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TERT promoter mutations and telomeres during tumorigenesis

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Telomerase regulation and telomere shortening act as a strong tumor suppressor mechanism in human somatic cells. Point mutations in the promoter of telomerase reverse transcriptase (*TERT*) are the most frequent non-coding mutation in cancer. These *TERT* promoter mutations (TPMs) create *de novo* ETS factor binding sites upstream of the start codon of the gene, which can be bound by different ETS factors. TPMs can occur early during tumorigenesis and are thought to be among the first mutations in melanoma, glioblastoma and hepatocellular carcinoma. Despite their association with increased *TERT* levels, TPMs do not prohibit telomere shortening and TPM-harboring cancers present with short telomeres. Their short telomere length combined with their high prevalence and specificity for cancer makes TPMs an attractive target for future therapeutic exploitation of telomerase inhibition and telomere deprotection-induced cell death.

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Introduction

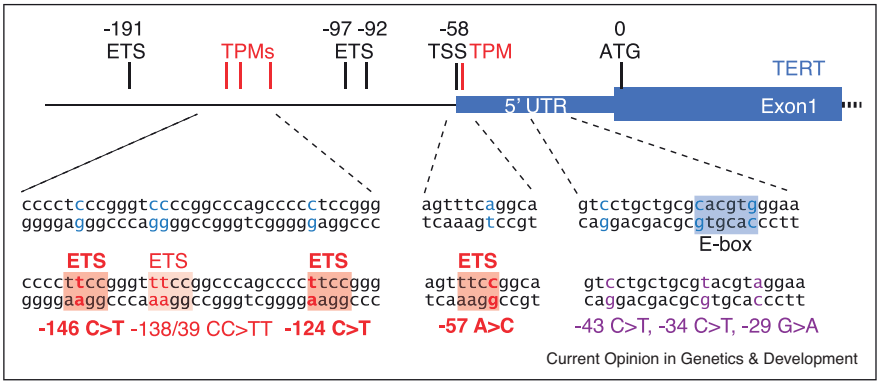
Telomeres are the repetitive elements at the end of linear chromosomes that cap the chromosome end from nucleolytic degradation and protect against a DNA damage response. They comprise tandem hexamers, which serve as a reservoir non-coding DNA, that can buffer terminal sequence loss. Cells can elongate their telomeres using the enzyme telomerase [1]. Most cell-types, however, downregulate telomerase by transcriptionally silencing telomerase reverse transcriptase (*TERT*), the catalytic subunit of telomerase [2]. In cells without telomerase, telomeres shorten and eventually signal as sites of DNA damage leading to the arrest of cells in a state called replicative senescence [3,4]. Induction of replicative senescence as a consequence of telomere shortening acts as a strong tumor suppressor mechanism in humans [5]. In order to overcome replicative senescence, cancer cells have to inactivate DNA damage signaling (i.e. p53 or CDKN2A) but also stabilize telomeres. More than 90% of all human cancers overcome this proliferative barrier by expressing or re-activating telomerase [6]. Several large scale chromosomal aberrations leading to aberrant *TERT* expression have been identified: amplifications of the *TERT* gene [7], transcriptional activation of the *TERT* gene through viral integrations [8,9], and rearrangements of the *TERT* locus [10–12]. Despite the discovery of these important mechanisms, it remained unresolved until recently how *TERT* is activated for a majority of cancer cases.

The discovery of *TERT* promoter mutations: prevalence and tissue specificity

A major breakthrough came in 2013, with two studies in melanoma that identified three high frequency hotspot mutations in the *TERT* promoter at position −57A/C, −124C/T, −146C/T relative to the ATG of the *TERT* gene [13••,14••] (Figure 1). Soon after, a survey of 60 different tumor types confirmed the high prevalence of *TERT* promoter mutations (TPMs) in many other cancers, although they occurred with different frequencies: for example, 51% in glioma and 44% in hepatocellular carcinoma (HCC) [15••]. A comprehensive analysis of a TCGA data set of 9127 patients and 31 cancer types determined that a remarkable 27% of all analyzed samples harbored one of these promoter mutations [16•]. This extremely high prevalence makes TPMs the most frequent non-coding mutation in cancer and places them among the most frequent cancer mutations overall.

