gene specific reverse transcription. qRT-PCR was performed with KAPA SYBR fast [KAPA Biosystems] or SYBR Select Master Mix [ABI] in 96-well or 384-well format with a total reaction volume of 20 µl or 10 µl respectively. 2 µl cDNA from the iScript reaction mixture was used for the detection of TERT mRNA. For measuring the expression levels of all other genes, cDNA was diluted 1:10 and 2 µl were used for qPCR.

In hESCs, expression levels of TERT, OCT4, CDKN2A and TR were normalized by expression of GAPDH. Due to different expression levels of GAPDH between hESCs and differentiated cells, GAPDH data are shown in the figures that required comparison of expression in different cell types. Relative expression levels were calculated based on Δ/Δ Ct and/or ΔCt analysis. qRT-PCR primers used in this study were:

TERT fw (TGTCAAGGTGGATGTGACGG)
TERT rev (GAGGAGCTCTGCTCGATGAC),
TR Fw (CCCTAACTGAGAAGGGCGTA)
TR Rev (AGAATGAACGGTGGAAGGCG).
GAPDH fw (CAGTCTTCTGGGTGGCAGTGA)
GAPDH rev (CGTGGAAGGACTCATGACCA),
OCT4 fw (CGTTGTGCATAGTCGCTGCT)
OCT4 rev (GCTCGAGAAGGATGTGGTCC),
COL1A1 fw (GTCACCCACCGACCAAGAAACC)
COL1A1 rev (AAGTCCAGGCTGTCCAGGGATG)
NESTIN fw (CTGCTACCCTTGAGACACCTG)
NESTIN Re (GGGCTCTGATCTCTGCATCTAC)
TUJ1 fw (GGCCAAGGGTCACTACACG)
TUJ1 Re (GGCCAAGGGTCACTACACG)
CDKN2A ex2 fw (CAACGCACCGAATAGTTACGG)
CDKN2A ex2 rev (ACCAGCGTGTCCAGGAAG)
SV40 TAg fw (GGTGGGTTAAAGGAGCATGA)
SV40 TAg rev (TAGTGGCTGGGCTGTTCTTT)

Northern blotting

RNA for northern blot was purified using TRIzol according to the manufacturer's protocol [Lifetech]. Northern blot detection of TR was performed as previously described (Fu and Collins, 2003). 7SL RNA was detected using ^{32}P end-labeled probe (TGAACTCAAGGGATCCTCCAG) under similar conditions as TR, except

hybridization took place at 37°C. Southern blots analysis was performed as previously described (Hockemeyer et al., 2009; Hockemeyer et al., 2011) using a 3'- probe for TERT (6280 bp -6846 bp downstream of the TERT 1st ATG) and probe T1 (amplified from hES genomic DNA with primers Fw: GTGACTCAGGACCCCATACC and Rev: ACAACAGCGGCTGAACAGTC).

Immunoblotting

After heating to 80°C for 5 minutes, protein samples were cooled to room temperature and resolved by SDS-PAGE. Protein was then transferred to nitrocellulose membrane and subsequently incubated with mouse a-tubulin (1:500, DM1A, [Calbiochem]) and mouse anti-TERT (Wu et al., 2015) in 4% nonfat milk [Carnation] in TBS buffer (150 mM NaCl, 50 mM Tris pH 7.5) overnight at 4°C. The membrane was washed in TBS and incubated with goat a-mouse Alexa Fluor 680 (1:2,000, [Life Technologies]) in 4% nonfat milk in TBS for 1 hr at room temperature. After extensive washing with TBS, the membrane was visualized on a LI-COR Odyssey imager (Fu and Collins, 2003).

Immunofluorescence

For analysis by immunofluorescence, cells were briefly rinsed with PBS, and fixed with 4% formaldehyde in PBS. Cells were blocked with PBS 0.3% Triton X-100 with 5% horse serum. Fixed cells were incubated with antibodies against NEUN (mouse, monoclonal, [Millipore], MAB377; 1:1500) and TUJ1 (B-III-Tubulin, chicken, polyclonal, [Millipore], AB9354, 1:500), in PBS 0.3% Triton X-100 with 1% BSA over night. After washing with PBS the cells were stained with secondary antibodies (Alexa Fluor 546 goat α mouse, Alexa Fluor 488 goat α chicken [Lifetech]; 1:500), for 1h in PBS 0.3% Triton X-100 with 1% BSA. Cells were then washed with PBS and stained with 1 ng/µl DAPI [Sigma] in PBS.

Detection of telomere length

For preparation of genomic DNA, hESC lines were washed with PBS, released from the feeder cell layer by treatment with 1.5 mg/ml collagenase type IV and washed 3x in wash media by gravitational sedimentation to minimize contaminating MEF cells. Genomic DNA was then prepared as described previously (Hockemeyer et al., 2005). While this method removes the vast majority of MEFs, the signal from mouse telomeres is disproportionate to human telomeres due to amplified relative length and concentration into a smaller area (Kipling and Cooke, 1990). Because MEF telomeres are size-resolved from human telomeres they do not interfere with analysis of hESC telomere length. Genomic DNA was digested with MboI and AluI overnight at 37°C. The resulting DNA was normalized and run on 0.75% agarose [Seakem ME Agarose, Lonza], dried under vacuum for 2h at 50°C, denatured in 0.5 M NaOH, 1.5 M NaCl for 30 mins, shaking at 25°C, neutralized with 1 M Tris pH 6.0, 2.5 M NaCl shaking at 25°C, 2x for 15 min. Then the gel was pre-hybridized in Church's buffer (1% BSA, 1 mM EDTA, 0.5M $NaP0_4$ pH 7.2, 7% SDS) for 1h at 55°C before adding a ^{32}P-end-labeled $(T_2AG_3)_3$ telomeric probe. The gel was washed 3x 30 min in 4x SSC at 50°C and 1x 30 min in 4x SSC + 0.1% SDS at 25°C before exposing on a phosphorimager screen.