Despite their high prevalence, TPMs are associated with a specific cancer spectrum. They are much more frequently found in cancers originating from tissues with low-self renewal rates such as melanomas, liposarcomas, and subtypes of gliomas, but are rare in cancers originating from highly regenerative tissues such as the intestine or blood [15••]. In a matched data set of whole genome sequencing data and RNA-Sequencing from 14 different cancer types, TPMs show an association with elevated *TERT* mRNA expression levels [17•]. Additionally, when engineered into an isogenic human embryonic stem cell (hESC) model, TPMs led to a failure of *TERT* transcriptional downregulation upon differentiation [18•]. The aberrant *TERT* expression in *TERT* promoter mutant cells and the pattern of TPM presentation suggests that

Figure 1



Mutations and endogenous ETS-factor binding sites in the *TERT* promoter.
Schematic overview of the *TERT* promoter with the endogenous ETS factor binding sites (black), *TERT* promoter mutations (TPM, red) and 5′ UTR mutations (purple). All numbers are relative to the first ATG of the *TERT* gene. (ETS = E26 transformation specific, TPM = TERT promoter mutation, TSS = transcription start site, 5′ UTR = 5′ untranslated region, E-box = enhancer box).

TPMs do not provide a selective advantage in tumors initiating from naturally telomerase-positive cells. The mutation rather confers an advantage to cancer cells that initiate from tissues with low or typically no TERT expression, where TPMs allow for extended proliferation [18•] (Figure 2a).

As cancer sequencing projects have expanded in read depth (number of sequencing reads) and number of samples analyzed, it remains a key question whether there are additional mutations in the promoter of *TERT* to be uncovered in specific cancer subtypes. Systematic sequencing of the *TERT* promoter region of skin cancer samples also revealed less frequent tandem mutations in the *TERT* promoter (−124/125 CC>TT, −138/139 CC>TT) [19,20]. Additionally, in clear cell renal cell carcinoma previously unknown mutations in the 5′ UTR of *TERT* were identified [21] (Figure 1). Though found in a regulatory region that was previously shown to be involved in the transcriptional regulation of the *TERT* gene [22], the relevance and mode of action of the 5′ UTR mutations is not clear and needs to be tested. In summary the discovery of TPMs provides a starting point of understanding how a large fraction of cancer cells gain immortality, but also raises the question — what are the mechanisms by which cancer cells without TPMs gain or retain TERT expression?

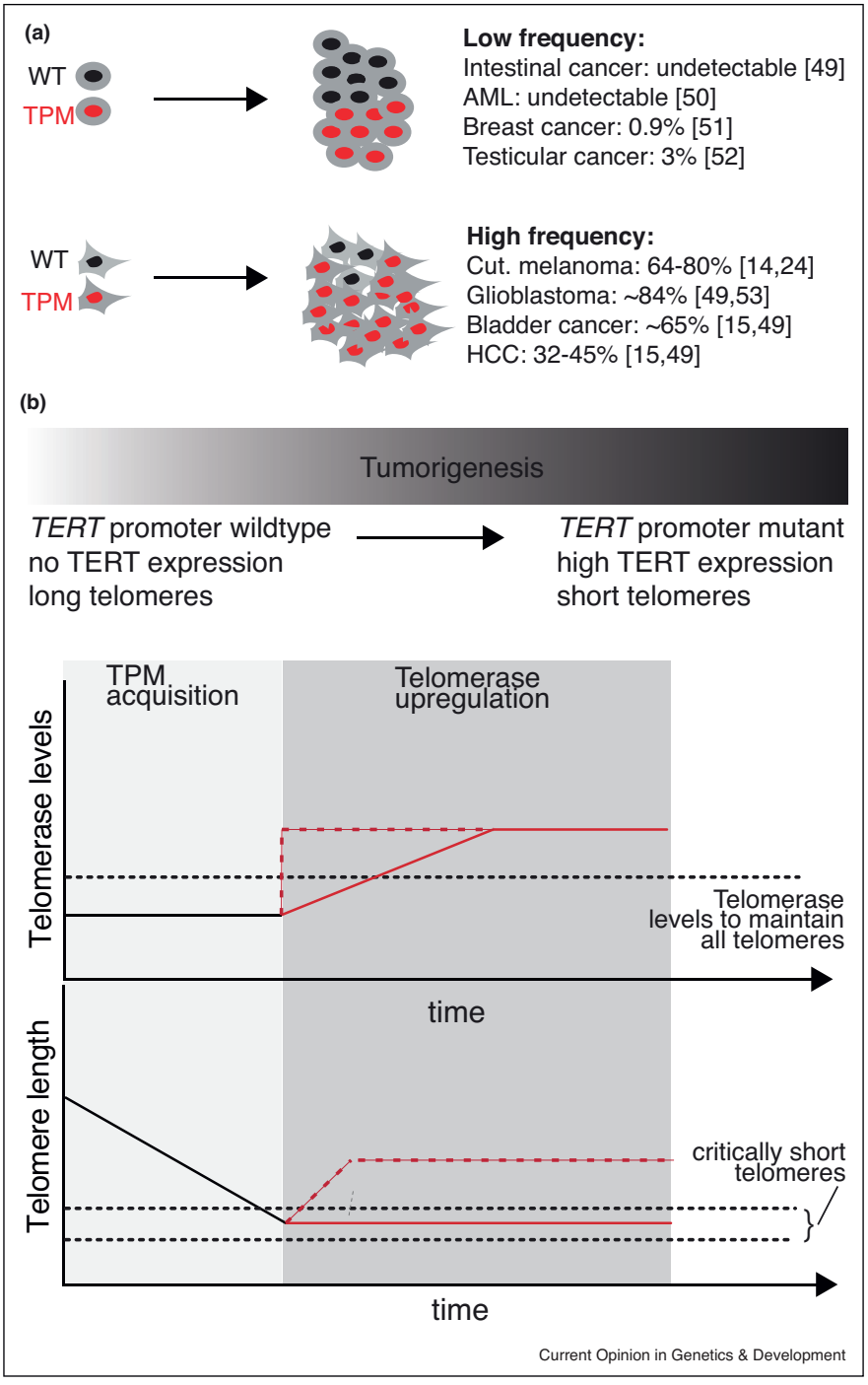
Molecular mechanisms of the mutant *TERT* promoter and ETS factor binding: an unresolved issue

Several studies have worked on elucidating the molecular mechanism of TPMs. TPMs occur heterozygously and in a mutually exclusive fashion [14••,23•,24]. Each of the hotspot mutations creates a *de novo* ETS (E26 transformation specific) factor binding site. The ETS transcription factor family members share a conserved DNA binding domain that recognizes unique sequences containing GGA(A/T). While some of the transcription factors are restricted to tissue specific expression, others are ubiquitously expressed [25].

In a screen of ETS factors the GA-binding protein α (GABPα) was identified as the ETS-factor with the greatest impact on TERT expression in glioma cells. The binding occurs tetramerically, formed by two GABPα/β heterodimers, requiring coordination between the *de novo TPM* ETS sites and the endogenous ETS sites of the *TERT* promoter [26•]. GABPα binding to the *TERT* promoter was independently validated in a study showing that TPMs lead to monoallelic expression of the mutant allele, with allele-specific changes in active histone marks upon GABPα depletion [27,28]. GABPα enhanced expression of TERT by mediating long-range interactions of the mutant *TERT* promoter. Upon GABPα silencing, these interactions as well as the active histone marks found in the promoter decreased [29]. However, hESCs lacking the endogenous cooperative ETS binding sites required for tetramerization still had higher TERT levels when also harboring TPMs, providing additional evidence that even without the GABPα/β tetramerization on the mutant *TERT* promoter ETS factor binding could stimulate TERT expression [30••].

Subsequent experiments found that the formation of the GABPα/β tetramer on the mutant *TERT* promoter specifically required the GABPβ1L isoform [31]. The disruption of GABPβ1L initially resulted in telomere shortening and cell death in cancer cells with TPMs. Cells depleted of the GABPβ1L isoform eventually continued to proliferate both *in vitro* and in *in vivo* xenografts