Mean telomere length was quantified as described previously (Kimura et al., 2010) using the following equation: Mean telomere length = $\Sigma(OD_i \times L_i)/ \Sigma(OD_i)$. OD_i = optical density at position i, L_i= telomere length at position i. To exclude skewing by MEF telomeres, signals above 23 kb were not incorporated into the quantification. Normalized pixel intensity showing the accumulation of short telomeres was calculated by quantifying pixel intensity and normalizing to the area under the curve for each lane using ImageJ and Prism7.

Assaying telomerase catalytic activity

PCR-based telomeric repeat amplification protocol (TRAP) was performed as previously described using TS (AATCCGTCGAGCAGAGTT) and ACX (GCGCGGCTTACCCTTACCCTTACCCTAACC) for amplification of telomeric repeats and TSNT (AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT) and NT (ATCGCTTCTCGGCCTTTT) as an internal control. Cell extracts were generated by repeated freeze-thaw cycles in Hypotonic Lysis buffer (HLB) (20mM HEPES, 2mM $MgCl_2$, 0.2mM EGTA, 10% Glycerol, 1mM DTT, 0.1mM PMSF) supplemented with 0.5% CHAPS. Protein concentrations were measured using the Bradford Protein Assay Kit [Biorad]. 200 ng of total protein were used for detection of fibroblasts and NPCs telomerase activity. For hESCs and cancer cell lines, a series of diluted lysate, 200, 40 and 8 ng was used. The TRAP products were resolved on a 10% polyacrylamide /1xTAE gel. Dried gels were visualized by phosphor imaging.

For enrichment of endogenous telomerase, lysates from hESCs were incubated with 10µl of streptavidin agarose resin [Sigma-Aldrich] conjugated with 5'-biotinylated telomeric template oligonucleotide (CTAGACCTGTCATCAGUUAGGGUUAG: the underlined nucleotides are 2'-O-Methyl RNA) (Schnapp et al., 1998) . The resin and lysate were incubated at room temperature for 2 hours. The resin was washed three times with HLB supplemented with 150 mM NaCl, 0.1% NP-40. Purified telomerase was eluted by 10 µl of the elution buffer (HLB supplemented with 150mM NaCl, 0.1% NP-40 and 30µM of the displacement oligonucleotide (CTAACCCTAACTGATGACAGGTCTAG with 2',3'-dideoxyguanosine modification at the 3'-terminal)) for 1 hour at room temperature. The resin was removed using Empty Micro Spin Columns [Harvard Apparatus]. Primer extension assay with oligo-purified telomerase was performed in 20 µl reaction containing 8 µl of the eluate fraction, 500 nM of the telomeric primer ($(T_2AG_3)_3$) in MTB buffer (50mM Tris-acetate, 3mM MgCl2, 50mM Potassium acetate, 1mM EGTA, 1mM spermidine, 5mM β-mercaptoethanol) with 250µM dTTP and dATP, 5µM dGTP and 2µl of α-32P dGTP (3000 Ci/mmol, 10mCi/ml, [Perkin-Elmer]). The reaction was incubated at 30°C for 40 minutes, then the reaction was stopped by adding 80 µl of TES buffer (50mM Tris pH7.5, 20mM EDTA, 0.2%SDS). After phenol/chloroform extraction and ethanol precipitation, the extension products were resolved on 10.5% polyacrylamide/7M urea/0.6x Tris borate-EDTA gel. As a recovery control, end-radiolabeled $(T_2AG_3)_2$ primer was added before ethanol precipitation, and end-radiolabeled $(T_2AG_3)_3$ primer was loaded as a size marker. Dried gels were visualized using a Typhoon phosphorimager.

Fusion PCR

Fusion PCR was performed as previously described(Capper et al., 2007; Takai et al., 2016) with the following modifications. Briefly, genomic DNA was extracted using phenol chloroform following overnight proteinase K digestion. Isolated DNA was solubilized by EcoRI and RNaseA digestion and phenol chloroform extracted followed by ethanol precipitation. Purified DNA was resolved in TE buffer and quantified. 100 ng of digested DNA were used per 25 μl Failsafe PCR reaction mix (Buffer H) with mixed primers of XpYpM (ACCAGGTTTTCCAGTGTGTT), 17p6 (GGCTGAACTATAGCCTCTGC) and 21q1 (CTTGGTGTCGAGAGAGGTAG). PCR was conducted at the following condition [95 °C for 2 min, (95 °C for 15 sec, 58 °C for 20 sec, 68 °C for 9 min) x35 cycles, 75 °C for 5 min]. PCR products were detected by southern blotting using probes amplified with the following primers: 17p probe: 17p7(CCTGGCATGGTATTGACATG) and 17p2 (GAGTCAATGATTCCATTCCTAGC), XpYp probe: XpYpE2 (TTGTCTCAGGGTCCTAGTG) and XpYpB2 (TCTGAAAGTGGACC(A/T)ATCAG).

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