Figure 2



TERT promoter mutant cancers occur with a high frequency in tissues of low self-renewal capacity and continue to shorten their telomeres.
(a) Model for *TERT* promoter mutations leading to a selective advantage depending on the telomerase status of the cell of origin of the cancer. In telomerase positive cells there is no selective pressure from shortening telomeres, *TERT* promoter mutant (TPM, red) and wildtype (WT, black) can proliferate equally well. Cancers originating from tissues of high self-renewal have a low rate of TPMs. In telomerase-negative cells, telomeres shorten and act as a strong tumor suppressor mechanism. Cells that have acquired a TPM (red) have a selective advantage over WT (black) cells, which undergo replicative senescence. Cancers originating from tissues with low self-renewal capacity have TPMs with a high frequency.
Low frequency: Intestinal cancer [49], acute myeloid lymphoma (AML) [50], breast cancer [51], testicular germ cell cancer [52].
High frequency: Cutaneous melanoma [14••,24], glioblastoma [49,53], bladder cancer [15••,49], hepatocellular carcinoma (HCC) [15••,49].
(b) Over the course of tumorigenesis cells that start out with a wildtype *TERT* promoter, no *TERT* expression and long telomeres turn into cancerous cells with high *TERT* expression and short telomeres. 'Classic' crisis model (dashed line): Acquisition of the TPM leads to immediate upregulation of telomerase and outgrowth of a small subpopulation by telomere elongation. The two-step model of immortalization by the

again suggesting redundancy in ETS factor binding to the mutant *TERT* promoter.

Different studies have provided further evidence that other ETS factors can bind the mutant *TERT* promoter and drive expression: Glioma cell line experiments found that the −124C/T and −146C/T TPM to not be functionally identical. The −146C/T mutation was bound cooperatively by ETS1/2 and p52, thereby stimulating *TERT* expression via non-canonical NFκB signaling [32]. Similarly, in thyroid cancer the ETS factor ETV5 can bind and activate TPMs [33]. An ATAC-Seq chromatin assay confirmed heterozygosity and differential accessibility of the mutated allele in a TCGA data set of 23 different cancer types. Motif analysis of the ATAC-Seq peaks yielded the ETS family members ELF1 or ELF2 as binding factors of the *de novo* binding site [34]. In nuclear extracts from melanoma cell lines ELF1 can bind the mutant promoter as detected by mass-spectrometry. In *in vitro* binding assays ELF1 also bound the mutant *TERT* promoter but competed for binding with the higher affinity GABPα/β tetramer on the endogenous ETS binding sites [35]. *TERT* expression from the mutant promoter can be further enhanced by active mitogen-activated protein (MAP) kinase signaling, for example, by the co-occurrence of a BRAFV600E mutation. This mechanism can similarly be mediated by upregulation of different ETS factors such as GABP [36] as well as ETV1, ETV4 and ETV5 [37]. Taken together, these studies suggest that the ubiquitously expressed GABPα/β plays a role for expression from the mutant promoter, but other ETS factors compete for binding and consequently can also drive *TERT* expression from the mutant promoter.

Why do cancer cells with TPMs generally have short telomeres and what are the implications?

Despite elevated *TERT* expression, TPM cancers have short telomeres compared to matched healthy controls. Sequencing-derived length estimates show that telomeres were shorter in TPM samples than in matched normal samples in glioma [38•], clear cell renal cell carcinoma [21] and melanoma [23•]. In the aforementioned TCGA cancer sequencing data of 31 cancer types, telomere length estimates of TPM tumors were lower than those of matched wildtype control samples for almost all analyzed cancer types [16•]. The short telomeres of TPM cancers could suggest that TPMs are a late event of tumorigenesis, when telomeres are exhausted. However, TPMs occur during early stages of tumorigenesis in glioma, hepatocellular carcinoma and melanoma. In glioblastoma TPMs are potentially even the earliest genetic event of cancer formation. A comparison of samples from the tumor-free subventricular zone (SVZ), the tumor, as well as matching normal tissue found that the non-tumorous SVZ samples already harbor TPMs and could be the site of origin of the cancer [39••]. Similarly TPMs were shown to be the most frequent somatic genetic alteration in HCC and also the first recurrent gene somatically mutated in preneoplastic cirrhotic lesions [40•]. Multi-region sequencing of multiple regenerative nodules (cancer precursor) and tumor samples from several patients further showed that TPMs are the initial and essential step for HCC carcinogenesis, as their frequency increased with the stepwise progression from dysplastic nodule to carcinoma [41•,42].

In cutaneous melanoma, TPMs are found frequently (∼70% of all cases [24]). For these cancers, samples from matched precancerous lesions as well as tumors have been analyzed for genetic and telomere length. Here, taking allele frequency into account, TPMs are among the earliest events of tumor formation and occur before the critical bi-allelic cell-cycle checkpoint inactivation [43•,44]. Measurements of relative telomere length by quantitative telomere FISH of patient samples with known genetic history, showed that telomeres shortened in patient samples even after acquisition of TPMs [30••]. Why then are telomeres short, if TPMs are acquired at a time when telomeres are expected to still be long?

This conundrum was resolved by employing an hESC model comparing isogenic TPM-harboring and wildtype hESCs following differentiation. When differentiated into fibroblasts, wildtype hESCs become telomerase-negative. In a comparison of differentiated wildtype and mutant cells into fibroblasts, the presence of TPMs did — similar to the wildtype — not prohibit telomere shortening, matching the patient data. Analysis of *TERT* expression dynamics showed that the transcriptional upregulation of *TERT* was gradual and only occurred when critically short telomeres were accumulating [30••]. Therefore, immortalization of precancerous cells by TPMs can be described as a two-step process, in which during the first phase of tumorigenesis, TPMs can be acquired but do not prohibit telomere shortening and are not intrinsically cancerous. In the second phase, TPMs convey a selective advantage, potentially only when all telomeres have become critically short (Figure 2b). In the classic crisis model telomeres become critically short following the inactivation of the DNA damage response and cells can emerge from this state by acquiring a mechanism to stabilize telomeres [45]. The early

acquisition of TPMs provides an explanation for these observations (solid line): During the first step a TPM is acquired by the pre-cancerous cell. In this first phase *TERT* and telomerase levels are marginal and do not prohibit telomere shortening. The second phase is characterized by critically short telomeres, which are accumulating. Cells with TPMs can now gradually upregulate *TERT* to stabilize critically short telomeres.

occurrence of TPMs before the loss of checkpoint genes in melanoma, glioblastoma and HCC, as well as the gradual upregulation of telomerase expression challenges the idea of immortalization of TPM cancers by emergence of cells from a 'classic' crisis. Indeed, TPMs can mediate stabilization of short telomeres independent of tumorigenesis. Dyskeratosis congenita patients with mutations in the coding region of *TERT* suffer from defects of the hematopoietic system because the proliferative capacity of the cells is limited due to short telomeres. In blood cells of these patients, TPMs on the wildtype allele of *TERT* act as a likely compensatory mechanism, resulting in higher telomerase expression without resulting in cancer [46].

Conclusions and future directions

To date, TPMs are unique in their high prevalence as non-coding mutations and early occurrence across cancer types. While the redundancy of ETS factor binding and the inherent difficulty of targeting a non-coding mutation remain an unresolved challenge in targeting TPMs therapeutically, telomerase inhibition has long been explored as a therapeutic strategy to interfere with telomere elongation and immortalization in cancer cells (reviewed in Ref. [47]). Most of the clinical evaluation for TERT inhibition performed to date preceded the discovery of TPMs. This is important to note, as the outcome of an early clinical trial of the telomerase inhibitor Imetelstat revealed a trend towards an improvement in median progression-free survival and overall survival of patients with shorter telomeres compared to patients with long or medium average telomere length [48]. The finding that cancer cells with TPMs generally have very short telomeres and proliferate for an extended period of time with marginal telomerase levels [30••] raises the intriguing possibility that telomerase inhibition will be an effective intervention in cells with TPMs. Therefore, revisiting telomerase inhibition as a cancer intervention should be considered.

Acutely, it seems important to revisit the therapeutic potential of chemical inhibition of telomerase activity in cancer cells with TPMs. It will be critical to study the shortening dynamics and TERT expression throughout tumorigenesis for each cancer type in a time-resolved manner in order to successfully exploit TPMs as a potential therapeutic target. Knowing when during tumorigenesis *TERT* promoter mutant cells outgrow non-mutant cells will aide finding a therapeutic window when cells are most vulnerable for such telomerase inhibition. Combining whole genome sequencing data and information on telomere length from different stages of cancer development will provide the guidance necessary for finding prognostic markers and more effective treatments.

Author contribution

FL and DH collaboratively wrote this review.

Conflict of interest statement

Nothing declared.

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